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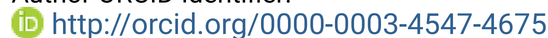
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UNDERSTANDING THE MOLECULAR MECHANISMS UNDERLYING *RSV1* MEDIATED RESISTANCE TO SMV IN SOYBEAN

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Mohammed Ali Ahmed Eid
Lexington, Kentucky
Director: Dr. Aardra Kachroo, Associate Professor of Plant Pathology
Lexington, Kentucky
2016
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ABSTRACT OF DISSERTATION

UNDERSTANDING THE MOLECULAR MECHANISMS UNDERLYING *RSV1* MEDIATED RESISTANCE TO SMV IN SOYBEAN

Like humans, viral diseases also affect plants. Of these, viruses belonging to the potyvirus genus are the most prolific. The potyvirus soybean mosaic virus (SMV) is an important pathogen of the crop plant soybean. SMV causes mosaic symptoms (yellow areas alternate with dark green areas on the leaves of the plant) and can affect yield by reducing seed quality. Few cultivars from soybean can resist different SMV strains. To understand soybean defense mechanisms to SMV, I identified soybean proteins that interact with the helper component protease (HC-Pro) of SMV, which also functions as the suppressor of host RNA silencing and thereby contributes to viral virulence. A genome wide yeast two hybrid screen identified two HC-Pro interactors; *BRI1*-associated receptor kinase 1 (*BAK1*) and ubiquitin conjugating enzyme 2 (*UBC2*). Interactions with HC-Pro were confirmed using bimolecular florescence complementation (BiFC), and co-immunoprecipitations (Co-IP) assays. HC-Pro showed co-localization with both *BAK1* and *UBC2* in planta. Six isoforms of *BAK1* were identified in soybean (*BAK1* a, b, c, d, e, and f). Functional analysis showed that silencing the gene encoding *BAK1a* resulted in breakdown of resistance derived from the resistance (*R*) locus *Rsv1*, against SMV. Consistent with the fact that *BAK1* is well known regulator of plant basal immunity, soybean plants silenced for *BAK1* exhibited enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae*. *BAK1*, a receptor-like kinase, functions as a co-receptor in plant defense signaling as well as brassinosteroid-derived signaling during plant growth. My data indicates that HC-Pro is phosphorylated in the presence of *BAK1* and this requires the T341 residue which regulates virus avirulence in *Rsv1* plants. This is an important finding because although *BAK1* is well known to phosphorylate *BRI1* and other defense-related receptors, its involvement in phosphorylating pathogen-derived proteins has not been reported. My work raises the possibility that *BAK1*-derived phosphorylation of HC-Pro may be important to trigger *Rsv1*-mediated resistance against SMV.

KEYWORDS: Soybean mosaic virus (SMV), HC-Pro SMV G5, *Rsv1* mediated resistance, *GmBAK1*, Extreme resistance, lethal systemic hypersensitive response (LSHR).

Mohammed Ali Ahmed Eid

December 7, 2016

UNDERSTANDING THE MOLECULAR MECHANISMS UNDERLYING *RSV1* MEDIATED RESISTANCE TO SMV IN SOYBEAN

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December 7, 2016

Date

DEDICATION

Dedicated with my love to

my honorable parents, FADIA and ALI

and my precious wife, FATMA ELZHRAA

ACKNOWLEDGEMENTS

In the name of ALLAH, Most Gracious, Most Merciful

It is my duty, as a start to praise ALLAH, Lord of the world, whose guidance, blessings and help in enabled me to take my first step on the path of improving my knowledge through this humble effort.

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CHAPTER 1

INTRODUCTION

1.1 Plant immunity

1.1.1 Pathogen-associated molecular patterns (PAMPs) trigger immunity (PTI)

The first line of defense in plants against a wide range of potential pathogens starts by perception of pathogen-associated molecular patterns (PAMPs) via pathogen recognition receptors (PRR), a large gene family in plants that is mostly located in the cell membranes, comprises group of leucine-rich repeat receptor-like kinases (LRR-RLKs) (Boller & Felix, 2009). PRR in turn activates efficient defense responses known as pathogen-associated molecular patterns (PAMP) trigger immunity (PTI) (Fig. 1.1) (Boller & Felix, 2009). For example, the initial response triggered by PTI in plant cells is the elevation of cytoplasmic Ca^{2+} levels, which plays a vital role in mediating other immune signaling pathways, including control of reactive oxygen species (ROS), salicylic acid (SA) production, and stomatal closure (Chiasson et al., 2005; Du et al., 2009; Kotchoni & Gachomo, 2006; Nomura et al., 2008; Nomura et al., 2012; Wang et al., 2009). In addition, the accumulation of callose, a plant β-1,3-glucan polymer, in different places inside the plant cells, at plasmodesmata (PD), and outside between the cell wall and the plasma membrane to prevent the dissemination and limit the penetration of pathogens, respectively, is a remarkable indicator of PTI (Bestwick et al., 1995; Brown et al., 1998; Seo et al., 2014). Plants also recognize abiotic threats by monitoring any changes in the cell. If that happens, endogenous danger associated molecular patterns (DAMPs) are actively or passively expressed and detected by PRR, resulting in PTI-like defense responses (Boller & Felix, 2009; Huffaker & Ryan, 2007; Krol et al., 2010).

1.1.2 Effector trigger immunity (ETI)

Beside PRR recognition patterns, some plant species or population of species can detect many pathogen effectors, known as avirulent proteins (*avr*- proteins), through specific *R* (resistance) proteins. This recognition will activate a strong defense responses known as effector trigger immunity (ETI) (Fig. 1.1) (Martin et al., 2003). The first phenotype of *R*-gene mediated resistance is hypersensitive response (HR), a visualized

form of programmed cell death "PCD". HR can be recognized by an oxidative burst, the physiological change that result in production of reactive oxygen species (ROS), and rapid ion flux across the plasma membrane (Morel & Dangl, 1997).

Majority of the encoded *R*-proteins in plant belong to NB-LRR family. NB is a nucleotide-binding site domain, and it is required for binding with ATP/GTP. Although ATP hydrolysis in R-protein function is still unclear, it has been shown that ATPase activity was associated with two *R*-gene products in tomato (Tameling et al., 2002). Leucine-rich repeats (LRRs) are required for *R* protein-pathogen effector interactions. It is important to initiate host defense responses (Bell et al., 2003). There are two types of NB-LRR proteins in plants. One is the TIR-NB-LRR proteins that have Toll- interleukin-1 receptor (TIR) homology domain in their N-terminal (Vidal et al., 2002; Whitham et al., 1994). The second is CC-NB-LRR proteins that have coiled-coil (CC) domain in their N-terminal (Dodds & Rathjen, 2010).

The role of LRRs as protein–protein interaction domains, led to the idea that the NB-LRRs might interact directly with their cognate Avrs. However, these interactions were not easily recognized, and thereby this fact suggested that NB-LRR proteins might monitor or guard other host proteins instead (Dangl & Jones, 2001; Van Der Biezen & Jones, 1998). In this model, the pathogen effector protein might mediate alterations to host target molecules, which in turn are perceived by the plant R-proteins. In such cases, these host targets or "guardees" were considered as co-factors in recognition. For example, the *RAR1* (required for *Mla12*-mediated resistance), and *SGT1* (suppressor of the G2 allele of skp1) proteins are well known to mediate the recognitions of many Avrs by their cognate *R*-proteins, in order to trigger immune defenses against a wide range of pathogens including viruses, bacteria, oomycetes, and fungi (Schulze-Lefert, 2004). *RAR1* and *SGT1* showed its importance in the resistance derived from *Rpg-1b* resistance protein against *Pseudomonas syringae* as well as the resistance conferred by *Rsv1* loci against soybean mosaic virus (SMV) in soybean (Fu et al., 2009). The nonrace specific disease resistance 1 (*NDR1*) is another host factor that played role in activation of many *R*-proteins against their cognate pathogens (Chandra-Shekara et al., 2004; Zhu et al., 2011). Arabidopsis *ndr1* mutant represented enhanced susceptibility to different varieties of *P. syringae* as well as

Hyalopernospora arabidopsidis (Aarts et al., 1998; Century et al., 1997). Selote et al. (2014) showed that two orthologues from *NDR1* in soybean (designated *GmNDR1a* and *GmNDR1b*) were important for the resistance derived from *Rpg-1b*, *Rpg3*, and *Rpg4* against different strains from *P. syringae.* Interestingly, some pathogens *Avrs* alter host proteins and the change in these proteins activates the cognate *R*-proteins. For example, in *Arabidopsis*, the activation of *RPM1* (resistance to *P. syringae pv. maculicola*) gene required phosphorylation of its co-factor *AtRIN4* (*RPM1*-interacting 4), which is mediated by the cognate *AvrB* protein. *AtRIN4* binds both *AvrB* and *RPM1*(Mackey et al., 2003; Selote et al., 2013).

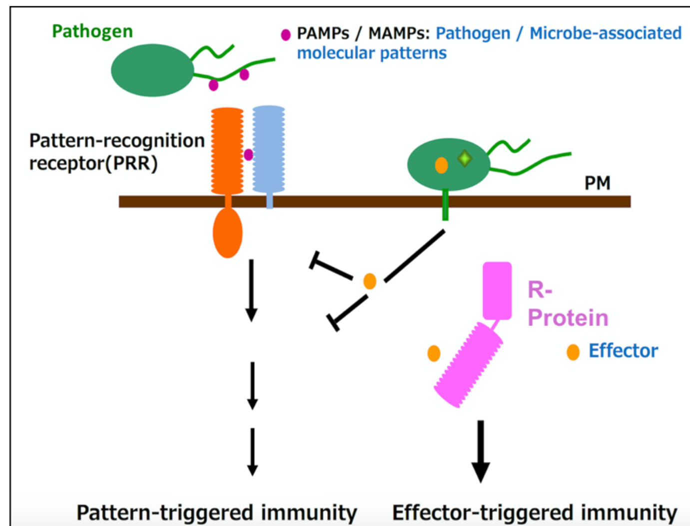

Fig. 1.1 The plant innate immunity starts via recognition of conserved pathogen / microbe-associated molecular pattern (PAMP / MAMP). These PAMPs are perceived by pattern recognition receptors (PRRs) located in the plasma membrane (PM), and promote an immune response known as PAMP-triggered immunity (PTI). On the other hand, pathogens develop effector proteins to inhibit this kind of resistance. Plants will then specify certain gene/s, known as *R*-genes, which directly or indirectly recognize such effectors, and trigger strong immune responses named as effector-triggered immunity (ETI). The figure was modified from the following website http://pgmkawasaki.web.fc2.com/English.html

1.1.3 Zigzag model; the plant immune system

Many pathogens avoid ETI by modifying their recognized effector genes, or by developing others. In response, the host plants can specify a new *R*-proteins to recognize such effectors and trigger ETI. Based on these hypotheses, Jones and Dangl (2006) represented four phases describing the plant immune system. In phase 1, PRRs in the plasma membrane perceive PAMPs and trigger PTI. In phase 2, the pathogens develop new effector to avoid such immune response resulting in effector triggered susceptibility (ETS). In phase 3, the plants deploy a new NB-LRRs protein to specifically recognize such new effector and promote ETI. This recognition could be directly or indirectly as described before. In phase 4, the pathogen will specify a new effector this process can continue.

1.1.4 Plant resistance to viruses

In order to understand the molecular mechanisms underlying the reaction between different viral strains and their host, we should give a hint, firstly, about the widely host–virus relationships. Two main types fall under this category: compatible host-virus relationship, and non-compatible host-virus relationship. In compatible host–virus relationships, viruses can infect the host cell, and cause both local and/or systemic symptoms on the compatible host. The symptoms can appear on all parts of the plant (leaves, roots, stems, flowers and/or fruits). Such symptoms that can be recognized by the naked eye are called external symptoms. These kind of relationships can be greatly affected by the environmental factors. For example, Tobacco mosaic virus (TMV) can easily move either from cell-to-cell or through the whole plant (long distance movement) in *Nicotiana tabacum 'Xanthi'*, at high temperature. But, it can only form local chlorotic and necrotic lesions at low temperature. Another type of compatible relationship exists when the virus can survive, and multiply inside the host cell, move from cell-to-cell, but without clear visible external symptoms. Some plants show another kind of compatible relationship by developing inclusion bodies inside the cytoplasm, in response to the viral infection. For example, pinwheel inclusion bodies are a very special pattern observed with potyviruses. In this type, the plant can recognize the virus at the site of infection, and prevent its movement by sacrificing this part, forming local necrotic lesions. Hindering the virus

movement results in symptomless pattern on all other plant parts. However, some viruses can escape and infect the adjacent cells or even the whole plant leading to lethal systemic hypersensitive reaction (LSHR) (Gaur et al., 2013).

In an incompatible host-virus relationship, the plant can completely resist the virus infection, and prevent its replication and movement. Kegler and Meyer (1987) divided this kind of resistance into two subcategories; qualitative or quantitative. The qualitative resistance; when the plant can specifically detect certain gene/s in the virus, through a very specific resistant gene/s, and trigger extreme resistance, HR, or prevent spreading of it. In the case of quantitative resistance; there is no specific gene to gene reaction. Such as, resistance to virus replication, and spreading. Understanding this kind of resistance will help us to control such devastating diseases and yield losses. In some cases this response is extreme so that no symptoms or viral particles can be detected in any plant parts upon infection (Gaur et al., 2013). This kind of resistance is known as extreme resistance (ER) (Fig. 1.2). Different mechanisms could explain ER against viral diseases; *R* genes are the most common and important candidate that would help explaining this kind of defense.

1.2 Soybean-SMV pathosystem:

1.2.1 Soybean:

Soybean (*Glycine max* (L.) Merr.) is one of the most important foods in many countries overall the world. It has a nutrient value due to its contents of protein, carbohydrates, minerals, essential fatty acids, numerous vitamins, isoflavones, and fiber. Soybean is the main protein source for animal feeding worldwide (John et al., 2016). The production of soybean in the top five producer countries (USA, Brazil, Argentina, China, and India) through 2014 were 259 million metric tons, approximately. The United States alone produced 99.7 million tons from 26% of its total cropland area (Food and Agricultural Organization).

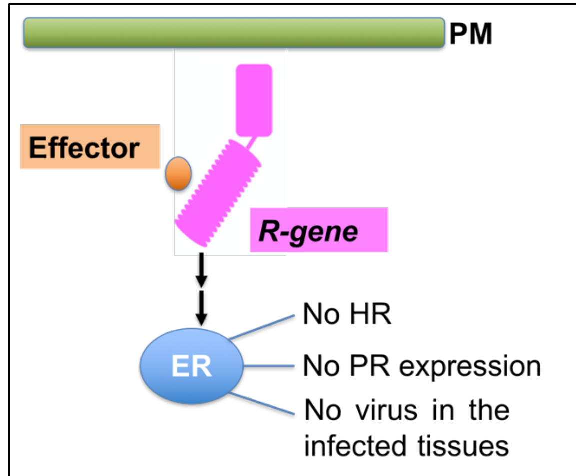

Fig. 1.2 Some resistance genes (*R*-genes) trigger extreme resistance (ER) after direct or indirect recognition of the viral effector protein. In such immune responses, no hypersensitive response (HR), virus multiplication, and pathogenesis related (PR) proteins expression could be detected in the infected tissues. PM refers to plasma membrane.

1.2.2 <u>Soybean diseases:</u>

Soybean diseases are a major problem worldwide and cause significant yield loss (Hill, 2003; Wrather et al., 1997). The yield loss in soybean due to disease was about 13 millions tones at $4.8 billon in USA during 2010 (Wrather & Koenning, 2011); $3.8 million of this loss was due to viral diseases alone (Hill & Whitham, 2014). Using the suitable fertilizer, and pesticide along with advanced management practices would help controlling these losses, and producing improved varieties from resistant soybean. However, controlling of viral diseases is much difficult, because some viruses are latent, but they still can cause yield losses. In addition, many factors contribute in such diseases, for example planting the soybean adjacent to alternative host plants, seeds are commonly main reason in these disease transmission, and viral vectors are big agent that can cause this problem. Deployment of soybean varieties with resistance genes is considered the most preferable method to control them. About 70 viruses can infect soybean; 20 of them are shown in (Table 1.)(Hema et al., 2013; Hill & Whitham, 2014; Tolin & Lacy, 2004). In this chapter, I will focus on potyviruses, and soybean mosaic virus (SMV).

Table 1.1 Some viral diseases of soybean:

Genome type **Family** **Genus**	**Type species**
Single stranded RNA, Positive sense	
Secoviridae	
Comovirus	Bean pod mottle
	Broad bean true mosaic
	Cowpea mosaic
	Cowpea severe mosaic
	Quail pea mosaic
Fabavirus	Broad bean wilt
Nepovirus	Soybean severe stunt
	Tobacco ringspot
	Tomato ringspot
Potyviridae	
Potyvirus	Azuki bean mosaic
	Bean common mosaic
	Bean yellow mosaic
	Blackeye cowpea mosaic
	Passion fruit woodiness
	Peanut chlorotic ring mottle
	Peanut mottle
	Peanut stripe

Table 1.1 continued

	Soybean mosaic
Virgaviridae	
Tobamovirus	Tobacco mosaic
	Sunn-hemp mosaic
Tobravirus	Tobacco rattle (soybean fleck)

The table is modified from Hill and Whitham (2014)

1.2.3 *Potyviridae*

All viruses in the potyviridae have single strand, flexuous, and filamentous positive sense RNA (+ve ssRNA). The viral protein genome-linked (VPg) of about 24 kDa is covalently linked to the 5' end of its genome along with a polyadenylated (20 to 160 adenosines) 3' terminus (King 2011). Nucleotide sequence analysis showed 5' untranslated region, a single open reading frame, and 3' untranslated region in all of its genera (Riechmann et al., 1992; Shukla et al., 1991). The genome encodes a polyprotein, with a conserved order, that are self-cleaved to single multifunction proteins. The virions in this family range from 11-15 nm in diameter, with no envelope. Viruses in the following genera, *Potyvirus, Ipomovirus, Macluravirus, Rymovirus, Tritimovirus, Brambyvirus,* are monopartite with a single strand RNA particle of 650-900nm in length. Members in genus the *Bymovirus* are bipartite with two RNA particles of 250-300 nm and 500-600 nm in length (King, 2011).

1.2.4 Genus Potyvirus: Type species: *Soybean mosaic virus*

Soybean mosaic virus (SMV) causes a devastating disease worldwide in soybean, leading to a huge loss in the yield production. It affects seed quality, by causing seed coat mottling symptoms (Chen et al., 2008; Kennedy & Cooper, 1967). The estimated loss is expected to be high at the regions where *Cerotoma trifurcata*, a beetle vector of bean pod mottle virus (BPMV), and *Aphis glycines*, a vector of SMV, are highly distributed (Burrows et al., 2005; Giesler et al., 2002; Wang et al., 2006). SMV, like all potyviruses,

has filamentous particles, approximately 750 nm in length and 11–15 nm in diameter. It is composed of approximately 2,000 copies of a 29.5 kDa coat protein(CP) arranged in a helix. The CP encapsidates one molecule of single stranded, positive-sense 9,588 nucleotide long RNA. A 3' poly A tail and a 5' linked VPg flankes this RNA molecule (Hunst & Tolin, 1982).

1.2.5 SMV genome sequence and organization:

The genomic map of SMV is shown in (Fig. 1.3). The genomic RNA has one long open reading frame (ORF) and another small one resulting from frame shift at P3 cistron, both together encode 11 mature multifunction proteins (Chung et al., 2008; Jayaram et al., 1992) From N to C these proteins are; P1 (the first protein): it is expected to have important role in virus replication. It has a serine protease domain towards the C-terminus, by which it cleaves itself from the polyprotein. HCPro (Helper Component Protease): cleaves itself from the polyprotein by the cysteine protease domain in the C-terminus. It also functions as a suppressor of gene silencing in the host and is involved in vector transmission. P3 (the third protein): It has very important role in viral replication, virulence and symptoms development. P3N-PIPO: resulting from the frame-shift in the P3 cistron. It facilitates the virus movement. 6K1 (the first 6 kDa peptide): unknown function. CI (Cylindrical Inclusion protein): It forms inclusion bodies in the cytoplasm of infected cells, and it has helicase activity. 6K2 (the second 6 kDa peptide): A small transmembrane protein that might help the virus to anchor its replication complex to the endoplasmic reticulum (ER). VPg (Viral Protein genome-linked): It is covalently attached to the 5' end of the genome. It interacts with one or more isoforms of the eIF4E translation initiation factor, requiring for virus translation and replication. Some results showed its incorporation in suppression of RNA silencing. NIa-Pro (nuclear inclusion "a" protein–protease): Serine-like cysteine protease that cleavages the remaining sites in the polyprotein, typically at Gln/Glu-(Ser/Gly/Ala). NIb (the nuclear inclusion "b" protein): The RNA-dependent RNA polymerase. CP (coat protein): it has roles in virus movement, genome amplification and vector transmission (King, 2011).

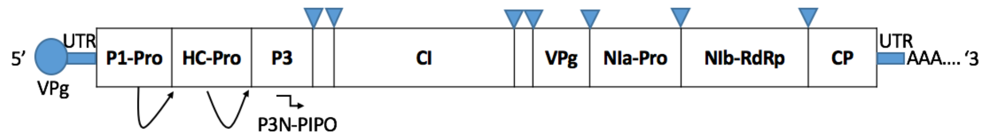

Fig. 1.3: Genome map of SMV showing mature proteins: P1: protein 1, a serine protease; HC-Pro: helper component-protease; P3: protein 3; CI: Cylindrical Inclusion protein, a helicase; VPg: genome-linked protein with primer activity; NIa: nuclear inclusion a, a protease; NIb: nuclear inclusionb–RNA dependent RNA plymerase; and CP: coat protein. The small triangles indicate the cleavage sites of NIb. VPg in the 5' terminal indicated by a circle which is attached to the untranslated region (UTR), as same as in the 3' terminal region. UTR at 3' end followed by poly A tail. The bent arrows represent domains of P1 and HC-Pro that have nuclease activity, and responsible for their release from the precursor poly-protein. Polymerase slippage at P3 cistron is remarked by zigzag shape arrow, which results in the production of the P3N-PIPO protein.

1.2.6 Classification of soybean mosaic virus in the United States (US):

Cho and Goodman (1979) characterized seven strains of SMV (G1-G7) in US, according to their virulence and reactions with eight different soybean cultivars; Clark, Rampage, Davis, York, Kwanggyo, Marshall, Ogden, and Buffllo, the lower number, SMV G1, showing the lower virulence among the different cultivars they used. SMV can only cause mosaic symptoms on the two susceptible cultivars Clark, Rampage. G2 has the same pattern like G1, in addition it can cause necrosis in Marshall. G3 not only cause necrosis in Marshall like G2, but also in Ogden. York and Davis are resistant cultivars to G1-G3, But G4 can break this resistance and form necrotic phenotype. G5 showing mosaic symptoms on York and Davis, along with necrosis on Kwanggyo. Beside the same reaction as G5, G6 can cause necrosis on Marshall as well. G7 can infect all cultivars, and showing mosaic on York and Davis; necrosis on Kwanggyo, Marshall, Ogden, as same as Buffllo.

1.2.7 *Rsv*; *R*-genes in soybean confer extreme resistance (ER) to SMV:

Kiihl and Hartwig (1979) showed a single dominant gene resistant to SMV in soybean. They used eight different soybean cultivars previously known that they are

resistant to SMV, and three susceptible ones to investigate their reactions against two isolates from SMV (SMV-1, and SMV-1B) that behave differentially. The segregation analysis in F2, and F3 showed two type of resistance; i) extreme resistance (ER) to both viral isolates, ii) ER in all homozygous cultivars against SMV-1, but necrosis to SMV-1B, and all the heterozygous cultivars confer necrosis to both SMV strains. A single gene conditioned these resistances in PI96983 and Ogden against SMV. Although, both cultivars were resistance to SMV-1B, only Ogden gave necrotic reaction, and PI96983 showed extreme resistance to both isolates. Using the necrotic reaction that observed in the progeny of resistance x susceptible segregation, they were able to detect the dominance of the genes incorporated in these two kind of resistances. They found that the gene, which confers resistance in PI96983, was completely dominant to the one that confers necrosis in Ogden. And the one that confers resistance in Ogden was dominant to the susceptible cultivars. Depending on this result, they concluded that the genes in both cultivars were allelomorphic and assigned them as ***Rsv*** in PI96983, and *rsv*^{t} in Ogden. The susceptible cultivars were assigned as *rsv*.

The single dominant genes in the soybean cultivar that confers ER to SMV G1, G2, G3, G4, and G5, usually gave necrotic reaction to SMV G6, and SMV G7. On the other hand, PI507389 cultivar, that triggered necrotic reaction to SMV G1, was susceptible to SMV G7 (Chen et al., 1994; Cho et al., 1977; Cho & Goodman, 1979). Beside the previous result, Ma et al. (1994) and Ma (1995) found that PI507389 gave a quick LSHR to SMV-G1, G2, G5, and G6, and they were susceptible to G3, G4 and G7. Furthermore, the segregation analysis of F1, and F2 after the following crosses; PI507389 x Lee 68, PI507389 x PI96983, PI507389 x York, and PI507389 x Marshall, showed that all homozygous progenies, carrying allele at locus *Rsv1*, conferred LSHR against SMV-G1, G2, G5, and G6. This allele was recessive to the resistance alleles in PI96983, York and Marshall. They assigned it in PI507389 as ***Rsv1*-n**.

Chen et al. (1991) investigated allelism among soybean cultivars that confer different resistant reactions against all known SMV strains (G1-G7); PI96983, Marshall, Kwanggyo, Ogden, and York. Because they noticed that each cultivar has a single dominant gene, and conditioned resistance to SMV, they supposed that those genes in each

cultivar were allelic to *Rsv1* locus, and they assigned them as *Rsv1*-k, *Rsv1*-y and *Rsv1*-m for Kwanggyo, York, and Marshall, respectively. *rsv*^{t} in Ogden was changed to *Rsv1*-t because of its dominance on the susceptible cultivars.

Buzzell and Tu (1984) conducted study on OX670, a breeding line, came after successive cross between different resistant cultivars; L78-379, Williams, PI96983, OX615, OX613, OX315, Harcor, and Harosoy. It is thought that the resistant gene it was carrying came from Radian. Radian carries a resistance gene against SMV, that was expected to be independent from *Rsv1*. Their study showed that the gene in OX670 conferred ER to all SMV strains (G1-G7, and G7A). Because of its different behavior than *Rsv1*, they assigned it as ***<u>Rsv2</u>***. However, later studies by Buss et al. (1995) proved that Radian gave ER to SMV strains G1, G2, G3, G4, and G7, but necrotic reaction to SMV G5, and G6. In addition, their further study on this cultivar proved that it carries a resistance locus which is allelic to *Rsv1*. This contradictory result postulated that the resistant gene in OX670 was not from Radian, but it may be from another resistant one they used.

Tu and Buzzell (1987) extended their study by using OX686, a breeding lines came from F2 plant of Columbia x Harosoy. Harosoy is a susceptible cultivar to SMV G1 and G4, but giving ER to SMV G2, G3, G5, and G7. OX686 conditioned stem tip necrosis (STN) to SMV-G1 and G4, which is dissimilar than Harosoy. Segregation analysis obtained from F2 and F3 progenies after the following crosses; OX686(STN) x L78-379 (*Rsv1*) and OX686 (STN) x OX670 (*Rsv2*), and with infection by SMV-G1 and G4, indicated that the STN gene is independent of both *Rsv1* and *Rsv2* loci, hence they assigned it as ***<u>Rsv3</u>***. L29, a selection line from Williams (6) x Hardee, showed resistance reaction to SMV G5 and SMV G7, and susceptibility to SMV G1, G2, G3, and G4. It is shown that this line carries a resistance locus allelic to *Rsv3* (Buss et al., 1999; Ma, 1995).

Lim (1985) reported that resistance in PI483084, PI96983 and PI486355 was conferred by a single dominant gene at independent loci. However, Chen et al. (1993) postulated the presence of two independent resistance genes in PI486355, and they conferred resistant to SMV-G1 through G7. They found one of these two genes was at the Rsv1 locus. Later Ma et al. (1995) crossed PI486355 with Essex in order to separate these

two genes. Those progenies that carried allelic form of Rsv1 were assigned as LR1, and those with the other one were assigned as LR2. Inheritance and allelic studies confirmed that each of these two lines possesses a single dominant resistance gene. Because R1 behaved differentially than the normal Rsv1 locus by triggering resistance to all SMV strains from G1 to G7, they named it Rsv1-s, hence it is the only Rsv1 locus that showed this pattern. LR2 showed complete resistance to strains SMV-G1 through G7 and exhibits complete dominance. In addition, it was independent of Rsv1 and Rsv3. Therefore, they gave it ***Rsv4*** symbol. Later, Buss et al. (1997) developed LR2 into a homozygous line, V94-5152, came from PI486355 x Essex.

From the previous studies, it is clear that there are three main independent loci, *Rsv1*, *Rsv3*, and *Rsv4* in different soybean cultivars interact compatibly, and non-compatibly with the seven different SMV strains. Hayes et al. (2004) and Suh et al. (2011) were able to map and sequence the proposed *Rsv1* and *Rsv3* loci. They found clusters of nucleotide-binding leucine-rich repeat characterizing both of them. Recent studies by Gunduz et al. (2004) suggested the role of *Rsv4* in resistance to all SMV virus strains (G1-G7), by restricting short and long distance movement of the virus. Gunduz et al. (2001) postulated that Harosoy has a resistance allele at the *Rsv3* locus and susceptible alleles at the *Rsv1* and *Rsv4* loci.

1.3 HC-Pro (a key protein):

Most of the encoded proteins are multifunction, especially HC-Pro. It is the main helper component in aphid transmission of SMV from the infected plant to the healthy one (Thornbury et al., 1985). Beside its role in vector transmission, it is also involved in cell-to-cell movement of the virus inside the host plant (Kasschau et al., 1997). It consists of three main domains N-terminal, central (core region), and the C-terminal. The two conserved boxes among potyviruses found in its N-terminal domain "KITC, and ID" (Thornbury et al., 1990), with the highly conserved histidine and cysteine residues showed the ability to form zinc finger shape that allow it to bind the unspecified nucleic acid sequenced (Maia & Bernardi, 1996). The mutational analysis in these conserved motifs had a great effect on the virulence of some potyviruses, the long distance movement, as well as aphid transmission (Atreya et al., 1992; Atreya & Pirone, 1993). The central region

of HC-Pro has RNA silencing suppressor activity (RSS) in many potyviruses (Plisson et al., 2003). Shiboleth et al. (2007) postulated that a conserved motif in HC-Pro 'FRNK' affect this pattern by sequestering the double form miRNA of the host. This region also is important in virus replication (100-300 AA), synergism with other viruses (IGN motif, 260-262 AA), and long distance movement (CC/SC motif, 292-295 AA) (Cronin et al., 1995; Kasschau et al., 1997). Beside the proteinase activity of the C-terminal domain that release it from the precursor polyprotein, there is a conserved motif (KTP) that affects aphid transmission, along with KITC motif (Huet et al., 1994). Mutational analysis showed that this region is important in cell-to-cell movement. For example, C-terminal deletion of 87 and 293 AA totally prevents *Bean common mosaic necrosis virus* (BCMNV) from cell-to-cell movement and cytoplasmic movement inside the host plant (Rojas et al., 1997). Recently, the transgenic soybean cultivar with HC-Pro experienced severe symptoms in comparison to the untransgenic lines, including deformed vegetative and reproductive development (Lim et al., 2007). The concurrent mutation in both P3 and HC-Pro of avirulent SMV is sufficient to convert it to virulent strain on a soybean resistant genotype (Eggenberger et al., 2008).

HC-Pro and P3 cistrons from avirulent SMV strain can be recognized by the *Rsv1* loci and elicit extreme resistance (ER), yet the incorporated pathway/s for this recognition is still an enigma (Eggenberger et al., 2008; Hajimorad et al., 2008; Hajimorad et al., 2005; Hajimorad et al., 2006; Hajimorad et al., 2011; Wen et al., 2013). One hypothesis has been proposed that both of them were recognized together as polypeptide by *Rsv1* locus (Hajimorad et al., 2008). However, this did not correlate with the findings of Hayes et al (2004), when postulated that the resistance conferred by *Rsv1* loci against SMV is derived from a multigenic locus. For example, the recombinant hybrid lines (RIL) L800; that included only one region from the *Rsv1* locus which is a member of a subfamily (the class G family) of nucleotide-binding leucine-rich repeat (NB-LRR) genes and designated as (*3gG2*), recognized only P3 csitron but not HC-Pro to trigger ER against the avirulent SMV strain (Hayes et al., 2004; WEN et al., 2011). Whereas, the other RIL L943; lacked this *3gG2* gene but contained other five class G CC-NB-LRR genes (*IeG30*, *5gG3*, *IeG15*, *6gG9*, and *IgG4*) from the same chromosomal region of the *Rsv1* locus in *PI96983*, recognized HC-Pro instead, to elicit the resistance against different avirulent SMV strains

(Wen et al., 2013). This result showed that the multigenic nature of *Rsv1* loci could specify different genes to recognize either HC-Pro or P3 cistrons to confer the resistance. Another hypothesis suggested that any of HC-Pro and/or P3 might interact with different host factor which are guarded by *Rsv1* and elicit the corresponding resistance (Dangl & Jones, 2001; Eggenberger et al., 2008).

1.4 RNA silencing:

The second form of defense, beside *R*-proteins, in plants against viruses is RNA silencing. RNA silencing is a very sophisticated system that has been developed by plants against viruses, and represent an ancient innate immune technique of defense. Beside its role in defense, it is important in gene regulation in all organisms (Baulcombe, 1999; Bosher & Labouesse, 2000; Catalanotto et al., 2000; Matzke et al., 2001; Waterhouse et al., 2001). This form of resistance is accomplished by small interfering RNAs (siRNA)-that recognize viral RNAs, and promote its degradation by the help of other proteins in the system. siRNAs which are specific to the viral RNAs (vsiRNAs) are generated by a group of RNase-III ribonuclease Dicer-like (DCL) proteins that detect viral double-stranded RNA products and secondary RNA structures, then cleaved them into small 21–25 nucleotides (Baulcombe, 1999; Ding & Voinnet, 2007; Molnár et al., 2005; Várallyay et al., 2010). In addition, RNA silencing pathway can be executed by microRNAs (miRNAs), a small sequence of RNAs 20–24 nucleotides, that regulate vital and important biological processes in all living organisms such as genome maintenance, hormone responses, beside biotic and abiotic stress responses (Mallory & Bouché, 2008; Voinnet, 2009). They are encoded by MIR genes in plant genome. Those genes are transcribed by DNA-dependent RNA polymerase II (PolII) giving primary miRNA (pri-miRNA). pri-miRNA form double-stranded hairpins which are processed by DCL1 producing mature miRNAs (Kurihara et al 2006). Strikingly, many plant viral infections are associated with altered levels of certain specific endogenous miRNA and their mRNA targets. For example, miR164, miR164a precursor and its target CUC1 mRNA showed high level of expression in response to oilseed rape mosaic virus (ORMV) or tobacco mosaic virus Cg (TMV-Cg) infections in Arabidopsis (Bazzini et al., 2009). Soybean resistance to SMV infection is associated with up-regulation of some miRNAs (miR160, miR393 and miR1510) (Chen et al., 2015; Yin

et al., 2013). Moreover, miR168 showed up-regulation as well as the expression AGO1 mRNA in *Nicotiana benthamiana* and Arabidopsis in response to many plant virus infections (Havelda et al., 2008; Várallyay & Havelda, 2013; Várallyay et al., 2010; Vaucheret et al., 2006).

Beside siRNA and miRNA, there are four main different proteins responsible for the whole RNA silencing machinery: Argonaute (AGOs), RNA-dependent RNA polymerases (RDRs), DCLs, and double-stranded RNA-binding proteins (DRBs). AGOs are other nucleases that recruit both kinds of small RNA and guide them to the single strand RNAs (ssRNAs) that have their complementary sequences. AGOSs and si/miRNA along with another endoribonucleases will form complex called RISC (RNA-Induced Silencing Complex) which is required for breakdown and destroying the target mRNA or viral RNA genome. RDRs use siRNA as a primer to synthesis more copies from double stranded RNA (dsRNA) using their complementary ssRNAs, which in turn be processed again by DCLs. Thereby, there will be new more copies from siRNAs. In addition to DCLs, a family of (DRBs) are also required for the processing of dsRNA substrates (Brodersen & Voinnet, 2006; Hammond, 2005; Vaucheret, 2006). Some other proteins have been found that involved in miRNA biogenesis, such as HEN1; “an enzyme that methylates the 2′OH of the 3′end nucleotide of miRNAs” and “SERRATE; a zinc finger protein”(Han et al., 2004; Kurihara et al., 2006; Yu et al., 2005).

Many viruses can counteract this system by developing viral RNA silencing suppressor (VRSS). VRSS restrains RNA silencing by either blocking si/miRNAs production or preventing their integration with RISC (Anandalakshmi et al., 1998; Burgyán, 2008; Pumplin & Voinnet, 2013). For example, P25 protein of Potato Virus X (PVX) blocks RNA silencing machinery by mediating the degradation of the corresponding AGO1 and AGO2 (CHIU et al., 2010). P6 protein of Cauliflower mosaic virus (CaMV, a DNA virus) impairs the production of siRNA by interacting directly with DRB4 (Haas et al., 2008). Moreover, Rice yellow stunt virus (RYSV) can hinder RNA silencing by its P6 protein, which interacts directly with RDR6 preventing formation of siRNA (Guo et al., 2013).

1.5 HC-Pro, the RNA silencing suppressor (RSS) of potyviruses:

HC-Pro, the RSS of the *Potyviridae* family (Urcuqui-Inchima et al., 2001), also suppresses host defense, and play an important role in viral pathogenicity (Brigneti et al., 1998; Bruening, 1998; Kasschau & Carrington, 1998; Pruss et al., 1997; Shi et al., 1997). Some symptoms of potyviruses can be caused by the expression of HC-Pro alone without virus infection. Soybeans overexpressing a transgene with HC-Pro showed severe developmental abnormalities, that significantly decreased in case of low expression (Lim et al., 2007). Later, (Lakatos et al., 2006); Mérai et al. (2006) showed the ability of HC-Pro to bind with the duplex form of mi/siRNA. Shiboleth et al. (2007) proved the importance of FRNK box of HC-Pro in duplex smRNA binding. Moreover, HC-Pro of Zucchini yellow mosaic virus (ZYMV) interacted with Hua Enhancer 1 methyltransferase (*HEN1*) and inhibited its activity (Jamous et al., 2011). *HEN1* is the RNA methyltransferase that is responsible for methylation of 2'-OH- or 3' terminal nucleotide of small RNA (sRNA) in Arabidopsis, drosophila and mouse (Horwich et al., 2007; Kirino & Mourelatos, 2007; Yu et al., 2005). This methylation of siRNA is required for its protection from the host exonucleases, and this happens before its incorporation with the Argonaute proteins (*AGOs*) in the RNA-induced silencing complex (RISC) (Fang & Spector, 2007; Ramachandran & Chen, 2008; Yang et al., 2006; Yu et al., 2005). It also can bind with calmodulin-like protein, rgs-CaM (an endogenous suppressor of gene silencing), and prevents methylation of virus-derived small RNAs (smRNAs) (Anandalakshmi et al., 2000; Ebhardt et al., 2005; Yu et al., 2006). These discoveries could explain the role of HC-Pro in viral pathogenicity and host defense suppression. Especially, the mutated plants (*dcl1*, *hen1*, *hyl1*, *ago1*, and *se1*) that are not able to accumulate miRNA, showed similar developmental deformities that are associate with virus infection (Lobbes et al., 2006), and this is similar to the effect of HC-Pro overexpression on transgenic plants.

In summary, SMV infection to soybean generally causes 8% to 35% yield losses every year, however in case of early infection and/or dual infection with other viruses the losses could be as high as 94% (Kolte, 1984). Although there are different loci (*Rsv1*, *Rsv3*, and *Rsv4*) that trigger resistance against SMV, no single locus provides resistance to all strains. HC-Pro of this virus has been shown to contribute to avirulence in plants containing

Rsv1, though the underlying mechanisms are still not understood. To address these issues, I identified soybean protein/s that interact with this SMV effector protein using yeast two-hybrid screening. The interactions between HC-Pro and the identified soybean was confirmed, followed by characterization of the defense-related functions of one of the identified proteins. The subsequent chapters present the data showing the requirements for one of the HC-Pro interactors in *Rsv1*-mediated resistance.

CHAPTER 2

MATERIAL AND METHODS

2.1 Plant growth conditions

Soybean (*Glycine max* (L.) Merr.) cvs. Essex, *Rsv1*, Harosoy, and V94-5152 were grown in a greenhouse at day and night temperatures of 25 and 20°C, respectively. The recombinant Bean Pod Mottle Virus BPMV vector, for *Glyma08G074500* "*GmBAK1a*" gene silencing, was inoculated at the VC stage. The one without cloning insert "vector (V)" was used as a negative control for each experiment. At least four to six even plants were inoculated with V or *GmBAK1a* silencing vectors. The secondary infections of different pathogens (soybean mosiac virus "SMV" G5 and G7 strains, bean yellow mosaic virus "BYMV", tobacco etch virus "TEV" or *Pseudomonas syringae "Psg"*) were done at the V2 stage, after analyzing BPMV symptoms phenotype. Arabidopsis plants were grown in MTPS 144 Conviron walk-in chambers at 22°C, and 14-h photoperiod under 65% humidity.

2.2 Yeast two-hybrid assay

A LexA-fused HC-Pro from SMV G5 (cloned in pEG202, used as bait) is expressed in yeast cells (EGY48), in which *lexA* operators are located upstream of a reporter gene (*LEU2*). This yeast cell strains lack histidine (HIS) and tryptophan (TRP) expressing genes as well, for easy selection of the incorporated plasmids. The soybean cDNA library cloned in pB42AD (Clonetech, CA), kindly provided by Dr. Madan Bhattacharyya, Iowa State University, expresses soybean cDNAs (1.2 X 106 original clones) fused to B42 acidic activator (AD) under control of the *GAL1* promoter. It is derived from the cultivar Harosoy (*rsv1*, *Rsv3*, susceptible to SMV-G2, resistant to SMV G5 and SMV-G7). The LexA-fused HC-Pro binds to the *lexA* operators but is unable to activate transcription of the reporter gene in the absence of interaction with the AD-fused partner. The low affinity interaction result in a very low expression of the reporter gene or not at all; the perfectly grown yeast cells indicate strong interactions and very good expression.

2.3 Sequencing

The sequencing reaction was done in 10 µL total volume containing 100-200 ng of PCR products or gel-purified DNA (Qiagen, CA, USA), 3 µL of 5 µM sequencing primer, 0.5 µL of Big Dye and 2 µL 5× sequencing buffer (Applied Biosystems, UK). The reaction product was precipitated with 2 µL 3 M NaOAc, pH 5.2, 2 µL 125 mM EDTA, pH 8 and 50 µL 100% ethanol, after incubating at -20 ^{O}C for about one hr. The precipitate was subjected to wash with 300 µL of 70% alcohol. The final cleaned product was air-dried and submitted to sequence facility at the Advanced Genetic Technologies Center (AGTC), University of Kentucky.

2.4 Agrobacterium mediated transient expression

Taking the characteristics of Ti-plasmid present in *Agrobacterium tumefaciens*, and using some modified version from that plasmid (ex. pGWB or pSITE), we can transiently express some foreign proteins in tobacco plants (*Nicotiana benthamiana*). *A. tumefaciens* strain LBA4404 carrying pGWB or pSITE vector, cloned with target tagged genes, was grown on LB broth containing suitable antibiotics at 29 ^{O}C overnight. The growing cells were settled down at 3,000 rpm for 10 min and re-suspended in an induction buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 150 µM acetosyringone). The mixture was incubated at room temperature for at least 3 hrs before infiltration into *N. benthamiana* leaves. Infiltrated plants were transferred into a growth chamber and samples were collected 12-48 h post infiltration.

2.5 Bimolecular fluorescence complementation assays

BiFC assay involved first cloning target proteins within the N/C terminal half-EYFP using pSITE-n/cEYFP vectors (Martin et al., 2009). Cloned vectors were transformed into electro-competent *A. tumefaciens* strain LBA4404. The positively transformed cells carrying various constructs were infiltrated into *N. benthamiana* plants expressing CFP-tagged nuclear protein H2B. After 36 - 48 h, a small part from the infiltrated *N. benthamiana* leaf was scanned using a water immersion PLAPO60XWLSM 2 (NA 1.0) objective on a FV1000 point- scanning/point-detection laser scanning confocal

3 microscope (Olympus) equipped with lasers spanning the spectral range of 405–633 nm. EYFP was excited using 488-nm laser line, giving yellow fluoresce upon its reconstitution in case of positive interaction. CFP and YFP overlay images (40× magnification) were acquired at a scan rate of 10 ms/pixel. Olympus FLUOVIEW 1.5 program was used to control the microscope, image acquisition and the export of TIFF files. This assay was repeated at least three separate times; different infiltrations were done for each interaction using both combinations of c/nEYFP fused proteins.

2.6 Protein localization *in planta*:

Green Florescence Proteins (GFP) or Red Florescence Protein (RFP) are required as tags for protein localization. They are fused to target proteins using pSITE-3CA-GFP or pSITE-3CA-RFP vectors. The cloned vectors were then electrically transformed into *A. tumefaciences* strain LBA4404. Those showing positive colony PCR and carrying various tagged proteins were infiltrated into wild-type *N. benthamiana* plants individually or mixing together. After 24 - 48 h, a small part from the infiltrated *N. benthamiana* leaf was scanned using a water immersion PLAPO60XWLSM 2 (NA 1.0) objective on a FV1000 point- scanning/point-detection laser scanning confocal 3 microscope (Olympus) equipped with lasers spanning the spectral range of 405–633 nm. GFP and RFP were excited using 488-nm laser line and 558 nm laser line, giving green and red fluoresce patterns, respectively. Olympus FLUOVIEW 1.5 program was used to control the microscope, image acquisition and the export of TIFF files.

2.7 Protein extraction, western blot analysis and co-immunoprecipitation assays

Total protein extraction from previously treated leaves starts by grinding them (50-200 mg), after immersing in liquid nitrogen, with 1-2 mL protein extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.5% Triton-X-100, and 1 ×protease inhibitor cocktail), followed by centrifugation at 4° C at 13,000 rpm for 10 min. The cleared supernatant then was transferred to a new 1.5 mL Eppendorf tubes. For detecting protein concentration, 2 µL from the extract was mixed with 998 µL of 5 x diluted Bio-Rad protein assay kit. OD was measured at 595 nm. For SDS-PAGE gel, equal amount of proteins from different samples were mixed with 3 × loading buffer (3.0 mL

H2O, 1.2 mL 1 M Tris-HCl pH 6.8, 2.4 mL glycerol, 0.48 g SDS, 60 µL 10% bromophenol blue and 1.5 mL β–mercaptoethanol). Mixtures were incubated at 100 ^{O}C for 5 min, and loaded directly to SDS-PAGE minigel (6 × 9 cm) at 100 V in 1 × running buffer (14.4 g glycine, 3 g Tris-base, 1 L H2O). Protein samples running was ended when the bromophenol blue reached the bottom of the gel, then were transferred to PVDF membrane (Immun-Blot, Bio-Rad). The membrane was pre-wetted in methanol before using, as well as the reaming required materials were pre-wetted at 1 × transferring buffer (3.2 g Tris-base, 15 g glycine, 1 L H2O). For efficient transferring, 400 mA for 1 h was used under cold conditions with the Bio-Rad mini-gel box electro- transfer unit. The transferred PVDF membranes were stained in Ponceau-S solution (40% methanol, 15% acetic acid, 0.25% Ponceau-S). The stain was removed by rinsing via deionized water, and the membrane was blocked by incubation with 5% non-fat dry milk dissolved in 1 × TBST buffer (5 mM Tris-base, 20 mM NaCl, pH 7.4, 0.1% Tween 20) for at least 1 h on a shaker. For immune detection of the specific tagged proteins, the membranes were incubated within primary corresponding antibodies in fresh 10 mL 5% non-fat dry milk dissolved in 1 × TBST buffer for 2-4 h. The secondary antibody (HRP-conjugated, Sigma) was applied for about 1 h in fresh 10 mL 5% non-fat dry milk dissolved in 1 × TBST buffer, after washing it for at least 3 times 10 min each one with 1 × TBST buffer. Three further washing times were done and bands were visualized using ECL kit (1 mL/membrane) (Super-Signal, Thermo Scientific) and exposed to autoradiography film (Santa Cruz Biotechnology, USA). For IP assays, beside the previous procedures the total protein extracts were firstly incubated with M2 FLAG-affinity beads (unless noted otherwise) for at least 2 h, followed by 3 times washing, 10 min for each one with extraction buffer lacking PVPP at 4 ^{O}C. Expected molecular weight of proteins: *GmBAKs1* ~ 68 kD, FLAG-*GmBAKs1* ~ 69 kD, MYC-*GmBAKs1* ~ 72 kD, *GmUBC2* ~ 16 kD, FLAG-*GmUBC2* ~ 17 kD, MYC-*GmUBC2* ~ 20 kD, HC-Pro ~ 52 kD, MYC-HC-Pro ~ 56 kD, FLAG-HC-Pro ~ 53 kD, SMV CP ~ 29 kD, TRSV-CP ~ 30 kD.

2.8 Primers, sequence accessions and phylogenetic analysis.

Database accessions for complete genes sequences used here are PR1 (AI930866), β-tubulin (M21297), GmBAKs1 family (Glyma08G074500 a, Glyma05G119600 b, Glyma08G180800 c, Glyma15G051600 d, Glyma05G119500 e, Glyma02G076100 f), AtBAK1 (AT4G33430.1), GmUBC2 (Glyma02g40330), GST (AF243364). Megalign program in the DNASTAR package was used for alignment and sequence analysis.

2.9 Construction of viral vectors, in vitro transcription and plant inoculation

For generating silencing vectors, specific primers with BamHI and MscI sites, forward 5'- GCAGGATCCAATTTGCTTGGAAATCGTT -3' and reverse 5' - CAGTTGGCCAATTTGAGTCATTAGGAGT -3', were used to amplify 204-bp DNA fragment encoding GmBAK1a at protein kinase domain from Essex soybean cDNA. The gel purified PCR products were digested with these restriction enzymes as well as pGG7R2-V (containing full length BPMV RNA2) (Zhang & Ghabrial, 2006a). Both were subjected to a ligation reaction (6 µL DNA fragment, 2 µL plasmid, 1 µL reaction buffer, and 1 µL ligase enzyme "New England Biolabs") at 15 ^{O}C overnight. The ligation mixture was transformed to chemically competent *E. coli* (NEB 5-alpha Competent "High Efficiency"), and the positively selected colonies were used to extract large quantity of sequence confirmed cloned plasmid. In vitro transcription reaction started with linearization of both pGHoR1 "containing full-length cDNA clone to type I RNA1, from strain K-Ho1" (Zhang & Ghabrial, 2006a), and cloned pGG7R2-V (containing recombinant RNA2) using SalI and NotI and SalI alone, respectivly. 5 µg of linearized plasmids were incubated in a 100-µL reaction mixture containing 40 mM Tris- HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, 50 units of RNasin (Promega Corp., Madison, WI, U.S.A.), 0.5 mM each ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM cap-analogue (m7G[5′]G) (New England Biolabs, Ipswich, MA, U.S.A.), and 50 units of T7 RNA polymerase (New England Biolabs) at 37°C for 2 h. Transcription yield and quality of transcripts were checked via electrophoresis on a 1.0% agarose gel. Both transcripts were mixed together and used to rub inoculate fully expanded unifoliate leaves of soybean (VC stage). BPMV symptoms should be recognized in the second emerging

trifoliate leaves. The successfully infected plants were used to collect leaves showing clear symptoms for freeze drying and further silencing experiments.

2.10 Pathogens infection and chemical assays:

For viral infection, infected plant tissues showing clear symptoms were homogenized in 0.01 M phosphate buffer and used for rub-inoculation of previously sprayed leaves with carborundum. For bacterial infection, we used *Pseudomonas syringae pv. glycinea* to analyze both basal and R-mediated resistance in wild type (mock M), previously silenced (BAK1 sil), or vector infected (V) soybean cv. Merit. *P. syringae* was grown on kanamycin (50 μg/ml) and rifampicin (750 μg/ml) selective King's B medium at 29 ^{O}C for at least 24 h. The infection was done by bacterial suspensions (1 × 105 CFU/ml in 10 mM MgCl2 plus 0.04% Silwett L-77) at the V3 stage after appearance of BPMV symptoms. Infected plants were grown in growth chamber at 22°C and 65% relative humidity with a 16-h photoperiod. Bacterial growth was analysed at 0, and 3 days postinoculation (dpi) by grinding 1-cm leaf discs with 10 mM MgCl2, and plating a diluted mixture on selective King's B plates. Experiments were repeated three independent times. For gene expression analyzing, leaf samples were collected at 0 and 2 dpi. For brassinosteroid (Br) and propiconazole (PPZ) treatments, plants (V2 stage) were sprayed with 1 μM Br or 0.2 μM PPZ 24 h prior SMV infections. Control plants were infiltrated with water.

2.11 ELISA assay:

For ELISA assays of SMV levels, 1 g of plant diseased tissues was homogenized in 5 ml of coating buffer (15 mM Na_2CO_3, 34 mM $NaHCO_3$, 3mM NaN_3, pH 9.6). Homogenates were centrifuged at 12,000 rpm for 5 min. Plates were washed three times with PBST buffer (0.14 M NaCl, 3 mM KCl, 4.5 mM Na_2HPO_4, 2 mM KH_2PO_4, 1% Tween 20, pH 7.4), and blocked with 0.5 % BSA buffer (0.5 g BSA in 100 ml PBS) before adding 200 μl of Homogenates for each well. 2 wells from negative (healthy) and positive saps were used as control. Plates were incubated at 37 °C for 1 h. The wells were washed 3 times with PBST, and 200 μL SMV CP specific antiserum (1:5000 in 0.5 % BSA) were pipetted in each well, and incubated at 37°C for 1 h. Plates were washed as above and 200

µL /well of goat anti-rabbit IgG-alkaline phosphatase conjugate (from Sigma) (1:1000 in 0.5 % BSA buffer), then incubated at 37 °C for 1 h. The plates were rewashed three more times and 200 µL/ well of freshly prepared substrate (Sigma 104 alkaline phosphatase in Diethanolamine buffer "97 ml diethanolamine, 800 ml water, 3mM NaN3, pH 9.8" were added for visualization. Plates were examined to measure the optical density (O.D.) at 405-nm wavelength with a spectrophotometer (EL800 Universal Microplate Reader, Bio-TEK Instruments, INC). The positive results were recorded over to 2-time fold of a negative control and less than it considered as a negative reaction.

2.12 Cell death assay

For ion leakage, M, V, and BAK1 silenced soybean leaves (cv *Rsv1*) were rub-inoculated with SMV G7 at V2 stage. 6 leaf discs (d = 0.7 cm) from infected leaves were collected 7 dpi from SMV G7 infection, then washed in distilled water for 30 min. The cleaned discs were then transferred to tubes containing 10 ml of distilled water for conductivity measurement every 4 h for 24 h by an NIST traceable digital Conductivity Meter (Fisher Scientific, Waltham, MA). Three replicates measurements per treatment per experiment were analyzed and used for calculation of standard deviation (SD).

2.13 Trypan-blue staining

Leaves from V, and BAK1 silenced soybean (cv *Rsv1*) were vacuum-infiltrated with trypan blue stain solution (10 mL acidic phenol, 10 mL glycerol, and 20 mL sterile water with 10 mg of trypan blue) and left at room temperature overnight. For de-staining, they were kept in chloral hydrate solution (25 g/10 mL sterile water; Sigma, USA) at least 3 h, for three times. They were mounted on a glass slide with glycerol, after ensuring the complete clearance from any extra dye. The samples were photographed using an AxioCam camera (Zeiss, Germany) and images were analyzed using Openlab 3.5.2 software (Improvision).

2.14 RNA extraction

RNA extraction was done using Trizol reagent (Invitrogen, Carlsbad, CA, USA). About 100 mg samples of soybean leaves were collected in 1.5 mL Eppendorf tubes and

frozen immediately in liquid nitrogen. The frozen samples were homogenized in 300 μL of Trizol using small pestles. After grinding the whole samples, another 700 μL of Trizole were added. The homogenates were vortex with 200 μL of chloroform, and centrifuged at 12,000 rpm for 17 min. Clear supernatants were collected in new autoclaved Eppendorf tubes, and mixed with equal volume of isopropanol then left 15 min at room temperature followed by centrifugation at 12,000 rpm for 12 min. RNA precipitate from each sample was washed once with 75% alcohol, air dried and re-suspended in 20 – 30 μL DEPC-treated water.

2.15 Complementary DNA synthesis (cDNA) and reverse transcriptase polymerase chain reaction (RT-PCR):

For cDNA synthesis, 5 - 7μg total RNA was annealed with 1 μL oligo dT17 (0.5 μg/μL) at 65 ^{O}C for 15 min in a water bath. After incubating in ice for 5 min, 1 μL 10 mM dNTPs, 2 μL 100 mM DTT, 1 μL reverse transcriptase (200 U/μL, Invitrogen, USA), and 1 μL RNAase inhibitor (40U/μL, Invitrogen, USA) were added, and the whole mixture was then incubated at 42 ^{O}C in a water bath for 1 h. The reaction was stopped at 65 ^{O}C for 15 min and the total volume was diluted by adding equal amount of DEPC treated water. The resulted cDNA was kept at -20 ^{O}C for further using. The RT-PCR was programed for 35 cycles to determine absolute levels of transcripts, and reduced to 21–25 for quantifying differences between them before saturation.

2.16 Quantitative real time polymerase chain reaction (qRT-PCR):

Three independent cDNA preparations were analyzed to quantify relative differences in transcripts levels. Gene specific primers were designed to generate PCR products of <200 bp (Table. 2.1). Endogenous actin transcript level was used to normalize the transcript level of each of target genes. 20 μL reaction (0.4 mM of each primer, 10 μL of SuperScript III SYBR Green (Invitrogen), 100 ng of cDNA, and sterile DEPC-treated water) was carried out in 96-well plates using an Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA, USA). The cycling conditions were: 30 s at 95 ^{O}C for preheating and enzyme activation, followed by 40 cycles (melt for 5 s at 95 ^{O}C, annealing and elongation for 20 s at 60 ^{O}C). The SDS RQ manager Applied Biosystems software was

used automatically to calculates baseline and threshold values.

2.17 Northern blot analysis

Extracted RNA was quantified using spectrophotometry at A260. For denaturation, 7 µg from the total extract of each sample was mixed with 16 µL denature mixture (1 mg/mL ethidium bromide, 0.39 X MOPS, 13.7% formaldehyde and 39% formamide) and incubated at 65 ^{O}C for 15 min then chilled immediately on ice for 5 min. Denatured RNA samples were then mixed with 2 µL of RNA loading dye (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol), and loaded directly on 1.5% agarose gel containing 3% formaldehyde and 1 X MOPS buffer (4.18 g MOPS, 680 mg NaOAc, 37 mg EDTA in 1 L sterile water and adjusted to pH 7.0). For northern blot analysis, RNA samples was capillary transferred onto Hybond-NX (GE Healthcare) nylon membrane at room temperature overnight via 20 X SSC buffer (3 M Sodium chloride and 300 mM tri-Sodium citrate dihydrate, pH 7.0), and cross-linked under UV for 0.9 min in a CL-1000 ultraviolet Cross-linker (UVP) for fixation. The membrane was washed in 2 X SSC buffer for 20 min, and dried at 65 ^{O}C for 10 min. It was incubated with hybridization buffer (sodium phosphate buffer "pH 7.0", 100 µg/mL sheared salmon sperm DNA, 7% SDS and 1.25 mM EDTA) at 65 ^{O}C for at least 1 h before hybridizing with the specific probe DNA fragment. For probe synthesis, specific primers (Table 2.1) was used to amplify the targeted DNA fragment from wild-type plant cDNA, which then gel-purified and confirmed by sequencing. The fragment was denatured by heat at 90 ^{O}C for 10 min, and immediately chilled on ice for 5 min, then incubated at 37 ^{O}C for 1 h with 1 µL Klenow enzyme (NEB, 2000 U/mL), 2 µL 10 X BSA and 10 µL labeling mixture (containing hexa-nucleotide primers, dATP, dGTP, dTTP) and 25 µCi α- ^{32}P-dCTP (specific activity 6000 Ci/mol, Perkin Elmer, USA). The resulted labeled DNA fragment was purified by MicroSpin G-50 Sephadex column (GE Healthcare). It was then denatured by 14 µL 2N NaOH for 15 min, followed by neutralization with 1M Tris pH 7.5 for 15 min and added to the hybridization buffer. Hybridization was carried out in oven (Labnet International Inc.) at 65 ^{O}C overnight. The hybridized membrane was washed twice with 2 X SSC, 0.5% SDS, and once with 1 X SSC, 0.1% SDS solutions at 65 ^{O}C, 20 min for each time. It was exposed to a Storage Phosphor Screen (Amersham Biosciences) overnight and scanned on a Typhoon 9400

Variable Mode Imager (GE Healthcare). The signal intensity was analyzed by ImageQuant TL V2005 software.

2.18 Site directed mutagenesis:

Approximately10 ng of plasmid DNA cloned with the gene of interest were used as template for PCR in a total volume of 50 µL reaction. The fragment of each gene was PCR amplified using two pairs of primers containing the required mutations to generate two PCR products (Table 2.1). The PCR products were gel-purified (Qiagen, CA, USA), and used as templates to produce a single PCR amplicon with a pair of primers containing full ATTB sequence. The full length PCR inserts containing mutation were, then, subjected to gateway cloning system (Esposito et al., 2009). The resulting plasmids were sequenced to confirm mutations and then used for further studies.

2.19 Band shift and *in planta* phosphorylation assays:

For band shift resulting from phosphorylation, total protein extraction was performed as mentioned above. The proteins were then separated on 8% SDS-PAGE at 20 V for 12-14 h, followed by western blotting using the same described methods. For in planta phosphorylation assay, MYC-tagged HC-Pro and FLAG tagged GmBAK1a proteins were transiently expressed in *N. benthamiana* using Agrobacterium. 24 h later I infiltrated 25 µCi of ^{32}P- dATP (specific activity 4500 Ci/mol, Perkin Elmer, USA) diluted by the induction buffer (10 mM MES, pH 5.6, 10 mM MgCl2, and 150 µM acetosyringone). Twelve hours post infiltration; the total protein extracts were firstly incubated with MYC-affinity beads for at least 3 h, followed by 3 times washing, 10 min for each one with extraction buffer lacking PVPP at 4 ^{O}C. SDS-PAGE electrophoresis of IP extracts was then performed, and the gel was exposed directly to a Storage Phosphor Screen (Amersham Biosciences) for 2 dyas and scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare) to detect ^{32}P-labeled proteins. The signal intensity was analyzed by ImageQuant TL V2005 software.

Table 2.1: List of primers were used in this study:

Prime name	Primer sequence (5' – 3')	Uses
pB42AD	ACCAGCCTCTTGCTGAGTGGA	pB42AD sequencing
GmBAK1a	AAAAAGCAGGCTTAATGACCAACATGGAGCGAATG	Gateway cloning
	AGAAAGCTGGGTATCATCTAGGACCTGAC	
GmBAK1a LRR domain	AAAAAGCAGGCTTAATGGGTGATGCCCTGATTGT	Gateway cloning
	AGAAAGCTGGGTATTGACTGGGACGTCTCC	
GmBAK1a PK domain	AAAAAGCAGGCTTAATGCTTGGTAAAGGTGGATT	Gateway cloning
	AGAAAGCTGGGTATCATCTCACCACCTCAGAC	
GmBAK1b	AAAAAGCAGGCTTAATGACCAATATGCAGCGAGTGGTT	Gateway cloning
	AGAAAGCTGGGTATCATCTAGGACCTGAC	
GmBAK1c	AAAAAGCAGGCTTAATGGATAGAGTGACTTCGTCT	Gateway cloning
	AGAAAGCTGGGTATCATCTAGGACCTGATAGTTC	
GmBAK1a	GCAGGATCCAATTTGCTTGGAAATCGTT	pGG7R2V vector

Table 2.1 continued

sil1	CAGTTGGCCAATTTGAGTCATTAGGAGT	cloning (BamH1, Msc1)
AtBAK1 4g	AAAAAGCAGGCTTAATGGAACGAAGATTAATG	Gateway cloning
	AGAAAGCTGGGTATTATCTTGGACCCGAGGGGTAT	
GmUBC2a	AAAAAGCAGGCTTAATGGCTTCGAAACGCATCCTG	Gateway cloning
	AGAAAGCTGGGTATCATCCCATGGCATACTTCTGGGT	
SMV G5 HC-Pro	AAAAAGCAGGCTTAATGTCCCAAAATCCTGAAGC	Gateway cloning
	AGAAAGCTGGGTAACCAACTCTGCAGAATTTCAT	
SMV G7 HC-Pro	AAAAAGCAGGCTTAATGTCCCAAAATCCTGAAGC	Gateway cloning
	AGAAAGCTGGGTAACCAACTCTGCAGAATTTCAT	
BYMV HC-Pro	AAAAAGCAGGCTTAATGTCAGCTGGTGACTTATTCTGG	Gateway cloning
	AGAAAGCTGGGTATCAACCAACTCTATAATACTTCAG	
TEV HC-Pro	AAAAAGCAGGCTTAATGAGCGACAAATCAATCTCTGAG	Gateway cloning
	AGAAAGCTGGGTATCATCCAACATTGTAAGTTTTCAT	
SMV G5 CP	AAAAAGCAGGCTTAATGTCAGGTAAGGAGAAGGAA	Gateway cloning
	AGAAAGCTGGGTACTGCTGTGGACCCATGCC	

Table 2.1 continued

SMV G5 HC-Pro T341H	GAATGCACCATTGATTTCCACAATGAACAA	PCR site-directed mutagenesis
	TTCACCACATTGTTCATTGTGGAAATCAAT	
SMV G5 HC-Pro K142I	AATAAGGCCCTGATGATAGGTCCATCG	PCR site-directed mutagenesis
	TGTTACCGATGGACCTATCATCAGGGC	
GmBAK1a K323E	GATGATGTAGCAGTAGAAAGACTTAAC	PCR site-directed mutagenesis
	GCTTTCAGGGTTAAGTCTTTCTACTGC	
GmBAK1a Y469F	CATATAGCACCAGAGTTTATGACAACT	PCR site-directed mutagenesis
	CCTTCCAGTTGTCATAAACTCTGGTGC	
GmPR1a	TTTGTGGGTGAATGAGAAAT	qRT-PCR and Probe synthesis
	GTATGGTCTTTGGCCAATAT	
Gm Actin	GAGCTATGAATTGCCTGATGG	qRT-PCR
	CGTTCATGAATTCCAGTAGC	
Gm ß-Tubulin	CCATTGGAGCGCATCAATG	RT-PCR
	ATACACTCATCAGCATTCTC	
M13	GTAAAACGACGGCCAG	pDonor sequencing
	CAGGAAACAGCTATGAC	

Table 2.1 continued

Full ATTB	GGGGACAAGTTTGTACAAAAAAGCAG	Gateway cloning
	GGGGACCACTTTGTACAAGAAAGCTGGG	
pGG7R2V	AGCTCAAATGGAAACAAA	pGG7R2V sequencing
	CGTCGTCCAATGAAAGCT	
GmBAK1a	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	CAGTTGGCCACAGTGGCTTCCTTCTATT	
GmBAK1b	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	GCAATCCGCAGTTCAGGCAGT	
GmBAK1c	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	TGCAACTTGCAGCTCACGCAAA	
GmBAK1d	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	TGCAACTTGCAACTCACGCAGA	
GmBAK1e	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	TGCAACTTGCAGTTCACGCAGT	
GmBAK1f	ACTGGAGCTATTGCTGGAGGA	qRT-PCR
	TGCAACTTGCAGTTCTCGTAAT	

CHAPTER 3
HC-Pro THE VIRAL RNA SILENCING SUPRESSOR OF SOYBEAN MOSAIC VIRUS INTERACTS WITH TWO KEY PROTEINS IN PLANT DEFENSE IN SOYBEAN

3.1 INTRODUCTION:

The multifunction helper components proteinase (HC-Pro) showed an important role in the survival and virulence of potyviruses inside the host plants (Maia et al., 1996). Besides its proteolytic activity by which it releases itself from the precursor polyprotein (Carrington & Herndon, 1992), HC-Pro is involved in aphid transmission, viral cell to cell movement, long distance movement, suppression of gene silencing, synergism between co-infecting viruses, symptoms development, and act as an avirulence/virulence determinant of many potyviruses (Govier et al., 1977; Llave et al., 2000; Moury et al., 2011; Pruss et al., 1997; Redondo et al., 2001; Rojas et al., 1997; Sáenz et al., 2002). HC-Pro of soybean mosaic virus (SMV) along with P3 cistron were determined as avirulence factors toward the *Rsv1* resistance loci in soybean (Eggenberger et al., 2008). They showed that the concurrent point mutations in both HC-Pro and P3 (M683R and I788R/T948A, respectively) were sufficient to convert the avirulent SMV-N strain to virulent on the *Rsv1* background. Moreover, several concurrent mutations in the same two cistrons on the avirulent SMV strain were shown to overcome the resistance from two different *Rsv* loci (*Rsv1* and *Rsv4)* (Chowda-Reddy et al., 2011). Wen et al. (2013) postulated that different point mutations in both HC-Pro and P3 cistrons were required to overcome the resistance of only *Rsv1* loci but in different backgrounds. For example, the avirulent SMV-N derived mutants (K321E+A947V and K321E+R945G) were able to overcome the resistance of *Rsv1* loci on L800 (*3gG2)* and L943 (*3gG2*) backgrounds. However, an additional point mutation in HC-Pro (T341I) was essential to convert the avirulent SMV –N strain to virulent on PI96983 background carrying the same loci. Suggesting that other host factors in the different backgrounds play role in resistance beside these loci. Interestingly, HC-Pro alone of potato virus Y (PVY) functioned as an avirulence factor toward the corresponding two resistance genes Nc_{spl} and Nc_{tbr} on potato *Solanum sparsipilum* and *Solanum tuberosum,* respectively (Moury et al., 2011). Indeed, HC-Pro showed the virulence

function, as well, by its ability to suppress the host RNA silencing machinery, through binding and sequestering the duplex form of micro RNA (miRNA) (Shiboleth et al., 2007).

Thereby, these functions of HC-Pro, probably, were achieved by its interactions with several host factors. For example, the amino acids substitutions in HC-Pro and P3 between the avirulent and virulent SMV, might change their conformational structures to enable interaction with different host factors, and this is sufficient to convert the avirulent strain to be virulent, and vice versa (Chowda-Reddy et al., 2011; Eggenberger et al., 2008; Wen et al., 2013). In addition, The FRNK box in HC-Pro showed its role in the complex formation with miRNA, where the derived mutant FINK abolished this function (Shiboleth et al., 2007). Moreover, HC-Pro of Zucchini yellow mosaic virus (ZYMV) interacted with Hua Enhancer 1 methyltransferase (*HEN1*) and inhibited its activity (Jamous et al., 2011). *HEN1* is the RNA methyltransferase that responsible for methylation of 2'-OH- or 3' terminal nucleotide of small RNA (sRNA) in Arabidopsis, Drosophila and mouse (Horwich et al., 2007; Kirino & Mourelatos, 2007; Yu et al., 2005). This methylation of sRNA is required for its protection from the host exonucleases, and this happens before its incorporation with the Argonaute proteins (*AGOs*) in the RNA-induced silencing complex (RISC) (Fang & Spector, 2007; Ramachandran & Chen, 2008; Yang et al., 2006; Yu et al., 2005). Interestingly, the FRNK box of HC-Pro played a role in this interaction, as well. The derived FINK mutant of HC-Pro showed weak interaction with *AtHEN1* compared to the wild type FRNK HC-Pro (Jamous et al., 2011). Thus, HC-Pro seems to suppress the host RNA silencing machinery not only through its binding with sRNA but also by its interaction with *HEN1* protein (Jamous et al., 2011; Shiboleth et al., 2007). Suppression of *HEN1* activity exposes the sRNAs to exonucleases resulting in their degradation (Yu et al., 2005).

HC-Pro is known to interact with many host proteins. For instance, HC-Pro of potato virus A (PVA) showed interactions with the RING finger protein (*HIP1*), an important host protein that is involved in protein-protein interactions, DNA repair and recombination, signal transduction, and viral infectivity and virulence (Guo et al., 2003; Saurin et al., 1996). HC-Pro of Lettuce mosaic virus (LMV) binds with the 20S proteasome complex in cauliflower, which may indicate its ability to abolish the protein degradation

function of the host cell in order to protect the viral proteins themselves (Ballut et al., 2005). The same behavior was recorded with HC-Pro of potato virus Y (PVY) by showing its interaction with many proteasome subunits of Arabidopsis plants (Jin et al., 2007). Cheng et al. (2008) postulated the ability of HC-Pro to interact with the chloroplast precursor of ferredoxin-5, that may affect photosynthesis, and in turn development of visualized mosaic symptoms upon viral infection and propagation. HC-Pro interacts with a calmodulin like protein; a host regulator of RNA silencing (Anandalakshmi et al., 2000). HC-Pro from different viruses (PVY, PVA, and tobacco etch virus "TEV") represented interactions with two host proteins, cap-binding translation initiation factor *eIF4E* and its isoform *eIF(iso)4E* that were characterized from potato and tobacco (Ala-Poikela et al., 2011). These two proteins are involved in the translation of host non-capped mRNA, as well as cap-independent translation of potyviral polyprotein (Gallie & Browning, 2001; Gallois et al., 2010). Moreover, HC-Pro interacts with viral genome linked protein (VPg) (Guo et al., 2001; Roudet-Tavert et al., 2007; Yambao et al., 2003), the viral coat protein (CP) (Blanc et al., 1997; Roudet-Tavert et al., 2002), the viral RNA helicase (CI) (Choi et al., 2000; Guo et al., 2001), and the first proteins (P1) (Merits et al., 1999), nuclear inclusion protein a (NIa) (Guo et al., 2001). HC-Pro forms dimers, tetramers, and hexamers inside the infected host cell (Guo et al., 1999; Merits et al., 1999; Urcuqui-Inchima et al., 1999; Yambao et al., 2003).

Because of these characteristics of HC-Pro, and the fact that other additional host factors are required for *R*-mediated resistance in many pathosystems (Banerjee et al., 2001; Jones & Dangl, 2006), I investigated the possible interacting partners of HC-Pro in soybean. I used HC-Pro as bait in the yeast two hybrid system (Y2H) to screen the soybean cDNA library for possible interactions. My results showed that one of the interacting proteins [BRI1-associated receptor kinase 1 (*BAK1*) (*Glyma08G074500*)] is a remarkable protein that recorded many significant roles in regulating plant defenses against a wide range of pathogens. It is a Leucine rich repeat-receptor like kinase (LRR-RLK), which belongs to the small embryogenesis receptor kinase (SERK) family that consists of five members in Arabidopsis. The other protein; Ubiquitin Conjugating Enzyme 2 (*UBC2*) (*Glyma02g40330*), a small protein with 128 amino acid residues, is one of the highly conserved proteins in eukaryotes that play significant roles in immune and abiotic stress

responses. The interaction of the full length proteins with HC-Pro was confirmed *in planta* using biomolecular fluorescence complementation (BiFC) and coimmunoprecipitation (CO-IP) assays. In addition, the results showed the co-localization of HC-Pro with both of BAK1 and UBC2 inside the plant cell. These two interactors are well known for their roles in the plant immune responses.

3.2 RESULTS:

3.2.1 Identification of SMV G5 HC-Pro partners in soybean using yeast two hybrid assay:

The full length HC-Pro cloned from SMV G5 was fused to a repressor *LexA* protein using pEG202 plasmid. A *LexA*-fused HC-Pro was then used as bait in yeast two hybrid (Y2H) assay to screen soybean cDNA library for possible partners that might interact. The library (kindly provided by Dr. Madan Bhattacharyya, Iowa State university) was derived from the cultivar Harosoy (*rsv1*, *Rsv3*, susceptible to SMV-G2, resistant to SMV G5 and SMV-G7), and cloned in pB42AD, expresses soybean cDNAs (1.2 X 10^6 original clones) fused to B42 acidic activator (AD) under control of the *GAL1* promoter. I used a yeast strain (EGY48), in which *lexA* operators are located upstream of a reporter gene leucine 2 (*LEU2*), for expressing both plasmids. This yeast strain lacks histidine (HIS) and tryptophan (TRP) expressing genes, for easy selection of cells transformed with the incorporated plasmids. The *LexA*-fused HC-Pro binds to the *lexA* operators but is unable to activate transcription of the reporter gene in the absence of interaction with the AD-fused partner from soybean cDNA library. The low affinity interaction result in a very low expression of the reporter gene or not at all; the perfectly grown yeast cells indicate strong interactions and very good expression. Thirty-five thousand transformants; those that showed successful transformation of both pEG202-HC-Pro and pB42AD-soybean cDNA library by their growth on the media lacking tryptophane, and histidine (-TRP, and –HIS), were then plated one by one to media lacking the previous two amino acids along with the reporter leucin (-HIS, -TRP, and –LEU). All the transformants failed to grow on this medium except six colonies, assigned as (C195-3, G289-3, H90-6, D169-4, B127-7, and B169-7), showed very good growth suggesting well positive interactions (Fig. 3.1). I, then, extracted and purified the incorporated original cDNA plasmids from these grown yeast

colonies. Only three plasmids were extracted and sequenced successfully. However, the remaining three plasmids showed difficulties in their extraction and sequencing, which may indicate false positive interactions. The sequence result primarily was aligned in the NCBI database. The data showed that clones derived from C195-3 and G289-3 colonies represented 98% identity to *BRI1*-associated receptor kinase 1 (*BAK1*). Whereas, the one that obtained from H90-6 colony showed 100% identity to Ubiquitin Conjugating Enzyme 2 (*UBC2*) (Table 3.1) (Figs. 3.2, 3.3, and 3.4). The full-length coding sequence (CDS), for each of them, was then derived from soybean database in phytozome website, that referred to (*Glyma08G074500)* for *GmBAK1*, and (*Glyma02g40330*) for *GmUBC2*. Using the sequence information, I designed primers for amplifying CDS of each gene, through soybean cDNA derived from Essex cultivar.

Table 3.1: Sequence similarities of HC-Pro interacting proteins from yeast two hybrid (Y2H) analysis:

Colonies Name	Number of times isolated	Gene ontology
C195-3, and G289-3	Two times	BRI1-associated receptor kinase 1 (*BAK1*)
H90-6	One time	Ubiquitin Conjugating Enzyme 2 (*UBC2*)
D169-4	One time	NA
B127-7	One time	NA
B169-7	One time	NA

Colony number indicates screened plate numbers, followed by number of the grown colony on that plates. Number of repeats indicates times the same sequence was isolated. NA: means there were difficulties extracting and/or sequencing the corresponding plasmids.

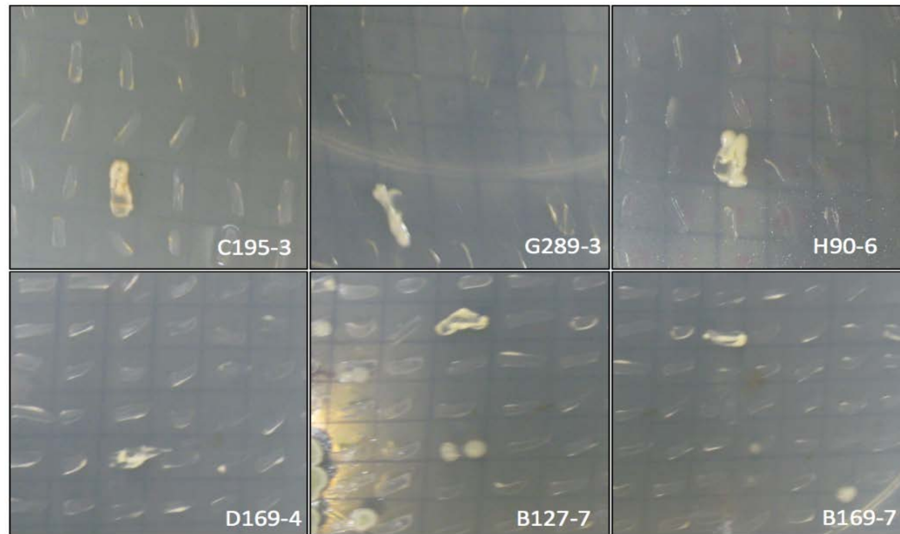

Fig. 3.1: Yeast two hybrid (Y2H) screening using HC-Pro as bait against the soybean cDNA library. Image showing Y2H grown on selective media (SD) –HIS (histidine)/ -LEU (leucine)/ -TRP (tryptophan). A LexA-fused HC-Pro from SMV G5 (cloned in pEG202) was expressed in yeast cells (EGY48), in which lexA operators are located upstream of a reporter gene (LEU2). This yeast cell strains lack HIS and TRP expressing genes as well, for easy selection of the incorporated plasmids. The soybean cDNA library cloned in pB42AD (Clonetech, CA), expresses soybean cDNAs (1.2 X 10^6 original clones) fused to B42 acidic activator (AD) under control of the GAL1 promoter. It is derived from the cultivar Harosoy (rsv1, Rsv3, susceptible to SMV-G2, resistant to SMV G5 and SMV-G7). The LexA-fused HC-Pro binds to the lexA operators but is unable to activate transcription of the reporter gene in the absence of interaction with the AD-fused partner. The low affinity interaction result in a very low expression of the reporter gene or not at all; the perfectly grown yeast cells indicate strong interactions and very well expression. The grown colonies shown in the image represent strong interaction between HC-Pro and a selected gene from soybean cDNA library.

```
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259  GTAATAATATAACTGGGGAAATCCCAGTCGAGCTTGGAAATTTGACAAACTTGGTGAGCTTGGATCTTTACATGAACAAA C195-3.seq
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400  ATTACTGGTCCCATTCCAGACGAATTGGCCAACCTTAACCAACTACAGTCCCTGCGTCTCAACGATAACAGTTTGTTGGG Glyma08G074500.seq

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1199 CTCTAGGAGCTGCTAGGGGGCTTGCTTATTTG-CATGATCATTGTGACCCAAAGATTATTCATCGTGATGTCAAAGCTGC Glyma08G074500.seq

1214 CTAAATAATTTGTTTGGCCTGGGAGG-GACGCCTCGAC                                           C195-3.seq
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1250                                                                                  C195-3.seq
1356 TCATGTTACTACCGCTATATGTGGTACGCAGGGCCATATAGCACCAGAGTATATGACAACTGGAAGGTCTTCAGAGAAGA Glyma08G074500.seq
```

Fig. 3.2: Nucleotide sequence alignment of the extracted pB42AD plasmid from C195-3 grown yeast colony. As shown, the sequenced result is identical to *Glyma08g074500* gene "*Glycine max BRI1*-associated receptor kinase 1 (*GmBAK1*)". The sequence result primarily was aligned in the NCBI website, that showed 98% identity to *GmBAK1*, then its full length sequence was derived from phytozome website version 11.0.7.

```
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1240                                                                                   G289-3.seq
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```

Fig. 3.3: Nucleotide sequence alignment of the extracted pB42AD plasmid from G289-3 grown yeast colony. As shown, the sequenced result is identical to *Glyma08g074500* gene "*Glycine max BRI1*-associated receptor kinase 1 (*GmBAK1*)". The sequence result primarily was aligned in NCBI website, that showed 97% identity to *GmBAK1*, then its full length sequence was derived from phytozome website version 11.0.7.

```
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447                                                                                   Glyma02g40330-1
```

Fig. 3.4: Nucleotide sequence alignment of the extracted pB42AD plasmid from H90-6 grown yeast colony. As shown, the sequenced result is identical to *Glyma02g40330* gene "Glycine max ubiquitin conjugating enzyme 2 (*GmUBC2*)". The sequence result primarily was aligned in NCBI website, that showed 100% identity to *GmUBC2*, then its full length sequence was derived from phytozome website version 11.0.7.

3.2.2 <u>HC-Pro G5 interacts with *GmBAK1 in planta*:</u>

Arabidopsis *BAK1* (*At4g33430*) encodes an LRR II RLK, that share sequence similarity with *Daucus carota* (carrot) somatic embryogenesis receptor kinase (*DcSERK*) (Schmidt et al., 1997; Shiu & Bleecker, 2001b). The deduced amino acid sequence analyses of *AtBAK1* referred to three main domains; I. the extracellular domain contains a predicted signal peptide at its N terminus, followed by four leucine zippers, five leucine rich repeats (LRRs), and a proline-rich region, II. a single transmembrane domain, and III. a serine/threonine protein kinase domain (PK) at the internal side of the cell membrane toward the cytoplasm (Li et al., 2002). Soybean *BAK1* (*GmBAK1a*, *Glyma08G074500*) showed 69.9% similarity when aligned with *AtBAK1*. Likewise, the amino acid analyses of *GmBAK1* represented the same domains; the extracellular domain at its N-terminal which included a predicted signal peptide, and LRRs, followed by single transmembrane domain, and the PK domain at its C-terminal (Fig. 3. 5). To confirm the interaction of HC-Pro with *GmBAK1*, I used biomolecular fluorescence complementation (BiFC) and co-immunoprecipitation (CO-IP) assays. For BiFC, I fused each protein as same as the LRRs and PK domains of *GmBAK1*, to examine which domain was required for this interaction, to N/C terminal half of enhanced yellow fluorescent protein (nEYFP and cEYFP) using pSITE-n/cEYFP vectors, and I transiently co-expressed them inside *Nicotiana benthamiana* leaves using *Agrobacterium tumefaciens*. The result showed green fluorescence patterns when nEYFP-HC-Pro (G5 strain) was co-expressed with cEYFP-*BAK1a* (*Glyma08G074500*) (Fig. 3.6a). Reconstitution of EYFP resulted in fluorescence indicated the interaction. The same result was shown when I co-epressed HC-Pro with PK domain, one isoform of *GmBKA1a* (*GmBAK1c*, *Glyma08G180800*), and with one orthologue in *Arabidopsis* (*AtBAK1*, *AT4G33430.1*). In contrast, I could not detect any fluorescence pattern when I co-expressed HC-Pro G5 with the other domain (LRR), other *GmBAK1* isoform that showed high similarity (*GmBAK1b*, *Glyma05G119600*), and glutathione-S-transferase (*GmGST*). To check if *GmBAK1a* could interact with other HC-Pro from other viruses, I co-expressed it with HC-Pro cloned from bean yellow mosaic virus (BYMV), and HC-Pro cloned from tobacco etch virus (TEV). The result showed no interaction with either of them, even though all proteins were adequately expressed (Fig. 3.6b) in *N. benthamiana*. Likewise, no interaction was detected with HC-Pro cloned from

SMV G7. For co-immunoprecipitation assay (CO-IP), proteins were transiently co-expressed in *N. benthamiana* leaves as MYC-HC-Pro and FLAG-*GmBAK1* derivatives. The total protein extracts from these leaves were, then, subjected to IP using antibodies specific to the tag on *GmBAK1*. Both HC-Pro and *GmBAK1* were detected in this IP (Fig. 3.7), indicating that both were able to interact in *planta*. This results showed further confirmation of the interaction between HC-Pro and *GmBAK1*. HC-Pro of potyviruses was well known to localize in cell periphery, cytoplasm, endoplasmic reticulum (ER) and the microtubule (MT) cytoskeleton when transiently expressed in *N. benthamiana* leaves without viral infection (del Toro et al., 2014). *BAK1* showed localization in the plasma membrane of a stable transgenic Arabidopsis plants expressing GFP-*BAK1* fusion protein (Li et al., 2002). Because Luan et al (2016) from our laboratory showed that P3 of SMV G5 strain interacted with soybean eukaryotic elongation factor 1A (*GmeEF1A*) and enhanced its localization inside the nucleus, I tried to investigate the effect of such interaction on the localization of HC-Pro and *GmBAK1 in planta* when they were co-expressed together. I used pGWB6, modified versions of pSITE vectors, to fuse *GmBAK1a* and *GmBAK1c* proteins, that showed interactions with HC-Pro, with green fluorescence protein (GFP), and pSITE-red fluorescence protein (RFP) for HC-Pro as a recognized different tag via gateway system. Cloned proteins were transiently expressed individually or with its partner as (GFP)- or (RFP) tagged derivatives in *N. benthamiana* leaves. GFP and RFP were excited using 488-nm laser line and 558 nm laser line, giving green and red fluoresce patterns, respectively. In consistence to the recorded results, HC-Pro showed localization in the cell periphery, as well as nucleus, however it localized only in the cell periphery when co-expressed with *GmBAK1a and c*. Both isoforms of *GmBAK1* showed localization in the cell periphery when individually expressed or with HC-Pro (Fig. 3.8, and Fig. 3.9). This result indicated that both proteins are co-localized together in *planta* and *GmBKA1* affected HC-Pro localization by moving it from nucleus toward the cell periphery.

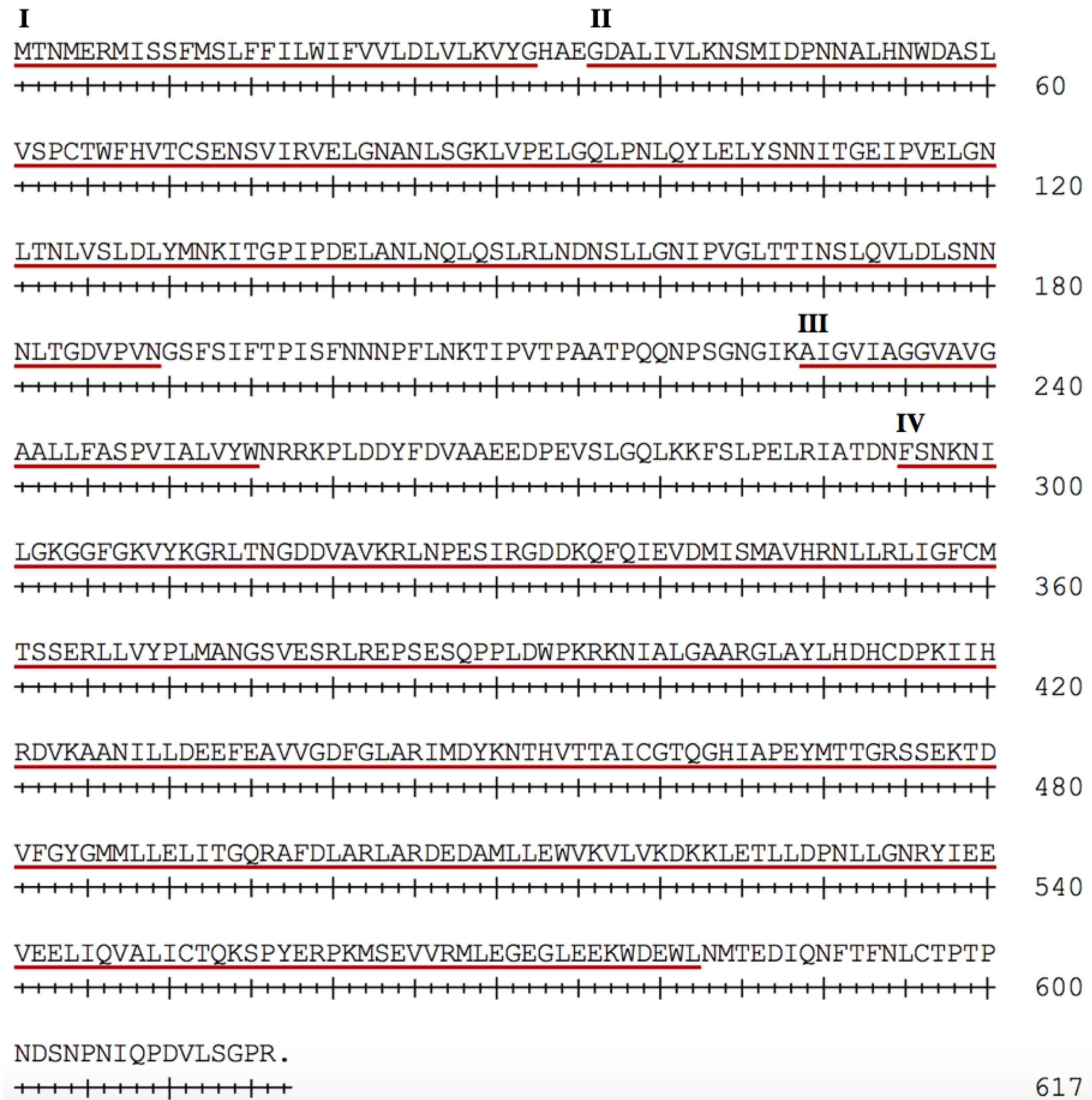
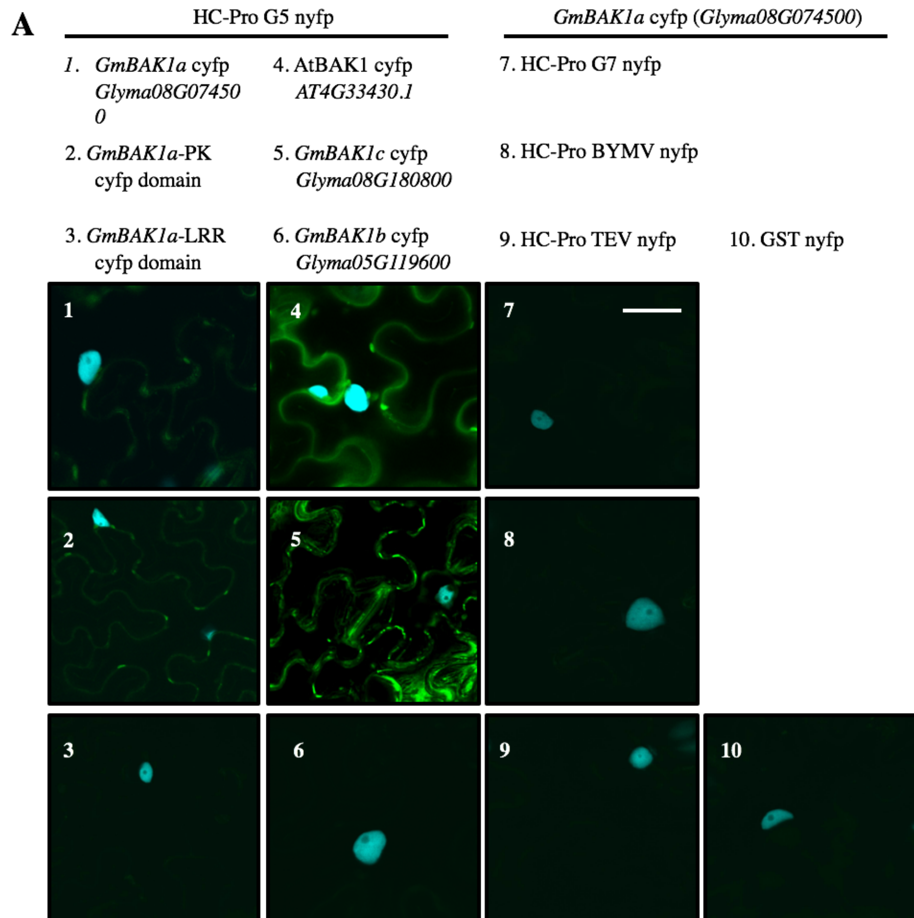


Fig. 3.5: Amino acid sequence analyses of *GmBAK1*. **I.** Referring to the predicted signal peptide, **II.** Leucine rich repeats domain located at the external side of the cell membrane, **III.** The transmembrane domain, and **IV.** Serine/threonine protein kinase domain (PK) at the internal side of the cell membrane. The underline starts at the beginning of each region and stops at its end.



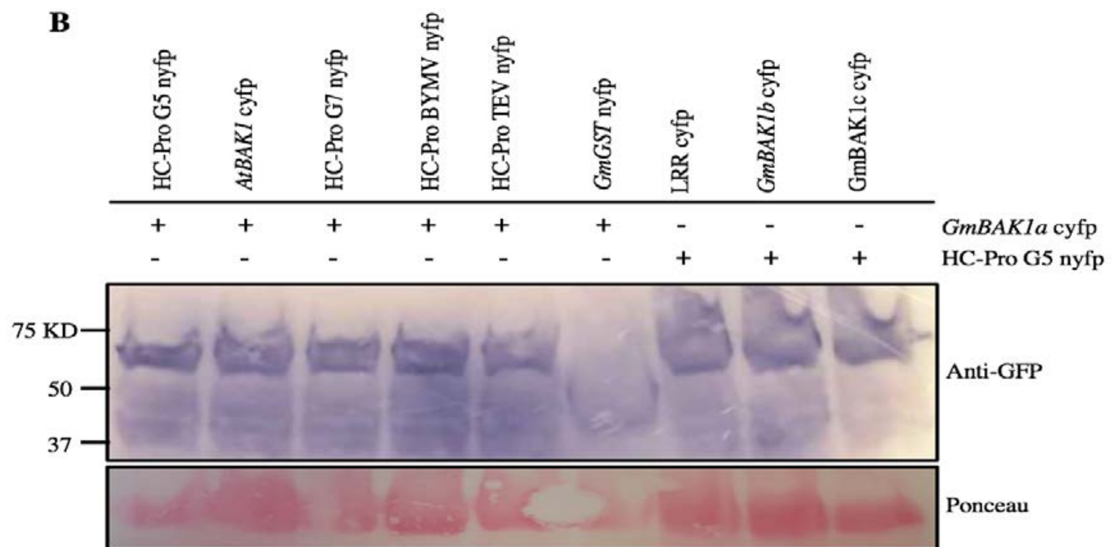


Fig 3.6: GmBAK1 interaction with different HC-Pro proteins from different potyviruses. **A:** Bimolecular fluorescence complementation (BiFC) assay showing the interaction in plant cells. The image showing 40× magnification of micrographs

from CFP-H2B (nuclear localized histone 2B) transgenic *Nicotiana benthamiana* plants co-expressing cYFP-*Glyma08g074500* (*GmBAK1* 8g) with nYFP-HC-Pro G5, nYFP-HC-Pro G7, nYFP-HC-Pro BYMV, nYFP-HC-Pro TEV, nYFP-P3, or nYFP-GST. cYFP-*Glyma08g180800* and cYFP-*Glyma05g119500* are other two isforms of *GmBAK1* were co-expressed with nYFP-HC-Pro G5 as well. The scale bar is 100 μM. This assay was repeated at least three separate times; different infiltrations were done for each interaction using both combinations of c/nYFP fused proteins. **B:** Western blot analysis showing expression of different nEYFP-HC-Pro proteins as well as nEYFP-*GST* for each combination using GFP primary antibody and visualized by the enzyme horseradish peroxidase (HRP) conjugated with a specific secondary antibody.

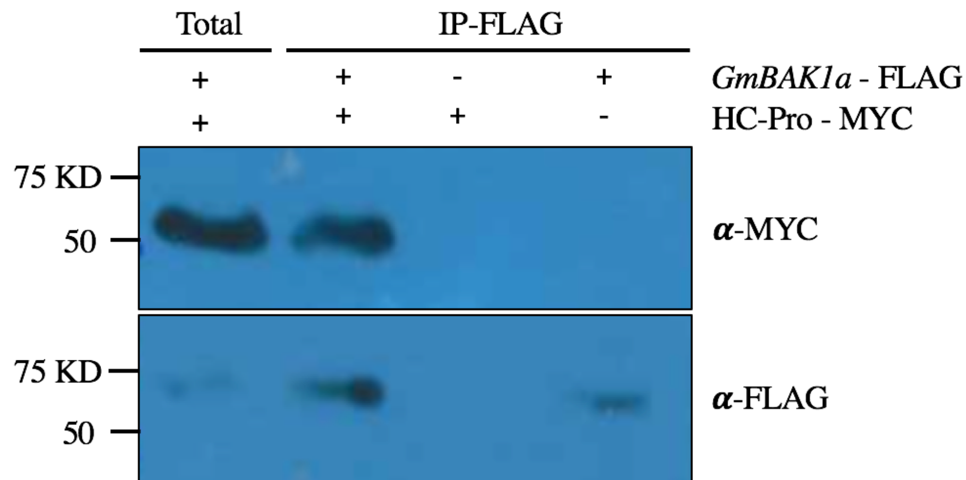


Fig 3.7: Immunoprecipitation assay between *GmBAK1a* and HC-Pro G5. MYC-tagged HC-Pro G5 and FLAG-tagged *GmBAK1a* proteins were coexpressed in *N. benthamiana* via Agro-infiltration. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using enzyme horseradish peroxidase (HRP) conjugated with a specific secondary antibody. This result represents two separate repeats with the same result.

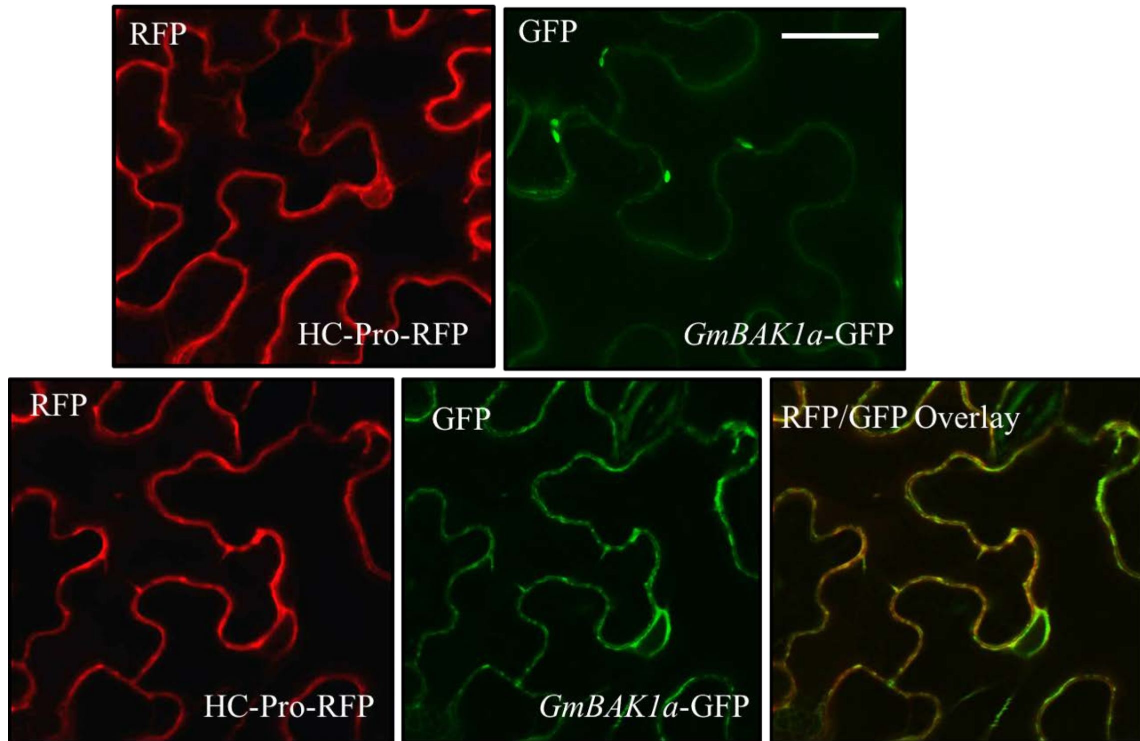


Fig 3.8: Confocal micrographs showing localization and co-localization of *GmBAK1a* and HC-Pro G5 in *planta*. N-terminal tagged RFP-HC-Pro and GFP-*GmBAK1* proteins were expressed individually or co-expressed in *N. benthamiana*. Bottom three panels show co-expressed proteins. The samples were analyzed 48 hr post infiltration. GFP and RFP were excited using 488-nm laser line and 558 nm laser line, giving green and red fluorescence patterns, respectively. This result is representative of three separate repeats with the same result. The scale bar is 100 µM.

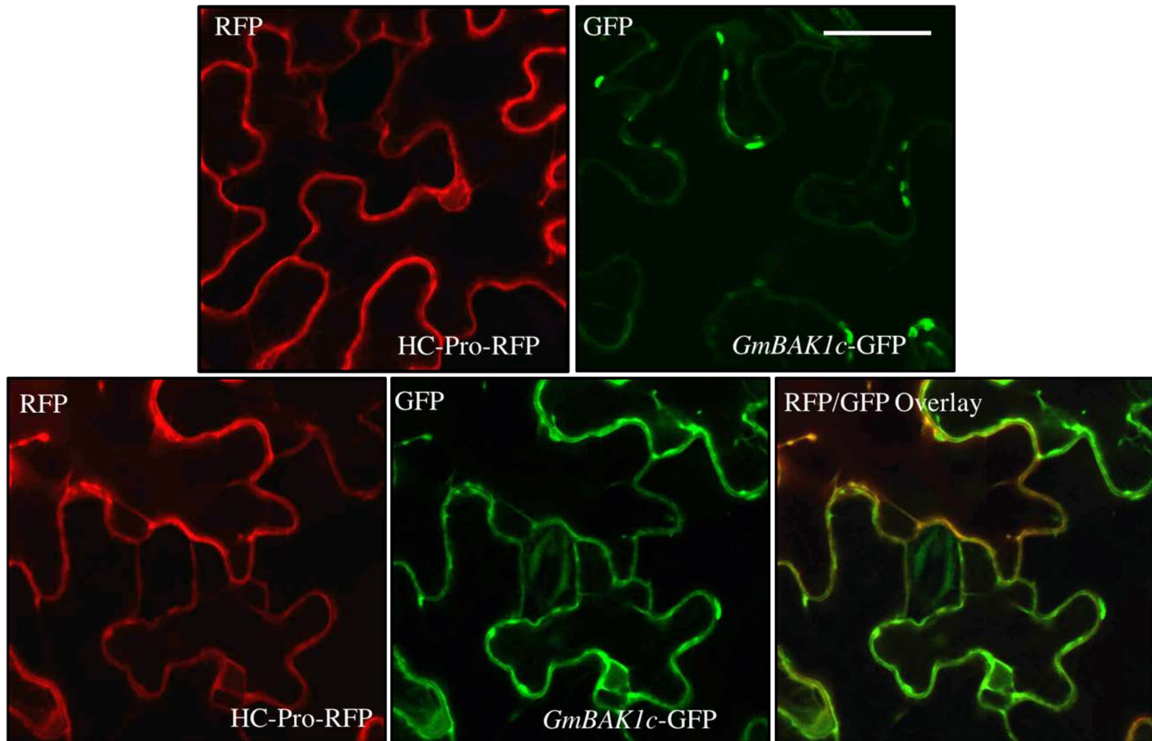


Fig 3.9: Confocal micrographs showing localization and co-localization of *GmBAK1c* and HC-Pro G5 in planta. N-terminal tagged RFP-HC-Pro and GFP-*GmBAK1c* (*Glyma08g180800)* proteins were expressed individually or co-expressed in *N. benthamiana*. The samples were analyzed 48 hr post infiltration. GFP and RFP were excited using 488-nm laser line and 558 nm laser line, giving green and red fluorescence patterns, respectively. This result is representative of three separate repeats with the same result. The scale bar is 100 µM.

3.2.3 HC-Pro G5 interacts with GmUBC2 in *planta*

The interaction between HC-Pro cloned for SMV G5 strain and *GmUBC2* was confirmed by using two different additional methods beside Y2H screening results; BiFC and CO-IP assays (Fig. 3.10, and Fig. 3.11). HC-Pro cloned from BYMV and TEV showed interaction with *GmUBC2* using BiFC assay as well (Fig. 3.10). *GmUBC2* showed localization in both the cytosol and nucleus of a stable transgenic *Arabidopsis* plants expressing EGFP-*GmUBC2* fusion protein (Zhou et al., 2010). By the same token, I tried to investigate the effect of such interaction on the localization of HC-Pro and *GmUBC2 in planta* when they were co-expressed together. In consistent with recorded results, HC-Pro showed localization in the cell periphery, as well as nucleus, in addition no differences in its localization were recognized when co-expressed with *GmUBC2* (Fig. 3.12). likewise, *GmUBC2* showed localization in the cell periphery, and nucleus when individually expressed or with HC-Pro. This results indicated that both proteins are co-localized together *in planta* and they did not affect each other on their recorded localization.

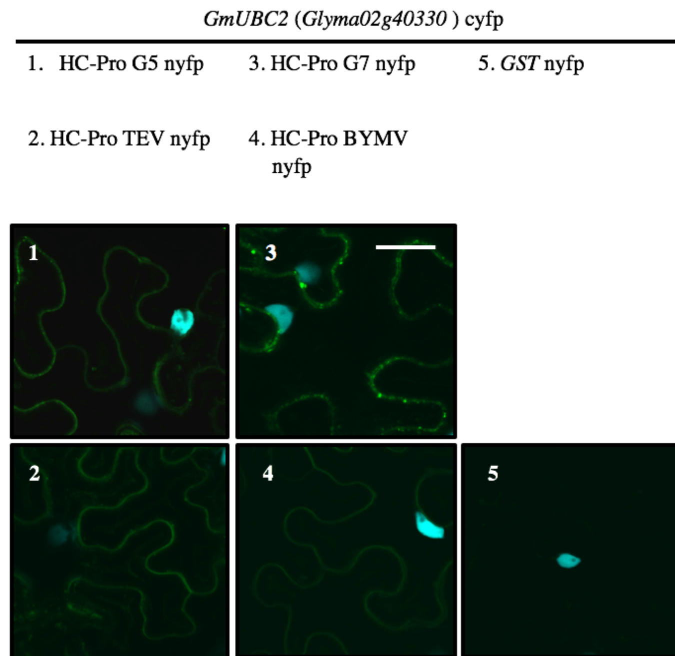


Fig. 3.10: Bimolecular fluorescence complementation (BiFC) assay showing the interaction between *Glyma02g40330* (*GmUBC2*) protein with SMVG5 HC-Pro protein in plant cells. The image showing 40× magnification of micrographs

from CFP-H2B (nuclear localized histone 2B) transgenic *N. benthamiana* plants co-expressing both combination of c/nYFP-fused *GmUBC2*, HC-Pro G5, and glutathione-S-transferase (*GST*) proteins. The scale bar is 100 μM. This assay was repeated at least three separate times; different infiltrations were done for each interaction using both combinations of c/nYFP fused proteins.

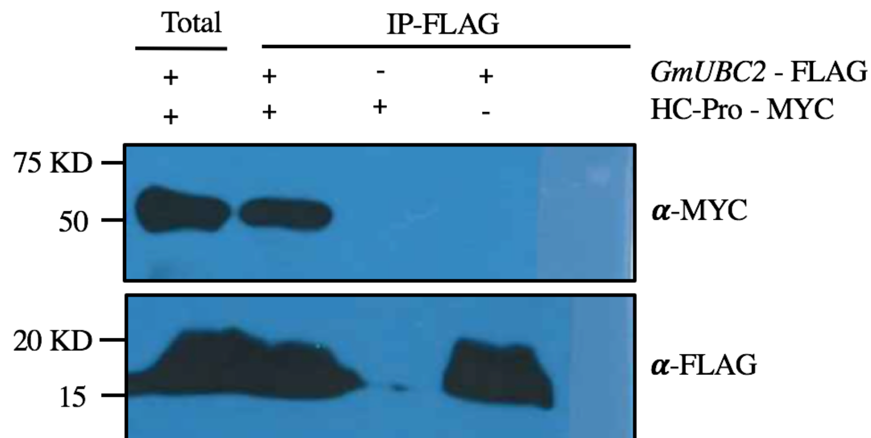


Fig 3.11: Immunoprecipitation assay between *GmUBC2* and HC-Pro G5. MYC-tagged HC-Pro G5 and FLAG-tagged *GmUBC2* proteins were coexpressed in *N. benthamiana*. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using the enzyme horseradish peroxidase (HRP) conjugated with a specific secondary antibody. This result is representative of two separate repeats with the same result.

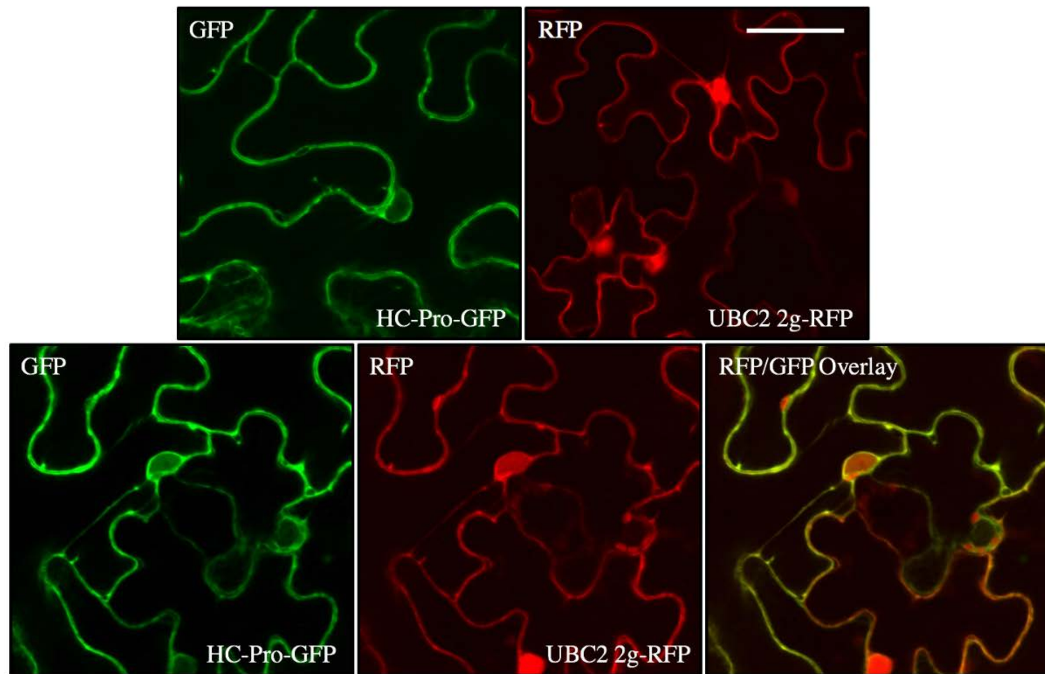


Fig 3.12: Confocal micrographs showing localization and co-localization of *GmUBC2* and HC-Pro G5 in planta. N-terminal tagged GFP-HC-Pro and RFP-*GmUBC2* proteins were expressed individually or co-expressed in *N. benthamiana*. Bottom three panels show co-expressed proteins. The samples were analyzed 48 hr post infiltration. GFP and RFP were excited using 488-nm laser line and 558 nm laser line, giving green and red fluorescence patterns, respectively. This result is representative of three separate repeats with the same result. The scale bar is 100 μM.

3.3 DISCUSSION:

The results in this chapter referred to novel interactors of HC-Pro from soybean (*GmBAK1* and *GmUBC2*). Both proteins are known to be involved in regulating plant defense against a wide range of pathogens (Alcaide-Loridan & Jupin, 2012; Chaparro-Garcia et al., 2011; Chinchilla et al., 2009; Chinchilla et al., 2007; Devoto et al., 2003; Mural et al., 2013; Shirsekar et al., 2010; Trujillo & Shirasu, 2010). The virulence role of HC-Pro in suppressing the host gene silencing machinery, and its role in symptoms development (Llave et al., 2000; Moury et al., 2011), along with the distinct functions of these two interactors could be a reason for these interactions. Although HC-Pro cistron from avirulent SMV strain can be recognized by the *Rsv1* loci and elicit extreme resistance (ER) (Eggenberger et al., 2008; Hajimorad et al., 2008; Hajimorad et al., 2005; Hajimorad et al., 2006; Hajimorad et al., 2011; Wen et al., 2013), the underlying mechanisms is still unknown. One hypothesis is that HC-Pro might interact with different host factor which are guarded by *Rsv1*. In accordance with this hypothesis, HC-Pro might be targeted by these two proteins to promote *Rsv1* loci and trigger resistance. On the other hand, HC-Pro might target these two proteins to suppress their functions in defense, analogous to its role in suppressing host RNA silencing machinery (Llave et al., 2000).

The incorporation of ubiquitin-mediated protein breakdown in plant defense has been established during recent years (Delauré et al., 2008; Devoto et al., 2003; Shirsekar et al., 2010). Ubiquitination refers to a covalently binding of ubiquitin, a highly conserved protein consists of 76 amino acid residues present in all eukaryotes, with a target host unwanted protein (Smalle & Vierstra, 2004; Welchman et al., 2005). Ubiquitination process occurs through sequential steps catalyzed by three enzymes; ubiquitin activating enzyme E1(*UBA1*), ubiquitin conjugating enzyme E2 (*UBC2*), and ubiquitin ligating enzyme E3 (Smalle & Vierstra, 2004; Vierstra, 2003). Both E1 and E3 had promising roles in regulating plant defense against a wide range of plant pathogens, especially E3 showed importance in eliciting *R*-mediated resistance, basal defense, programmed cell death, as well as systemic immunity (Goritschnig et al., 2007; Kim & Delaney, 2002; Shirsekar et al., 2010). However, the exact role of *UBC2* in plant defense has not be elucidated. Indeed, HC-Pro showed interaction with two components from ubiquitin proteasome system (UPS); the system describing the incorporation of ubiquitin and 26S proteasome for degradation

the unwanted or damaged proteins, and interfered with their functions. Guo et al. (2003) showed the interaction of HC-Pro from potato virus Y (PVY) with potato RING finger-type E3 ubiquitin ligases (*HIP1*) (Guo et al., 2003). This interaction was supposed to promote ubiquitination and degradation of HC-Pro as one defensive way from the plant against PVY. Whereas, HC-Pro from other potyviruses, lettuce mosaic virus (LMV), showed interaction with the 20S core of 26S proteasome and interfered with its endonuclease activities but not with its proteolytic activity (Ballut et al., 2005). The endonuclease-associated activity of 20S proteasome core was evolved by many plants to target viral RNAs, such as the RNA genome of tobacco mosaic virus (TMV), as well as some cellular mRNA (Ballut et al., 2003; Gautier-Bert et al., 2003). Taken together, this results suggest that HC-Pro counteracted one possible defense mechanism in plants against viruses by its virulence function and modulating this RNAase activity. Similarly, my result for the first time showed the interaction of HC-Pro G5 with another UPS component, *GmUBC2*, in soybean. Since *UBC2* only catalyzed the conjugation of ubiquitin to the substrate, which is normally performed by E3 ligase enzyme (Shirsekar et al., 2010), it was more likely that HC-Pro might target *UBC2* in order to impair this system, for protecting viral protein and RNA from degradation. Thereby, it could facilitate the viral survival and propagation. This finding suggested further evidence of the virulence function of HC-Pro in interfering with UPS system in plants, that need more investigation to unveil the fact of this role.

CHAPTER 4

SOYBEAN BRI1-ASSOCIATED RECEPTOR KINASE 1 (*BAK1*) AFFECTS *Rsv*-MEDIATED RESISTANCE TO SOYBEAN MOSAIC VIRUS IN SOYBEAN

4.1 INTRODUCTION:

Leucine-rich repeat receptor-like kinases (LRR-RLKs) pathogen recognition receptors (PRRs) include the flagellin-sensing 2 (*FLS2*) receptor (Boller & Felix, 2009). *FLS2* recognize a specific part in bacterial flagelline known as flg22 and trigger effective immune responses, including the activation of mitogen-activated protein kinase (MAPK) cascades (Asai et al., 2002; Gómez-Gómez & Boller, 2000; Zipfel et al., 2004). Brassinosteroid-insensitive 1 (*BRI1*) is another LRR-RLK receptor that is well characterized. *BRI1* is the receptor for steroid phytohormone brassinosteroids (BRs). *BRI1* binds to BRs through its extra cellular LRR-domain and initiates different BR-dependent plant growth and development pathways (He et al., 2000; Kinoshita et al., 2005; Li & Chory, 1997). *BRI1*-associated kinase 1 (*BAK1*)/SERK3; the leucine rich repeat-receptor like kinase (LRR-RLK), belongs to a somatic embryogenesis receptor kinase (SERK) family that consists of five members in Arabidopsis, forms a ligand-inducible complex with *BRI1* and FLS2 resulting in their full activation in order to trigger the corresponding signaling pathways (Chinchilla et al., 2007; Li et al., 2002; Nam & Li, 2002). The *BRI1*-*BAK1* ligand complex leads to sequential reciprocal receptor transphosphorylation, which increases the kinase activity of *BRI1* to promote the downstream signaling cascades (Wang et al., 2008). Likewise, *BAK1*-FLS2 heteromerize after few seconds from *flg22* perception result in their phosphorylation which in turn activate the immune response (Chinchilla et al., 2007; Heese et al., 2007). Heese et al. (2007) showed that the level of ROS and the mitogen-activated protein kinase 6 (MPK6) were increased upon perception of flg22. In contrast, they recorded a significant reduction in their level in *bak1* mutant Arabidopsis and *BAK1*-silenced *N. benthamiana* plants (Heese et al., 2007). *BAK1* also showed a functional role in responses triggered by the bacterial PAMPs 18-aa peptide derived from the translational elongation factor Tu (*elf18*), the oomycetes elicitor *INF1*, and the bacterial cold-shock proteins (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008). Thereby, *BAK1* was proposed to serve many PRRs and thus it is an important partner for many PAMP-elicited immune responses (Chinchilla et al., 2009). Arabidopsis and *N.*

benthamiana plants that are null in *BAK1* function showed enhanced susceptibility to bacterial, fungal and oomycete pathogens infections than plants with normal *BAK1* function (Heese et al., 2007).

Many researchers postulated that *BAK1* positively regulates and act as a decision node between different pathways. On one hand, it positively and negatively regulates many PRR dependent responses in case of innate immunity (Chaparro-Garcia et al., 2011; Chinchilla et al., 2007; Gao et al., 2009; Heese et al., 2007; Shan et al., 2008). On the other hand, it positively regulates the hormone brassinosteroid signaling pathway by interacting with the brassinosteroid (BR) receptor, the LRR-RLK *BRI1* (Chaparro-Garcia et al., 2011; Nam & Li, 2002). Noteworthy, in some cases, *BAK1* showed its preference to regulate innate immunity likely more than BR signaling pathway. For example, Albercht et al. (2012) suggested that *bak1-5* mutant is not impaired in BR signaling pathway, but PAMP associated response was remarkably reduced (Albrecht et al., 2012). On the other word, *BAK1* showed its associations in the negative regulation of some immune responses. For example, in Arabidopsis, the LRR-receptor kinase *BAK1*-interacting receptor-like kinase 1 (*BIR1*), which is a pseudokinase, was found to dynamically associate with *BAK1* and negatively regulate *BAK1*–FLS2 complex formation. Absence of *BIR1* was more likely to facilitate complex formation between FLS2 and *BAK1* (Blaum et al., 2014; Gao et al., 2009; Halter et al., 2014). *bir1-1* mutants showed a constitutive cell death phenotype resembled what associates with *R* protein activation phenotype. In addition, this phenotype was found to be partially dependent on phytoalexin deficient4 (*PAD4*) and enhanced disease susceptibility1 (*EDS1*) proteins (Gao et al., 2009). *PAD4* and *ESD1* are proteins required for the activation responses governed by many TIR-NB-LRR types of *R*-Proteins, as well as a regulator for many basal meditated defenses (Falk et al., 1999; Jirage et al., 1999; Wang et al., 2014; Wiermer et al., 2005). Based on these results they concluded that knock out of *BIR1* is responsible for the activation of resistance pathways that activated by other *R*-proteins. In addition, this role of *BIR1* and its association with *BAK1* gave a clue that both are working together to negatively regulate cell death and defense responses. On the other words, *BAK1* may positively regulate the basal defense by its association with *FLS2* and other RLKs-PRRs on one side, and negatively regulate other *R* mediated defenses as well as basal defenses by its association with *BIR1* on the other side. They build a

conclusion from their data that *BAK1* and *BIR1* complex might be a guardee of one or more *R* proteins, and losing of *BAK1* or *BIR1* functions will trigger the activation of them.

BAK1 not only showed an important role in immune response against bacterial pathogen (*Pseudomonas syringae pv. tomato,* DC3000) (Chinchilla et al., 2007), but also it played a significant role against fungal infection. Arabidopsis plants had a defective allele *bak1-5* displayed enhanced accumulation of *Plectosphaerella cucumerina* BMM (PcBMM) fungi comparing to the wild type plant (Col-0) (Jordá et al., 2016). In addition, *BAK1* positively regulated the immune responses against a wide range of plant viruses (Kørner et al., 2013).

Here, I tested the role of *BAK1* in soybean defense to SMV, I found that *BAK1* is an important partner in immune defense against bacterial infection in soybean. In addition, I determined that *BAK1* regulates the *Rsv1*-SMV mediated resistance but not the basal defense against SMV. The *GmBAK1* silenced soybean plants that contain the *Rsv1* loci showed susceptibility to the infection by SMV G5, which is normally incompatible on *Rsv1* plants. I also identified a possible role for brassinosteroid (BR) in SMV defense. BR or propiconazole (PPZ), a specific biosynthesis inhibitor (Hartwig et al., 2012) treated soybean plants showed normal susceptibility to the compatible infection of SMV in susceptible background. However, only PPZ treated plants that carrying *Rsv1* loci exhibited resistance to SMV G7. Interestingly, I identified the phosphorylation of HC-Pro in the presence of *GmBAK1* in planta. Moreover, I detected the importance of a single amino acid residue T341 (Wen et al., 2013), which has significant role in SMV avirulence, in the phosphorylation of HC-Pro. My data for the first time, highlighted the importance of *BAK1* in *R*-mediated resistance against plant virus infection. This role is achieved and initiated by the phosphorylation of a multifunction effector protein that plays important role in virus survival and virulence. This work gave a new understanding of the robust *Rsv1*-mediated resistance, that needs more research for investigating the downstream signaling of this mysterious relationship.

4.2 **RESULTS:**

4.2.1 **Regulation of *BAK1* genes expression in soybean upon SMV infection:**

To test if *GmBAK1* is associated with SMV infection, I first searched the soybean genome for sequences resembled to *GmBAK1a* (*Glyma08G074500*), the one that was identified from Y2H screen. I identified five isoforms encoding putative *GmBAK1*, designated *GmBAK1b* (*Glyma05G119600*), *GmBAK1c* (*Glyma08G180800*), *GmBAK1d* (*Glyma15G051600*), *GmBAK1e* (*Glyma05G119500*), and *GmBAK1f* (*Glyma02G076100*). Amino acid sequence alignment showed that *GmBAK1b* had highest percentage similarity to *GmBAK1a*, which is 87% similar (Fig. 4.1A and Fig. 4.2). *GmBAK1c* and *GmBAK1d* were 96.9% identical to each of them. Whereas, these two isoforms showed low similarity to *GmBAK1a*, as well as *GmBAK1e* and *GmBAK1f* (Fig. 4.1A). I, then, examined the mRNA levels of all isoforms in both Essex (susceptible) and Essex-*Rsv1* (resistance) cultivars upon infection of G5 strain from SMV (virulent on Essex and avirulent on Essex-Rsv1). Samples were collected from local infected leaves at 0 and 2 days post infection (dpi), then used for qRT-PCR analysis (Fig. 4.3). The analysis showed three isoforms (*GmBAK1c*, *d*, and *e*) were significantly induced in plants carrying the *Rsv1* loci, but only *GmBAK1d* showed significant induction in susceptible plants. In contrast, expression of *GmBAK1f* and b was significantly reduced in *Rsv1* infected plants, and a significant reduction was recorded only to the last one in Essex. Unexpectedly, *GmBAK1a* decreased non-significantly in both cultivars after SMV G5 infection. My data showed regulation of *BAK1* genes expression in soybean upon SMV infection, which gave the first indication of a possible involvement of *BAK1* in *Rsv1*-SMV mediated resistance.

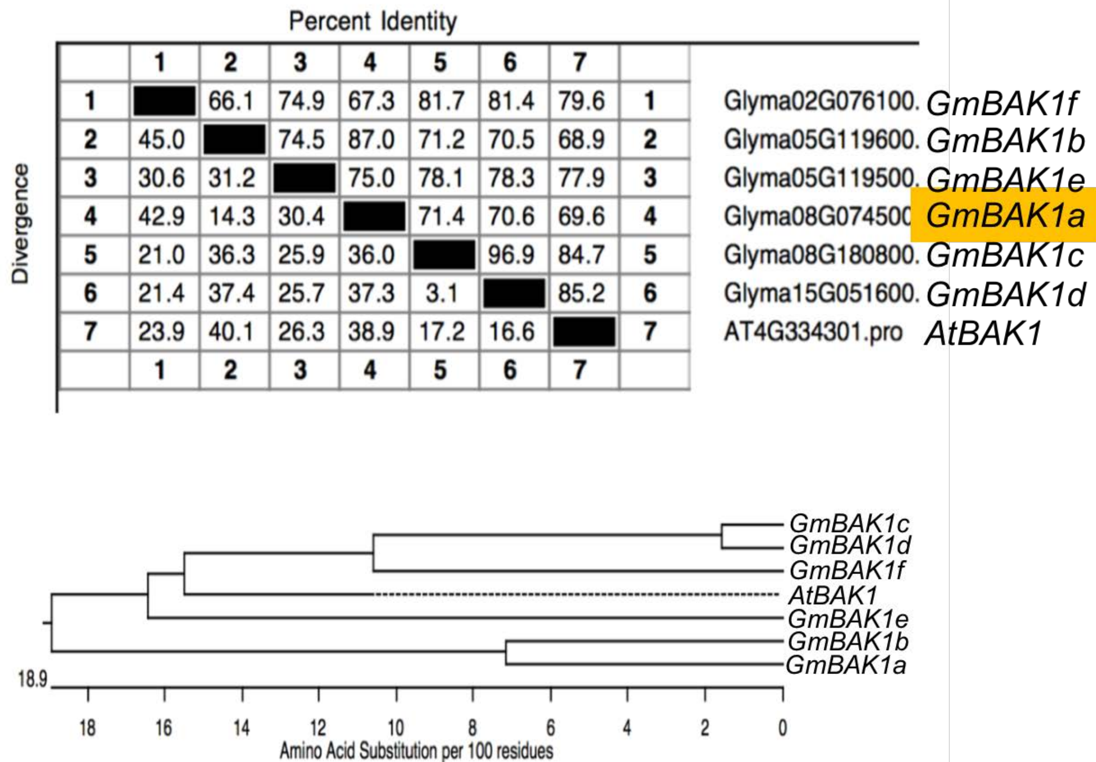


Fig. 4.1: Sequence distance between the different *GmBAK1* isoforms using Megalign program in the DNASTAR package. A; the percentage identity and the divergence between the different isoforms. B; the phylogenetic tree between these isoforms. The lower amino acid substitution per 100 residues, the higher similarity between the corresponding isoforms. The colored isoform *GmBAK1a* is the one that was identified by yeast two hybrid screening.

```
1   ---MERE---LWGSVFI-YWVLLARPLWLVSANMEGDALHSLRTNLQDPNNVLQSWDPTLVNPCTWFHVTCNNDNSVIRV Glyma02G076100.
1   MTNMQRVVSSFMSLFLILWMFVVLDLVIKVSGNAEGDALMALKNNMIDPSDALRSWDATLVHPCTWLHVFCNSENSVTRV Glyma05G119600.
1   MANMERVISDFMSWFPL-WAILVLDLLLKVSGNTEGDALTALKNSVSDPNNVLQSWDSTLVDPCTWFHVTCNNENSVTRV Glyma05G119500.
1   MTNMERMISSFMSLFFILWIFVVLDLVLKVYGHAEGDALIVLKNSMIDPNNALHNWDASLVSPCTWFHVTCS-ENSVIRV Glyma08G074500.
1   ---MDRVTSSFMGSFFV-WAILVLDLVLKASGNQEGDALNALKSNLQDPNNVLQSWDATLVNPCTWFHVTCNSDNSVTRV Glyma08G180800.
1   ---MERVTSSFMGSFFF-WAILVLDLVLKASGNQEGDALNALKSNLQDPNNVLQSWDATLVNPCTWFHVTCNSDNSVTRV Glyma15G051600.
1   ---MERR---LMIPCFF-WLILVLDLVLRVSGNAEGDALSALKNSLADPNKVLQSWDATLVTPCTWFHVTCNSDNSVTRV AT4G334301.pro

74  DLGNAVLSGQLVPQLGQLKNLQYLELYSNNISGPIPNDLGNLTNLVSLDLYLNRFSGPIPESLGKLSKLRFLRLNNNSLT Glyma02G076100.
81  DLGNENLSGQLVPQLGQLPNLEYLELYSNNITGEIPVELGSLTNLVSLDLYLNKITGPIPDGLANLKKLKSLRLNNNSLS Glyma05G119600.
80  DLGNANLSGQLVPQLGQLPNLQYLELYSNNITGKIPDELGSLRNLVSLDLYSNNITGPISDNLANLKKLRFLRLNNNSLS Glyma05G119500.
80  ELGNANLSGKLVPELGQLPNLQYLELYSNNITGEIPVELGNLTNLVSLDLYMNKITGPIPDELANLNQLQSLRLNDNSLL Glyma08G074500.
77  DLGNADLSGQLVPELGQLTNLQYLELYSNNITGKIPEELGNLTNLVSLDLYLNTLDGPIPTTLGNLAKLRFLRLNNNSLT Glyma08G180800.
77  DLGNADLSGQLVSQLGQLTNLQYLELYSNKITGKIPDELGNLTNLVSLDLYLNTLNGPIPTTLGKLAKLRFLRLNNNSLT Glyma15G051600.
74  DLGNANLSGQLVMQLGQLPNLQYLELYSNNITGTIPEQLGNLTELVSLDLYLNNLSGPIPSTLGRLKKLRFLRLNNNSLS AT4G334301.pro

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161 GNIPVGLTTINSLQVLDLANNNLTGNVPVYGSFSIFTPISFKNNPFLYQTTP------------VTPAATPQQNPSGNGI Glyma05G119600.
160 GKIPVRLTTVDSLQVLDLSNNNLTGDIPINGSFSSFTPISFRNNPSLNNTLV------------PPPAVTPPQSSSGNGN Glyma05G119500.
160 GNIPVGLTTINSLQVLDLSNNNLTGDVPVNGSFSIFTPISFNNNPFLNKTIP------------VTPAATPQQNPSGNGI Glyma08G074500.
157 GGIPMSLTNVSSLQVLDLSNNKLKGEVPVNGSFSLFTPISYQNNPDLIQPKN------------TPSPVSPTPPAASSGN Glyma08G180800.
157 GGIPISLTNVSSLQVLDLSNNHLKGEIPVNGSFSLFTPISYQNNLGLIQPKY------------TPSPVSPTPPPASSGN Glyma15G051600.
154 GEIPRSLTAVLTLQVLDLSNNPLTGDIPVNGSFSLFTPISFANTKLTPLPAS------------PPPPISPTPPSPAGSN AT4G334301.pro

234 SATGAIAGGVAAGAALLFAAPAIVFAWWRRRKPQEFFFDVPAEEDPEVHLGQLKRFSLRELQVATDTFSNKNILGRGGFG Glyma02G076100.
229 TAIGVIAGGVAVGAALLFASPVIAIVYWNRRKPPDDYFDVAAEEDPEVSFGQLKKFSLPELRIATDNFSNNNILGKGGYG Glyma05G119600.
228 RAIVIIAGGVAVGAALLFAAPVIVLVYWKRRKPRDFFFDVAAEEDPEVHLGQLKRFSLRELQVATDTFNNKNILGKGGFG Glyma05G119500.
228 KAIGVIAGGVAVGAALLFASPVIALVYWNRRKPLDDYFDVAAEEDPEVSLGQLKKFSLPELRIATDNFSNKNILGKGGFG Glyma08G074500.
225 SNTGAIAGGVAAGAALLFAAPAIALAYWRRRKPQDHFFDVPAEEDPEVHLGQLKRFSLRELQVATDNFSNKHILGRGGFG Glyma08G180800.
225 SNTGAIAGGVAAGAALLFAAPAIALAYWRRRKPQDHFFDVPAEEDPEVHLGQLKRFSLRELQVATDNFSNKHILGRGGFG Glyma15G051600.
222 RITGAIAGGVAAGAALLFAVPAIALAWWRRKKPQDHFFDVPAEEDPEVHLGQLKRFSLRELQVASDNFSNKNILGRGGFG AT4G334301.pro

314 KVYKGRLTDGSLVAVKRLKEERTPGGELQFQTEVEMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPAH Glyma02G076100.
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302 KVYKGRLADGTLVAVKRLKEERTQGGELQFQTEVEMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPES AT4G334301.pro

394 QQPLDWPTRKRIALGSARGLSYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGTIGHIAP Glyma02G076100.
389 KPPLEWPMRKRIALGAARGLAYLHDHCDPKIIHRDVKAANILLDDEFEAVVGDFGLARIMDYQNTHVTTAVCGTHGHIAP Glyma05G119600.
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385 QPPLGWPERKRIALGSARGLAYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGTIGHIAP Glyma08G180800.
385 QPPLGWPERKRIALGSARGLAYLHDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGTIGHIAP Glyma15G051600.
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474 EYLSTGKSSEKTDVFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKEKKLEMLVDPDLHSNYIDAEVEQLIQV Glyma02G076100.
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468 EYMTTGRSSEKTDVFGYGMMLLELITGQRAFDLARLARDEDAMLLEWVKVLVKDKKLETLLDPNLLGNRYIEEVEELIQV Glyma08G074500.
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465 EYLSTGKSSEKTDVFGYGVMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKDRKLETLVDADLQGSYNDEEVEQLIQV Glyma15G051600.
462 EYLSTGKSSEKTDVFGYGVMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKEKKLEALVDVDLQGNYKDEEVEQLIQV AT4G334301.pro
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Fig. 4.2: Amino acids sequence alignment of the different *GmBAK1* isoforms proteins along with *AtBAK1* 4g (*Arabidopsis thaliana BAK1* 4g) using Clustal W in the Megalign program in the DNASTAR package.

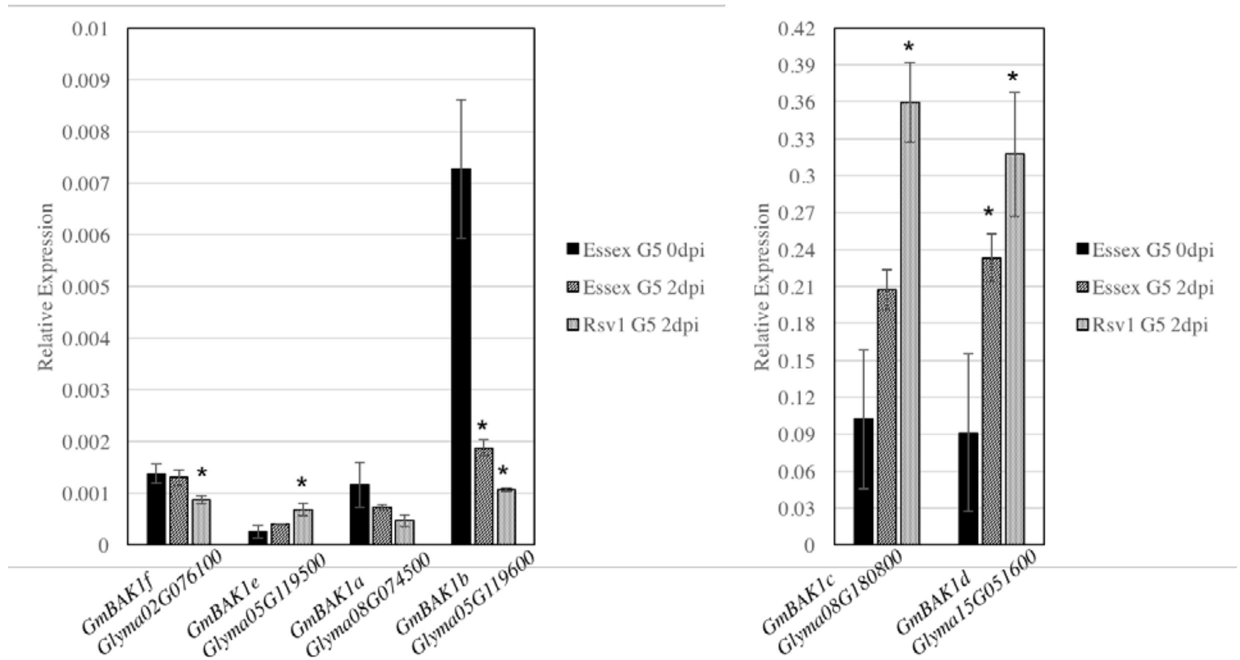

Fig. 4.3: Relative mRNA levels of the different *GmBAK1* isoforms genes in SMV G5 infected soybean plants (Essex, and Essex-*Rsv1*) at 0 and 2 days' post infection, as determined by qRT-PCR. Asterisks denote significant difference from the corresponding control (Essex G5, 0 dpi), t test, P value<0.0001.

4.2.2 Knocking down of *GmBAK1* expression in soybean:

At the moment, no data were provided about the role of *BAK1* in *R*-mediated resistance against viruses. To test if resistance derived from *Rsv1* loci against SMV required *GmBAK1*, I knocked down its expression in soybean (cvs, Essex) using the bean pod mottle virus (BPMV)-based VIGS (virus-induced gene silencing) vector (Kachroo & Ghabrial, 2012; Zhang & Ghabrial, 2006b). To generate the *GmBAK1* silencing vector, I selected a region (224 bp, A1591-1815C) in protein kinase domain of *GmBAK1a* that shared high percentage identity with the different isoforms. The goal was to knockdown expression of all *GmBAK1* isoforms simultaneously (Fig. 4.4). Plants were infected with the RNA (in vitro transcription) of the vector along with the RNA1 of the BPMV virus. Control plants were inoculated with buffer (M, mock) or empty BPMV vector (V, control). Essex plants infected with the vector were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) and qRT-PCR analysis to test *GmBAK1* transcript level. RT-PCR

data showed no transcript of the full length *GmBAK1a* gene in *GmBAK1*-knockdown plants (*BAK1* sil) compared to plants infected with BPMV control vector (contains a nonspecific sequence, V) and mock infected plants (M) (Fig. 4.5A). Consistent with this result, qRT-PCR analysis showed only a significant reduction in the mRNA level of *GmBAK1a* and *GmBAK1b* isoforms, those that shared the highest identity, in *BAK1* sil plants compared to V and M plants (Fig. 4.5B). However, no significant reduction was recorded in the mRNA levels of the rest of the isoforms.

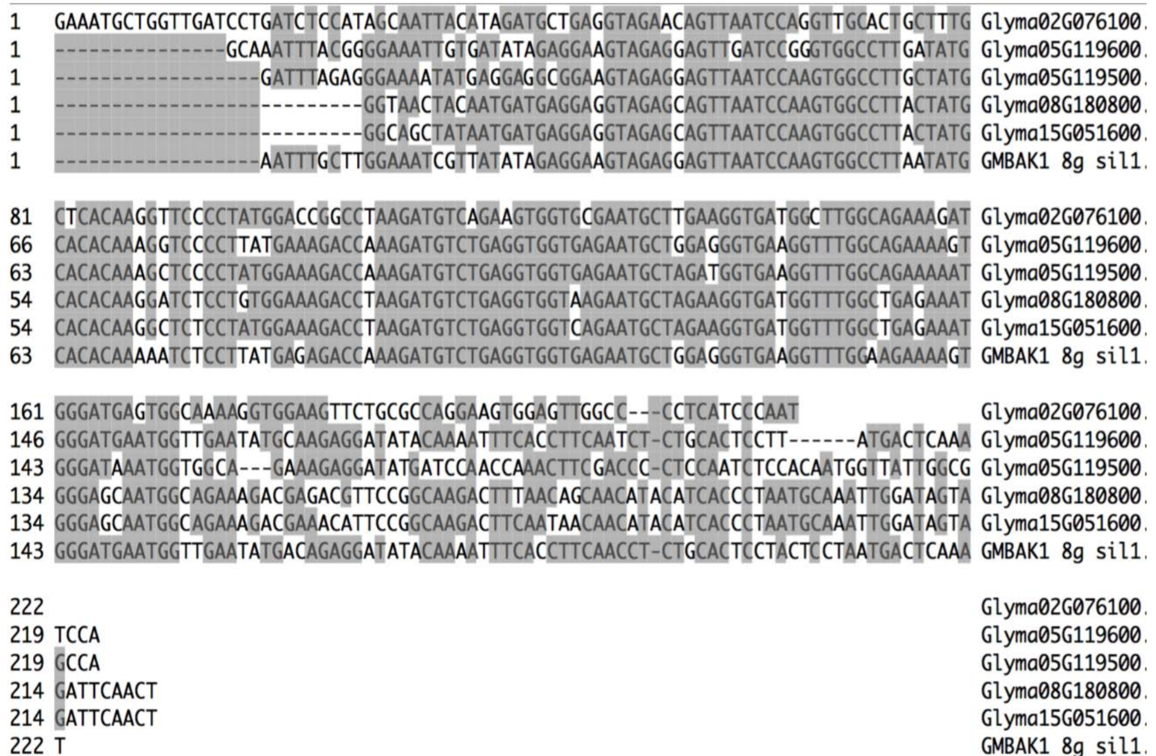

Fig. 4.4: Nucleotides sequence alignment of the selected silencing insert at protein kinase domain of *GmBAK1a* along with the same area in other different *GmBAK1* isoforms genes using Clustal W in the Megalign program in the DNASTAR package.

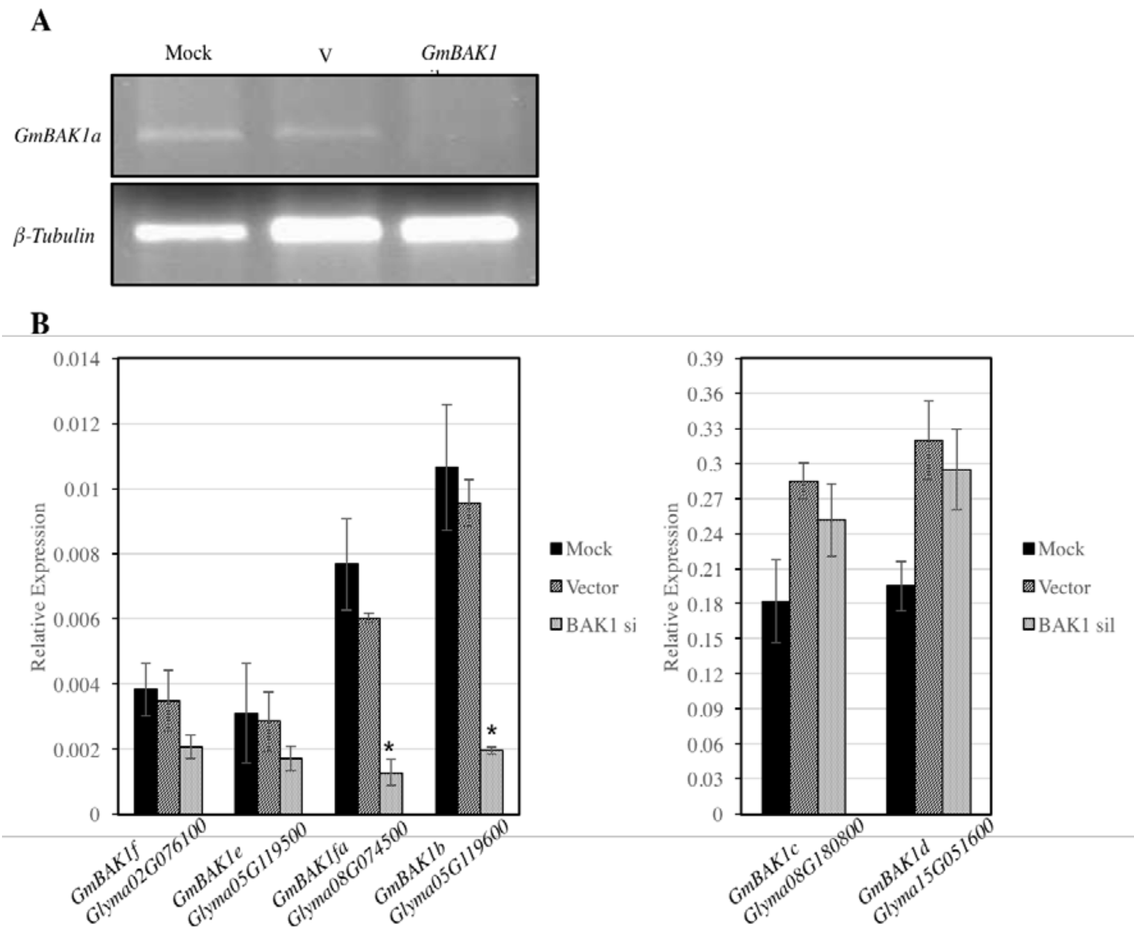

Fig. 4.5: Knocking down of *GmBAK1* expression in soybean. **A,** *GmBAK1a* expression in mock, vector, and BAK1a silenced plants. Reverse-transcription polymerase chain reaction (RT-PCR) analysis was carried out using cDNA prepared from total RNA extracted from leaves. Full length *GmBAK1a* was amplified by using its specific primers and *β*–tubulin levels were used as internal control for cDNA amounts. **B,** Relative mRNA levels of the different *GmBAK1* isoforms genes in silenced soybean plants (Essex). The silencing was done by viral induced gene silencing (VIGS) mechanism, using BPMV as a vector to deliver the silencing insert inside the plant cells. The data were recorded after 2 days' post BPMV infection, and determined by qRT-PCR. Asterisks denote significant difference from the corresponding control (Mock, Essex plants without any infection). t-test was used to determine statistical significance, P value<0.0001. Results represent 2 repeated times of the same experiment.

4.2.3 <u>Knocking down of *GmBAK1a* expression affects basal defense in soybean:</u>

The findings that *BAK1* initiates the innate immunity in plant by developing a complex with a number of RLK or receptor like proteins (RLP) (Schulze et al., 2010; Schwessinger et al., 2011), and its role in PTI in Arabidopsis (Chinchilla et al., 2007), prompted me to investigate first its role in the basal defense in soybean. For this, I knocked down its expression in soybean (cvs, Merit) using the bean pod mottle virus (BPMV)-based VIGS (virus-induced gene silencing) vector (Kachroo & Ghabrial, 2012; Zhang & Ghabrial, 2006b). The silenced *BAK1* plants showed significant susceptibility to *Pseudomonas syringae pv. glycinea* virulent (*Psg. vir*) by accumulating more than one-fold compared to V plants, as expected (Fig. 4.6A). This result in consistent to data shown in Chinchilla et al. (2007); (Heese et al., 2007; Schulze et al., 2010) in *Arabidopsis* and *N. benthamiana*, gave an evidence about *GmBAK1* role in the basal defense in soybean. *BAK1* is also known to regulate PTI against three different RNA viruses, namely oilseed rape mosaic virus (ORMV), tobacco mosaic virus (TMV), and tobacco crinkle virus (TCV) were mainly governed by *BAK1*-PRR signaling system. They showed that Arabidopsis plants mutated in genes that encode *BAK1* represented more susceptibility to these three viruses (Kørner et al., 2013). To test that role of *GmBAK1* in soybean, V and *GmBAK1* sil plants (cvs. Essex) were infected with tobacco ring spot virus (TRSV), compatible in Essex soybean plants. The protein gel blot analysis of total protein extracts showed that silenced plants accumulated less TRSV in comparison to V treated plants, in both local inoculated and systemic un-inoculated leaves, (Fig. 4.6B). This result proposed possibly contrasting functions of *GmBAK1* in the regulation of the basal defense against different pathogens in soybean.

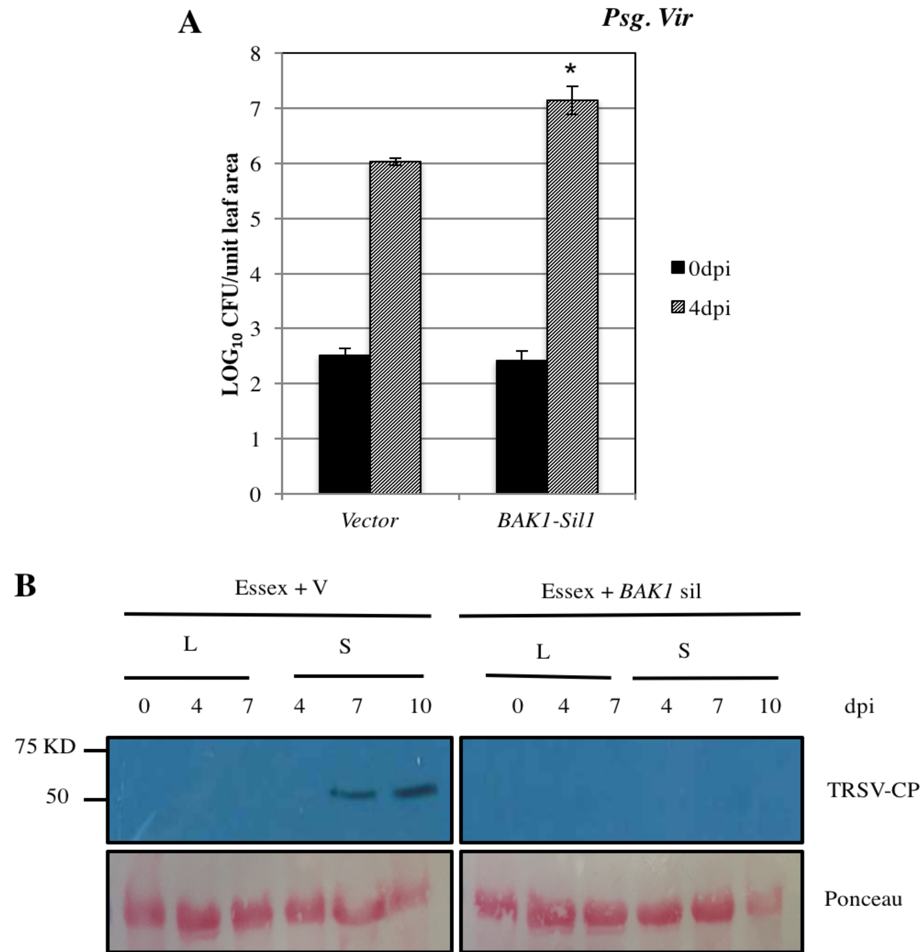

Fig. 4.6: *GmBAK1* affects basal defense in soybean. **A,** Bacterial counts in soybean (*Glycine max,* cv Merit) plants inoculated with empty BPMV vector (V), or those silenced for *GmBAK1*. Plants were infiltrated with *Psg Vir* (10^5 cfu/ml). Log_{10} values of colony-forming units (cfu) per unit leaf area from infected leaves at 0 (black bars) or 4 d post inoculation (gray/ black bars) are presented. Error bars, ± SD (n= 4). t-test was used to determine statistical significance. Asterisks denote significant difference between *BAK1* sil and V infected plants ($P < 0.0001$). **B,** *BAK1* negatively affect soybean susceptibility to tobacco ring spot virus (TRSV). The image showing western blot analysis of protein extracts from TRSV infected Essex plants. V, represents the plants previously infected with the empty BPMV vector and *BAK1* sil for those infected by the cloned BPMV vector with the silencing insert of *GmBAK1*. Lane numbers indicate days post SMV infection (dpi) from local inoculated leaves (L) and un-

inoculated systemic leaves (S). TRSV CP were visualized using coat protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. Results represent 2 repeated times of the same experiment.

4.2.4 Knocking down of *GmBAK1a* expression results in breakdown of *Rsv* resistance in soybean:

BAK1 showed a critical role in immune responses against a wide range of plant viruses (Kørner et al., 2013). However, its role in *R*-mediated resistance is still under investigation, for this I first tested its role in *R*-mediated resistance against bacteria. I knocked down the expression of *GmBAK1a* using bean pod mottle virus (BPMV)-based VIGS (virus-induced gene silencing) vector (Kachroo & Ghabrial, 2012; Zhang & Ghabrial, 2006b) in soybean plant (*cvs*, Merit), carrying *Rpg1-b* gene which is *R* protein that belonging to the coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR) class and provide specificity against *Pseudomonas syringae pv. glycinea* avirulent protein B (*Psg. avrB*) (Ashfield et al., 2003; Ashfield et al., 2004; Bisgrove et al., 1994). My result showed that Gm*BAK1a* silenced plants enhanced significant susceptibility to *Psg. avrB* compared to V control (Fig. 4.12). To address the functional role of *GmBAK1* in soybean *Rsv1*-mediated resistance against SMV. I knocked down the expression of *GmBAK1a* in both susceptible and resistance cultivars. Both V and *GmBAK1* silenced plants (Essex, Essex-*Rsv1*, Harosoy-*Rsv3*, and V94-5251-*Rsv4*) were infected with SMV G5 (virulent on Essex, avirulent on *Rsv1, Rsv3,* and *Rsv4*). Samples were collected from local infected and systemic leaves at 0, 4, 7 and 4,7,10, respectively. The protein gel blot analysis of total protein extracts showed no effect on the virus accumulation in the Essex susceptible plants when *GmBAK1a* was knocked down in comparison with V infected ones (Fig. 4.12B). Interestingly, *GmBAK1* silenced plants carrying *Rsv1* loci exhibited abnormal susceptibility to SMV G5, by accumulating the virus in both local infected and systemic leaves in comparison to V infected plants (Fig. 4.12A). Whereas plants carrying *Rsv3*, and *Rsv4* loci showed accumulation of the virus only in the local infected leaves (Fig. 4.9). ELISA analysis postulated the previous result by recording a significant accumulation of SMV G5 in local infected leaves of silenced plants carrying *Rsv1* loci at 4 and 7 dpi comparing to those that were infected with V at the same time points (Fig. 4.8A). SMV RNA analysis showed accumulation of the virus in local infected area of plants carrying

Rsv1 and *Rsv4* in comparison to V control plants, but no difference was detected in the plants carrying *Rsv3* loci (Fig. 4.8C, and 4.11, respectively).

The plants carrying *Rsv1*-loci develop extreme resistance (ER) against SMV (G1 – G6 strains but not G7) by neither induce *PR* gene expression nor develop hypersensitive reaction (HR), a characteristic feature of *R*-mediated resistance (Buzzell & Tu, 1984; Lim, 1985; Nimchuk et al., 2003). Consistent with this result, *Rsv1* plants showed no induction of *GmPR1* after infection with an incompatible SMV G5 in comparison to a compatible infection of SMV G5 and G7 strains in Essex and *Rsv1* cultivars, respectively (Fig. 4.9D). To confirm the breakdown of this resistance, I knocked down the expression *GmBAK1* in plants carrying *Rsv1* loci, and examined the expression of *GmPR1* in response to the incompatible infection of SMV G5. My data showed expression for *GmPR1* in *GmBAK1* silenced plants as well as V controls before SMV G5 infection (Fig. 4.9C). This expression hindered me to compare its induction in response to SMV G5 infection. However, *GmBAK1* silenced *Rsv1* plants showed the same patterns as the positive control (*GmBAK1* silenced Essex plants infected with the compatible SMV G5 by expressing the same low level of *GmPR1* in contrast to those that infected with V control which showed high level of the same gene after SMV G5 infection (Fig. 4.9C).

Beside *PR* expression, those plants exhibiting lethal systemic hypersensitive (LSHR) during SMV G7 infection as a result of breaking down its robust function in resistance, while plants lacking this loci develop mosaic symptoms in response to the same virus (Ma, 1995; Ma et al., 1994). Presence of LSHR is a remarkable indicator of the functional role of *Rsv1* resistance loci. Therefore, to confirm the role of *GmBAK1a* in this pathosystem, I evaluated HR- associated cell death during SMV G7 infection. Both V and *GmBAK1 Rsv1* silenced plants were infected by SMV G7. Systemic leaves were collected 7 dpi and subjected to trypan blue staining. The result indicated the breakdown of this resistance by showing significantly less systemic HR detected as microscopic cell death in the silenced plants in comparison to those that were infected with V control (Fig. 4.11B). Consistent with their microscopic phenotype, *GmBAK1* silenced plants exhibited reduced ion leakage as well (Fig. 4.11C). However, the protein gel blot analysis of total protein extracts represented no significant differences in the viral accumulation in both local

infected and systemic leaves in V and *GmBAK1* silenced plants at 0, 4, 7, and 4, 7, 10 dpi, respectively (Fig. 4.11A). Accumulation of SMV G7 virus in the plant leaves was expected because of its ability to breakdown this resistance. Together, these results suggested that *GmBAK1* is an important partner in *Rsv1*-SMV mediated resistance in soybean to trigger the corresponding extreme resistance.

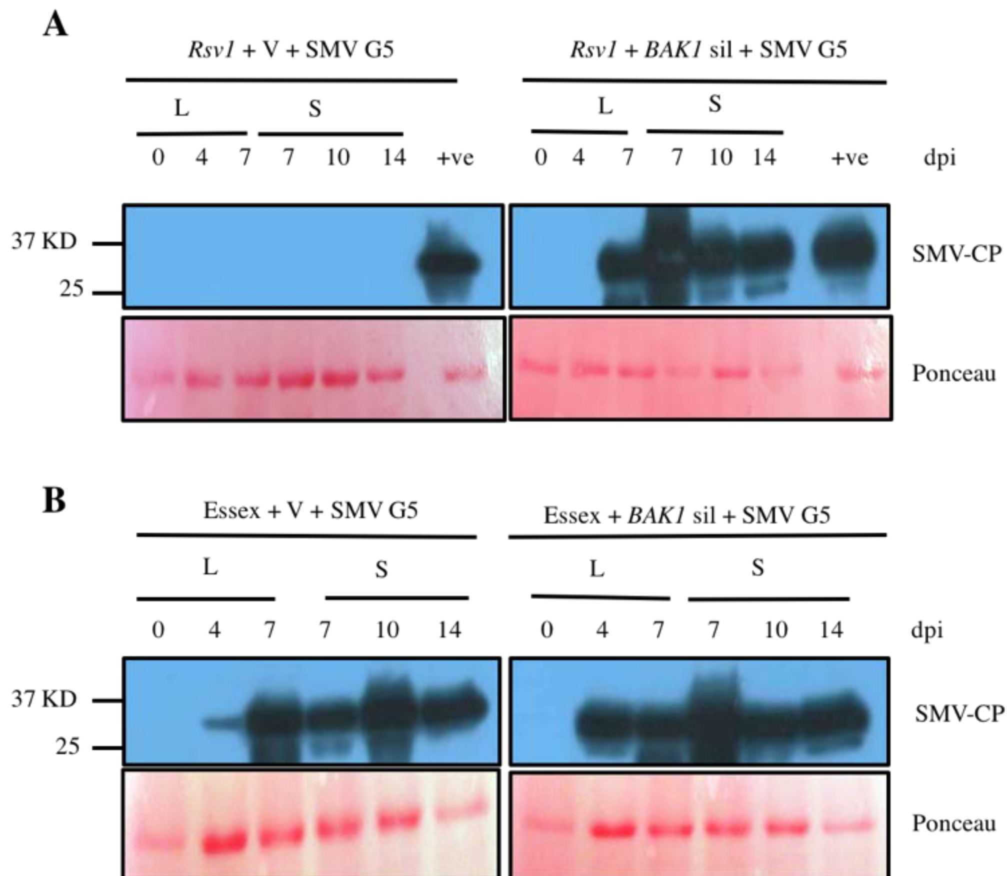

Fig. 4.7: *GmBAK1* silencing affects *Rsv1*-mediated resistance to SMV G5 in soybean. The image showing western blot analysis of protein extracts from SMV G5-infected *Rsv1* (**A**) and Essex plants (**B**), respectively. V, represents the plants previously infected with the empty BPMV vector, and *BAK1* sil, for those infected by the cloned BPMV vector with the silencing insert of *GmBAK1*. Lane numbers indicate days post SMV infection (dpi) from both local inoculated leaves (L) and un-inoculated systemic leaves (S). SMV CP was visualized using coat protein-specific primary antibodies and HRP-conjugated specific

secondary antibodies. *GmBAK1* silenced Essex plants were used as +ve control for SMV G5 infection. Results represent three repeated times of the same experiment.

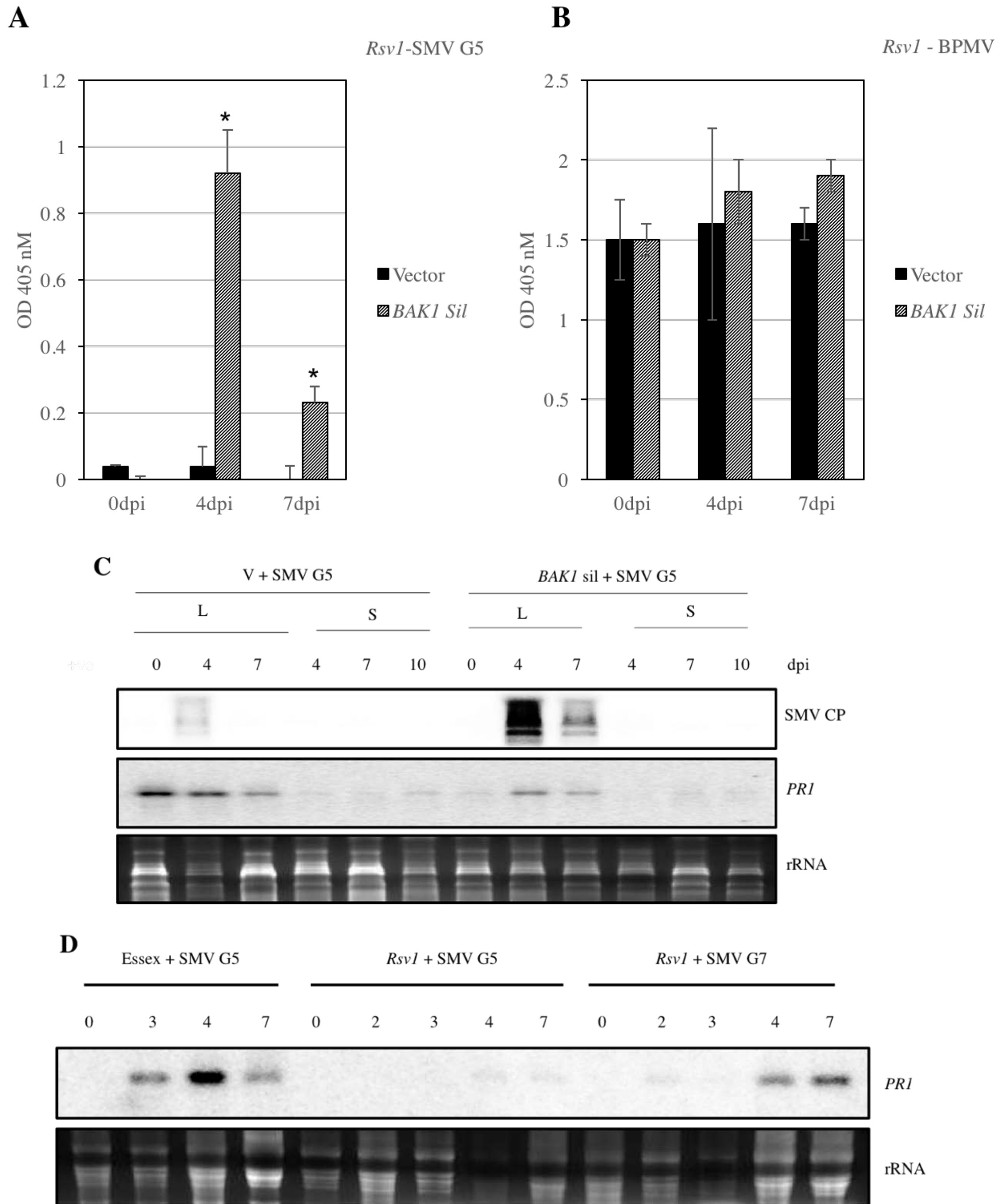

Fig. 4.8: A; ELISA of SMV G5 levels in Vector (V) and *GmBAK1* sil soybean plants (cv Essex-*Rsv1*). Error bars, ± SD (n= 4). t-test was used to determine statistical

significance. Asterisks denote significant difference between *BAK1* sil and V infected plants (P < 0.0001). **B;** ELISA of BPMV levels in Vector (V) and *GmBAK1* sil soybean plants (cv Essex-*Rsv1*). SMV G5 is avirulent on Essex-*Rsv1*. The samples were collected at indicated dpi. **C;** Northern analysis of mRNA level of SMV and pathogenesis-related (*PR1*) gene, from SMV G5 infected plants carrying *Rsv1* loci. V, represents the plants previously infected with the empty BPMV vector and *BAK1* sil, for those infected by the cloned BPMV vector with the silencing insert of *GmBAK1*. Lane numbers indicate post SMV infection (dpi) from both local infected leaves (L) and systemic leaves (S). Ethidium bromide staining of rRNA was used as a loading control. **D;** Northern analysis of mRNA level of pathogenesis-related (*PR1*) gene, from SMV G5 and SMV G5, G7 infected Essex plants and infected Essex plants carrying *Rsv1* locus, respectively. Lane numbers indicate days post SMV infection (dpi) from local infected leaves. Ethidium bromide staining of rRNA was used as a loading control.

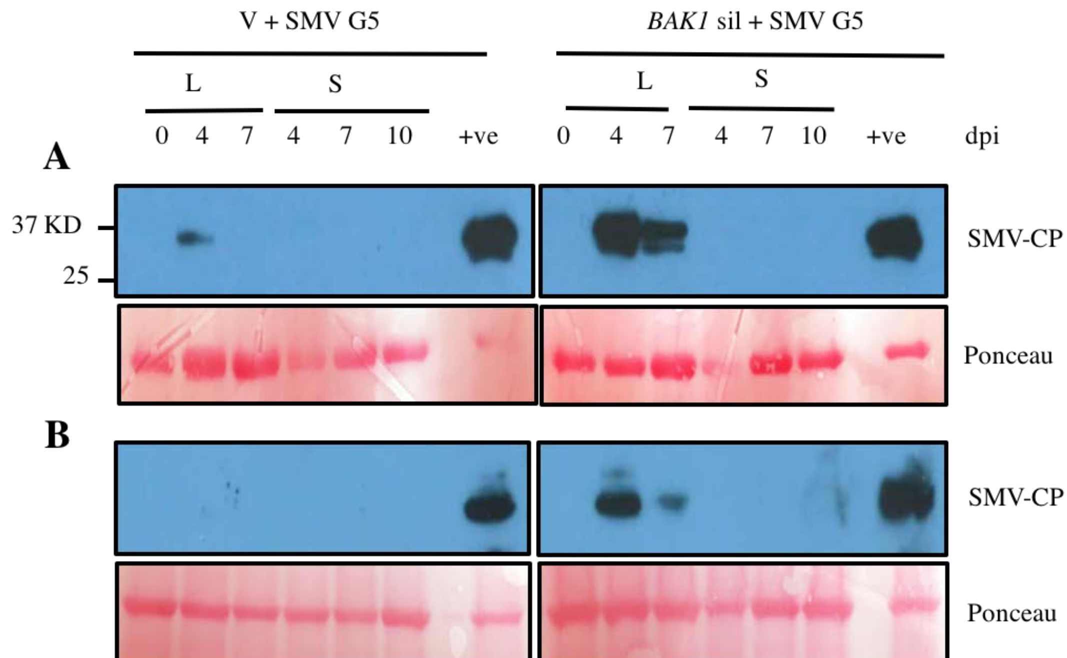

Fig. 4.9: Western blot analysis of protein extracts from SMV G5-infected plants carrying *Rsv3* (**A**) and *Rsv4* (**B**) resistant loci, respectively. V, represents the plants previously infected with the empty BPMV vector and *BAK1* sil, for those

infected by the cloned BPMV vector with the silencing insert of *GmBAK1*. Lane numbers indicate days post SMV infection (dpi) from both local inoculated leaves (L) and un-inoculated systemic leaves (S). SMV CP was visualized using protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. *GmBAK1* silenced Essex plants were used as +ve control for SMV G5 infection. Results represent 2 repeated times of the same experiment.

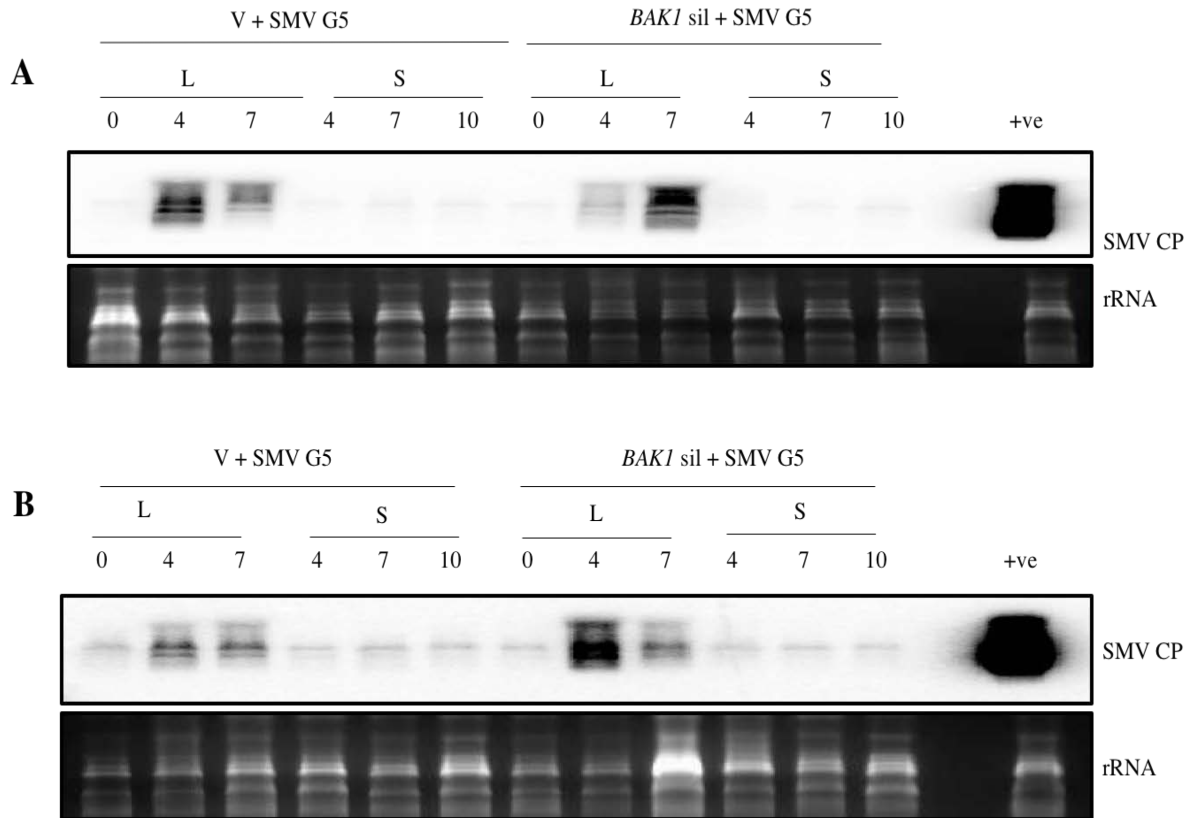

Fig. 4.10: Northern analysis of mRNA level of SMV, from SMV G5 infected plants carrying *Rsv3 (***A***)* and *Rsv4* (**B**) resistant loci, respectively. V, represents the plants previously infected with the empty BPMV vector and *BAK1* sil, for those whom infected by the cloned BPMV vector with the silencing insert of *GmBAK1*. Lane numbers indicate days required for sample collections post SMV infection (dpi) from both local infected leaves (L) and systemic leaves (S). Ethidium bromide staining of rRNA was used as a loading control.

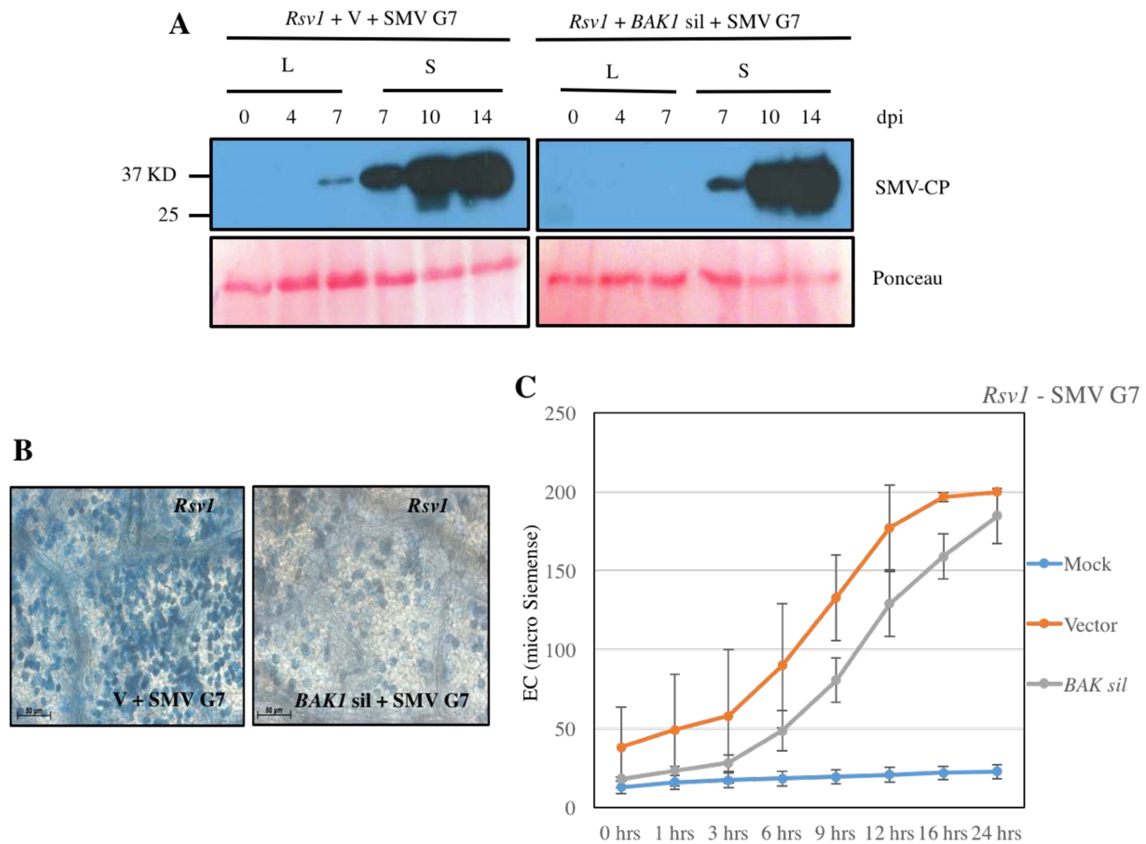

Fig. 4.11: *GmBAK1* silencing affects cell death response in soybean (cv Essex-*Rsv1*) aginst SMV G7. **A,** western blot analysis of protein extracts from SMV G7-infected Essex-*Rsv1*. V, represents the plants previously infected with the empty BPMV vector, and *BAK1* sil, for those whom infected by the cloned BPMV vector with the silencing insert of *GmBAK1* 8g. Lane numbers indicate days post SMV infection (dpi) from both local infected leaves (L) and un-infected systemic leaves (S). SMV CP was visualized using coat protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. Results represent 3 repeated times of the same experiment. **B;** Trypan blue staining showing microscopic cell death in V and *GmBAK1* sil leaves (cv Essex-*Rsv1*) infected with SMV-G7. **C;** Electrolyte leakage in mock (M), plants without any infection, V and *GmBAK1* silenced Essex plants carrying *Rsv1* locus at the indicated time points post SMV G7 infection. Error bars indicate SD (n = 5).

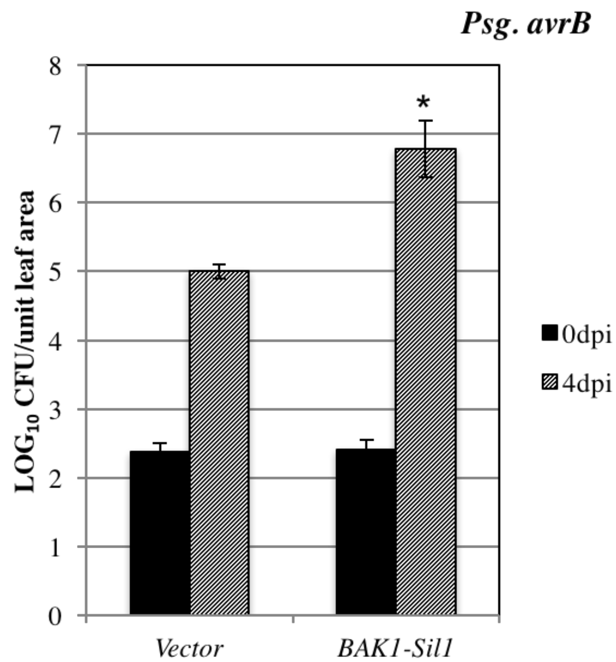

Fig. 4.12: *GmBAK1* is required for *Rpg1*-b-mediated resistance. Bacterial counts in soybean (Glycine max, *Rpg1*-b cv Merit) plants inoculated with empty BPMV vector (V), or those silenced for *GmBAK1*. Plants were infiltrated with *Psg avrB* (10^5 cfu/ml). Log_{10} values of colony-forming units (cfu) per unit leaf area from infected leaves at 0 (black bars) or 4 d post inoculation (gray/ black bars) are presented. Error bars, ± SD (n= 4). t-test was used to determine statistical significance. Asterisks denote significant difference between *BAK1* sil and V infected plants ($P < 0.0001$).

4.2.5 *GmBAK1* induces phosphorylation of HC-Pro *in vivo*:

BAK1 positively regulates the plant immune response and BR signaling pathway through its transphosphorylation with the corresponding receptor like kinases (RLKs) (Lin et al., 2014). Based on this fact, I attempted to understand how *GmBAK1* contributed in *Rsv1*- derived resistance against SMV. I considered the possibility that HC-Pro might be phosphorylated in presence of GmBAK1. To investigate the role of specific kinase activity of *BAK1*, I constructed respective kinase-dead site-directed mutant by replacing the 323 lysine residue in the kinase domain (KD) with glutamic acid (K323E) and 469 tyrosine residue that inhibit the auto-phosphorylation properties of *BAK1* with phenylalanine (Y469F) (Li et al., 2002; Oh et al., 2010) (Fig. 4.13). Conversely, I mutated the 341 threonine and 142 lysine residues of HC-Pro which is essential for SMV avirulence in the *Rsv1* background, or is critical for the silencing suppression function, respectively (Urcuqui-Inchima et al., 2000; Wen et al., 2013). I generated this site directed mutant by replacing the 341 threonine and 142 lysine residues with histidine and isoleucine (T341H and K142I), respectively (Fig. 4.14). First, I tested the possible interaction between these mutants with each other. For that purpose, I used biomolecular fluorescence complementation (BiFC) and coimmunoprecipitation (CO-IP) assays. For BiFC, I fused each protein to N/C terminal half of enhanced yellow fluorescent protein (nEYFP and cEYFP) using pSITE-n/cEYFP vectors, and I transiently co-expressed them inside tobacco leaves using *A. tumefaciens*. The result showed no fluorescence pattern when I co-expressed both HC-Pro mutants (T341I and K142I) with the wild type *GmBAK1a*, even though they were all adequately expressed. The vice versa, no fluorescence patterns were detected in case of co-expression of both *BAK1* mutants (K323E and Y469F) with the wild type HC-Pro. I could not detect the expression of both *BAK1* mutants, which may indicate their instability *in planta*. In contrast, very clear florescence patterns were visualized when HC-Pro mutants and *GmUBC2* were co-expressed, which indicating the reconstitution of EYFP by their interaction. The same result was observed when I co-expressed the wild type HC-Pro with the wild type *GmBAK1a* (Fig. 4.15). For further confirmation I used CO-IP. Proteins were tagged by MYC or FLAG and transiently co-expressed in *N. benthamiana* leaves as MYC-HC-Pro mutants and FLAG-GmUBC2 derivatives. The total protein extracts from these leaves were, then, subjected to IP using antibodies specific to the tag

on GmUBC2. Both HC-Pro mutant derivatives and GmUBC2 were detected in this IP (Figs. 4.16 and 4.17), indicating that both of them were able to interact *in planta.* Likewise, both HC-Pro T341H and GmBAK1c (*Glyma08g180080)* proteins were detected with IP FLAG, indicating protein interaction (Fig. 4.18). Consistent to the BiFC result no interaction was recorded between HC-Pro T341H mutant and the wild type GmBAK1a (Fig. 4.19). These results showed the importance of the T341 residue of HC-Pro in its interaction with GmBAK1a. Next, I tested HC-Pro phosphorylation in presence of GmBAK1a by analyzing these proteins when transiently co-expressed in *N. benthamiana* leaves. I tested the possible mobility shift of HC-Pro that could result from post translation modification. Protein expression was detected at 12, 24, 36, 48, and 72 h post infiltrations (hpi). Interestingly, HC-Pro showed mobility shift at 36 h, and that correlated with the high expression level of GmBAK1a (Fig. 4.20A). Incubation of protein extracts with calf intestinal phosphatase (CIP) restored the mobility of HC-Pro suggesting that phosphorylation contributed to the mobility shift of HC-Pro. In contrast, The T341H mutant version of HC-Pro showed no mobility shift when co-expressed with GmBAK1a, which supported the previous finding that indicated its importance in the association of HC-Pro with GmBAK1 (Fig. 4.20B). *in planta* phosphorylation assay by co-infiltrating ^{32}P-dATP with the various proteins in *N. benthamiana* leaves, was used as further confirmation for phosphorylation. MYC-tagged HC-Pro or T341H mutant derivative and FLAG tagged GmBAK1a or GmBAK1c proteins were transiently expressed in *N. benthamiana* leaves using *A. tumefacien*. 24 h later, I infiltrated 25 μCi of ^{32}P- dATP inside each infiltrated leaf. 12 h post infiltration, the total protein extracts were subjected to IP using the antibodies specific to the tag on HC-Pro and T341H mutant. SDS-PAGE electrophoresis of IP extracts was then performed, and the gel was exposed directly to a storage phosphor screen for 2 days and scanned to detect ^{32}P-labeled proteins. The wild type HC-Pro, but not the T341H mutant version, detected ^{32}P label when co-expressed with the wild type GmBAK1a, indicating its phosphorylation in the presence of GmBAK1a (Fig. 4.21A). In contrast, HC-Pro phosphorylation was not detected when co-expressed with the other GmBAK1 isoform (*GmBAK1c*, *Glyma08g180080*) as represented by the lack of ^{32}P signal compared to the one co-expressed with GmBAK1a (Figs 4.21B). This result for the first time showed the possible phosphorylation of a SMV effector protein (HC-Pro) by BAK1 which might play role in

promoting the corresponding immune response.

Fig. 4.13: Amino acid sequence of the full length *GmBAK1a*. The asterisks are denoting to the exchanged amino acid residue (K323E, and Y469F) required for the mutation.

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                                    * T341H
SQNPEAQFFRGWKKVFDKMPPNVENHECTIDFTNEQCGELAAAISQSIFPVKKLSCKQCR   60

QHIKHLSWEEYKQFLLAHMGCHGTEWETFQEIDGMRYVKRVIETSTAENASLQTSLEIVR  120

                         * K142I
LTQNYKSTHMLQIQDINKALMKGPSVTQSELEQASKQLLAMTQWWKNHMALTDEDALKVF  180

RNKRSSKALLNPSLLCDNQLDKNGNFVWGERGRHSKRFFANYFEEVVPSEGYSKYVIRKN  240

PNGQRELAIGSLIVPLNFERARMALQGKSVPREPITMSCISRQDGNFVYPCCCVTHDDGK  300

AFYSELKSPTKRHLVIGTSGDPKYIDLPATDADRMYIAKEGFCYLNIFLAMLVNVNEDEA  360

KDFTKMVRDVIVPQLGKWPTMLDVATAAYMLTVFHPETRNAELPRILVDHAGQTMHVIDS  420

FGSLTVGYHVLKAGTVNQLIQFASNDLQSEMKFCRVG                         457
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Fig. 4.14: Amino acid sequence of the full length HC-Pro G5. The asterisks are denoting to the exchanged amino acid residue (T341H, and K142I) required for the mutation.

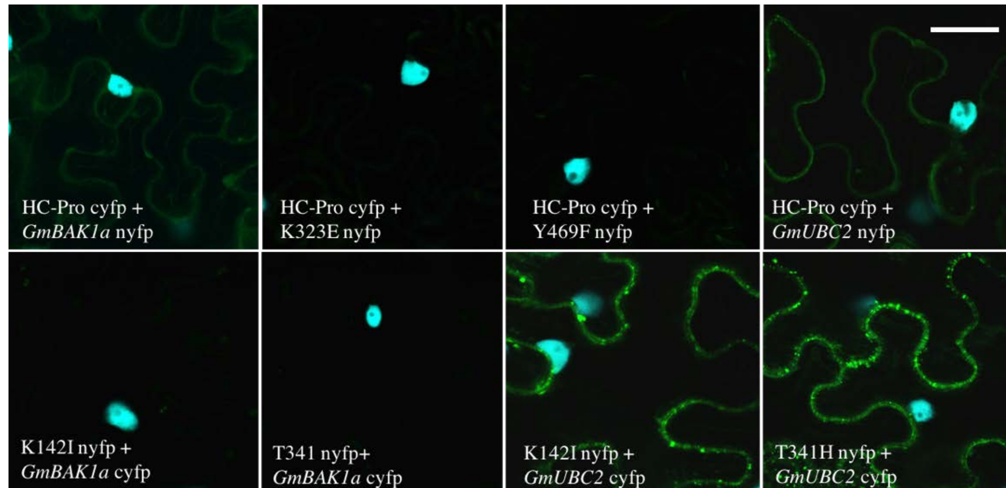

Fig. 4.15: Bimolecular fluorescence complementation (BiFC) assay showing the interaction between different mutations of SMVG5 HC-Pro protein and different mutations of *GmBAK1a* protein in plant cells. c/nyfp-*GmUBC2* protein was used as a positive control. The image showing 40× magnification of micrographs from CFP-H2B (nuclear localized histone 2B) transgenic *N. benthamiana* plants co-expressing both combination of c/nYFP-fused *GmUBC2*, HC-Pro $G5_{K142I}$, HC-Pro $G5_{T341H}$, $GmBAK1_{K323E}$ and $GmBAK1_{Y469F}$ proteins. The scale bare is 100 µM. This assay was repeated at least three separate times; different infiltrations were done for each interaction using both combinations of c/nYFP fused proteins.

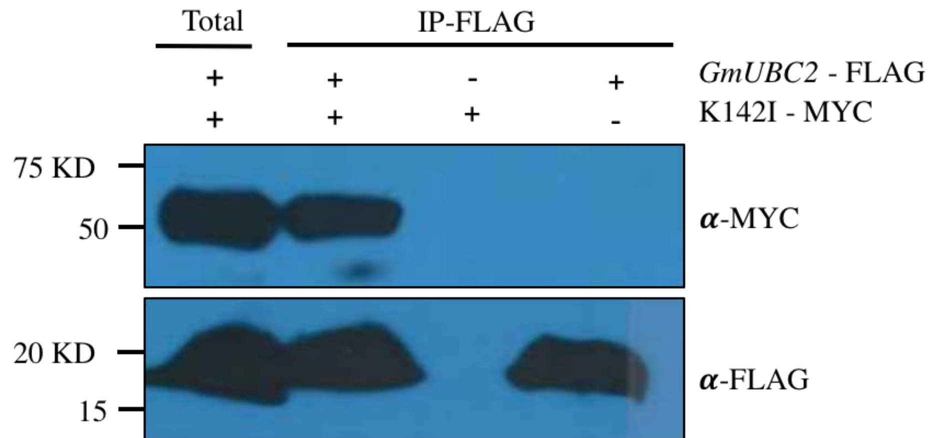

Fig 4.16: Immunoprecipitation assay between *GmUBC2* and HC-Pro $G5_{K142I}$. MYC-tagged HC-Pro $G5_{K142I}$ and FLAG-tagged *GmUBC2* proteins were co-expressed in *N. benthamiana*. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using HRP-specific secondary antibodies. This result represents two separate repeats with the same result.

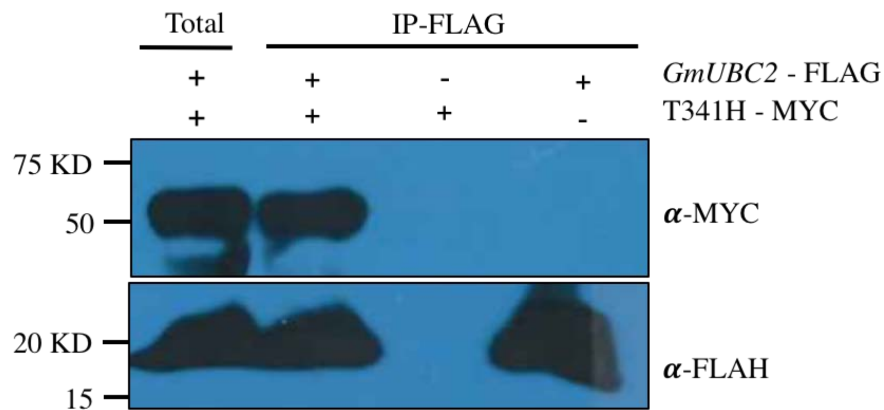

Fig 4.17: Immunoprecipitation assay between *GmUBC2* and HC-Pro $G5_{T341H}$. MYC-tagged HC-Pro $G5_{T341H}$ and FLAG-tagged *GmUBC2* proteins were co-expressed in *N. benthamiana*. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using HRP-specific secondary antibodies. This result represents two separate repeats with the same result.

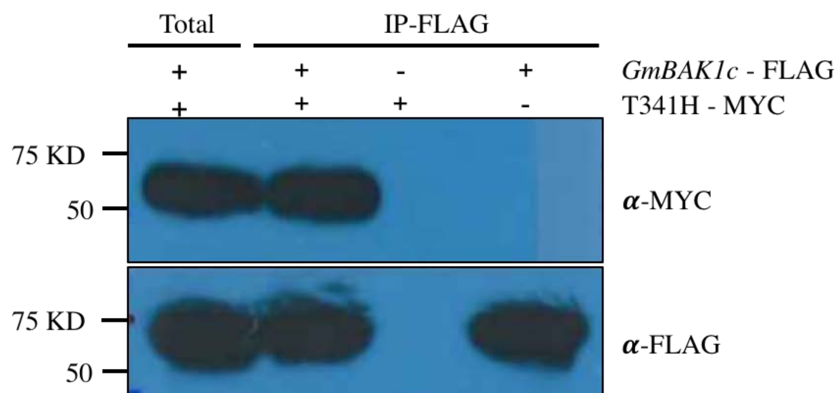

Fig 4.18: Immunoprecipitation assay between *GmBAK1c* and HC-Pro $G5_{T341H}$. MYC-tagged HC-Pro $G5_{T341H}$ and FLAG-tagged *GmBAK1c* proteins were co-expressed in *N. benthamiana*. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using HRP-specific secondary antibodies. This result represents two separate repeats with the same result.

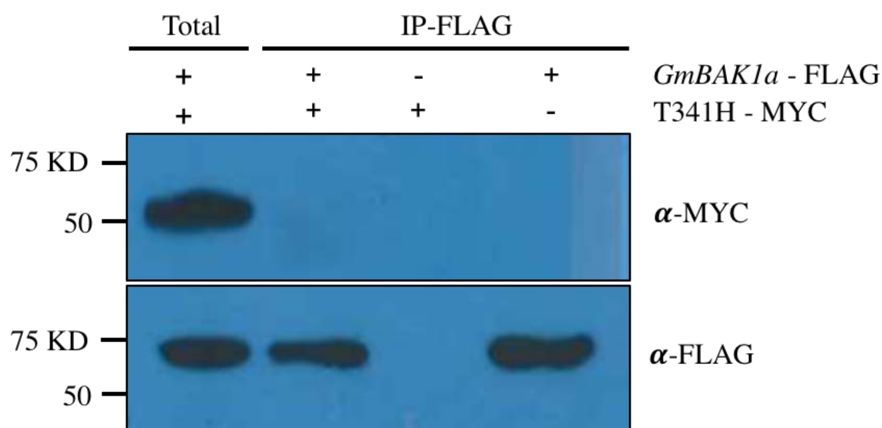

Fig 4.19: Immunoprecipitation assay between *GmBAK1a* and HC-Pro $G5_{T341H}$. MYC-tagged HC-Pro $G5_{T341H}$ and FLAG-tagged *GmBAK1a* proteins were co-expressed in *N. benthamiana*. Anti-FLAG beads were used to immunoprecipitate (IP-FLAG) proteins from total extracts. Visualization of both two proteins was done using HRP-specific secondary antibodies. This result represents two separate repeats with the same result.

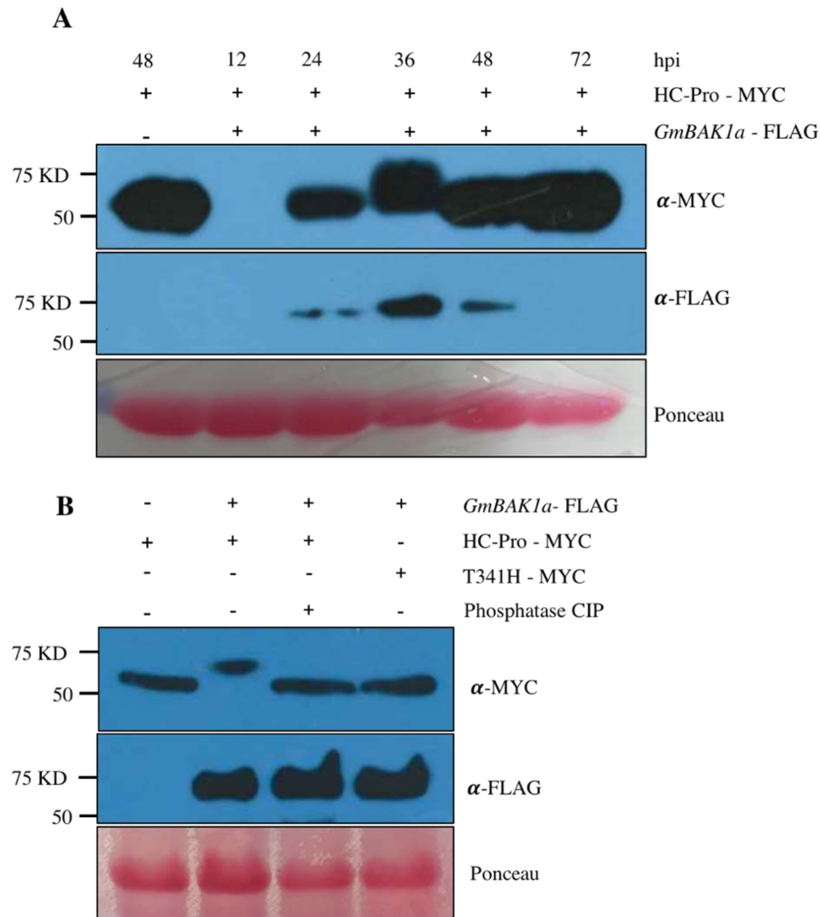

Fig 4.20: *GmBAK1* induces the phosphorylation of HC-Pro cloned form SMV G5 strain. **A;** Western blot analysis of MYC-tagged HC-Pro and FLAG-tagged *GmBAK1a* proteins transiently expressed in *Nicotiana benthamiana* leaves. To better recognize the reduced mobility of HC-Pro when co-expressed with *GmBAK1a*, proteins were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 20 V for 12–14 h. Proteins were visualized from total extracts using FLAG- or MYC specific antibodies, and HRP-specific secondary antibodies. Lane numbers indicate hours post infiltration (hpi). **B;** Showing the incubation of protein extracts with calf intestinal phosphatase (CIP) restored the mobility of HC-Pro. Protein extracts were incubated with buffer or with CIP before western blot analysis. Samples were collected 36 h PI. As same as no mobility shift was detected with HC-Pro T341H mutants when co-

expressed with *GmBAK1a*. – sign means absence and + sign means included. This result represents two separate repeats with the same result.

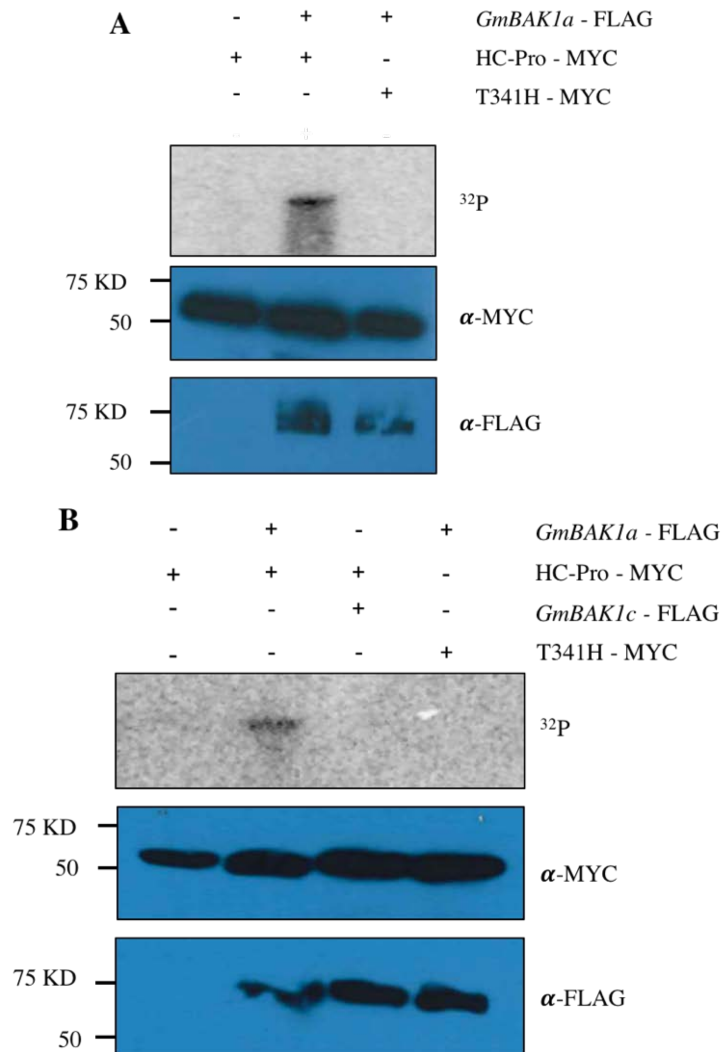

Fig 4.21: In planta phosphorylation assay. **A;** ^{32}P-dATP was infiltrated in *N. benthamiana* leaves 24 h after co-expression of *GmBAK1a*-FLAG and HC-Pro-MYC, *GmBAK1a*-FLAG and T341H, or expression of HC-Pro-MYC alone. HC-Pro and HC-Pro-T341 derived mutant were immunoprecipitated (IP) from total extracts using MYC-affinity beads. The radiolabel was visualized using Phosphoimager detection. The western blot analysis showed the expression of MYC-tagged HC-Pro/HC-Pro-T341H mutant and FLAG-tagged *GmBAK1a* proteins which were transiently co-expressed in *Nicotiana benthamiana* leaves. **B**; A second repeat of the same experiment with the incorporation of another

GmBAK1 isoform (*GmBAK1c*) that showed interaction with HC-Pro. – sign means absence and + sign means included.

4.2.6 Brassinosteroids (BR) negatively regulate the *Rsv1* mediated resistance against SMV G7:

BR worked as inducer of disease resistance against a wide range of many pathogens in tobacco and rice (Nakashita et al., 2003). It is also known to inhibit many PTI responses. For example, activation of the transcription factor *BZR1* by BR repressed promotors of many immune genes (Sun et al., 2010). These findings along with mine that showed the role of *BAK1* in *Rsv*-mediated resistance against SMV raised the questions "does BR possess a role in this pathosystem, or is there a link between the role of *BAK1* in BR signaling and its role in this pathosystem, that is controlled by its decisions? Trying to find the answers for these questions, I externally applied either brassinolide (BL), or propiconazole (PPZ), a specific brassinosteroid (BR) biosynthesis inhibitor (Hartwig et al., 2012), and tested its effect on virus resistance. The susceptible and resistance plants (Essex, and Essex-*Rsv1* soybean, respectively) were sprayed with BR or PPZ 24 h before inoculation with compatible SMV (SMV G5 and G7 on Essex, and SMV G7 on Rsv1). Samples were collected from local infected and systemic leaves post inoculation at 0, 4, 7 and 4, 7, 10 dpi, respectively. The protein gel blot analysis of total protein extracts showed no effect of BR on SMV G5 and G7 accumulation in Essex treated plants comparing to water sprayed control ones. Likewise, Essex plants sprayed with PPZ showed no effect on SMV G5 accumulation in both local and systemic leaves, however SMV G7 showed less insignificantly accumulation in comparison to water treated plants (Figs 4.22 and 4.23). Interestingly, PPZ treated *Rsv1* plants exhibited abnormal resistance against SMV G7 by recording significantly reduced viral accumulation in both local infected and systemic leaves. In addition, no difference was recorded in the same virus accumulation in case of BR treatment (Fig. 4.24). These results showed no significant effect of BR on this pathosystem. However, the enhanced resistance against SMV G7, in *Rsv1* background through the application of PZZ, gave another evidence of the possible role of *BAK1* in activation of *Rsv1* mediated resistance against SMV infection.

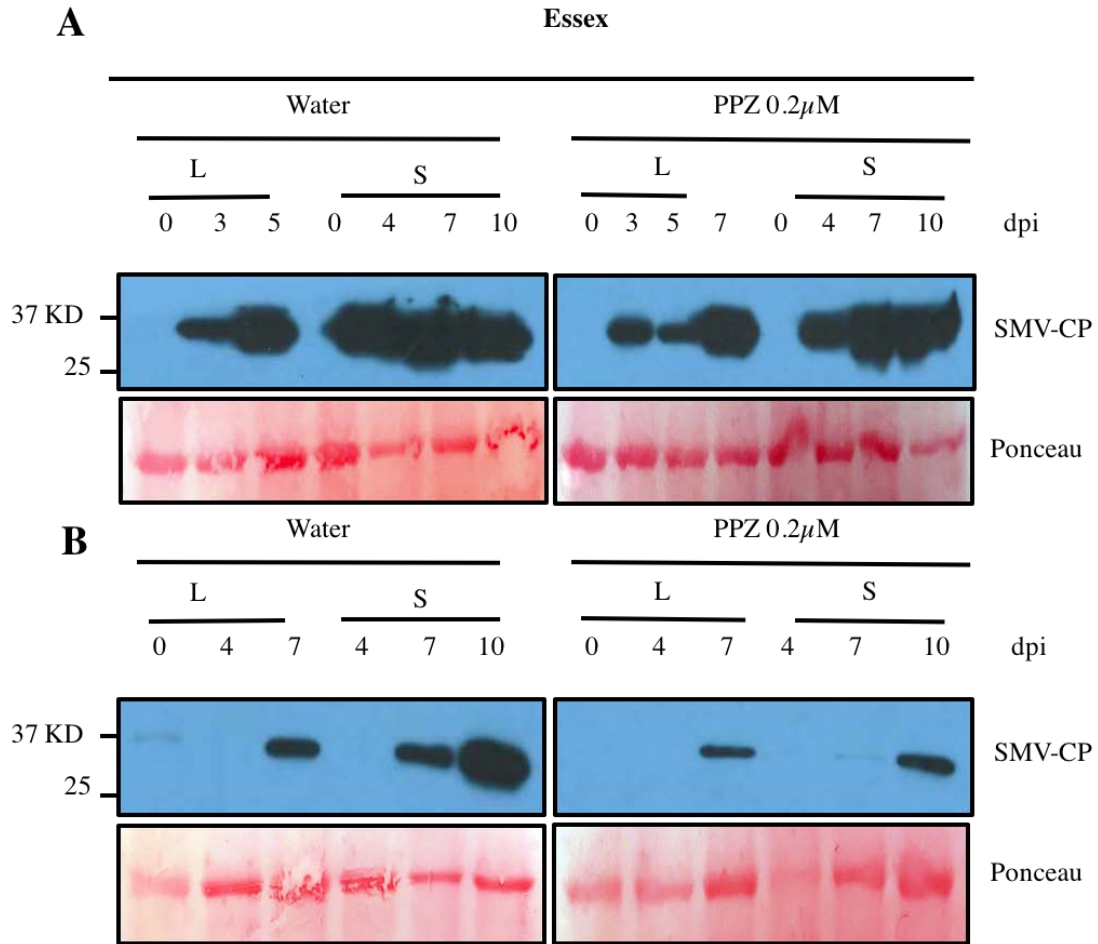

Fig. 4.22: Western blot analysis of protein extracts from SMV G5 (**A**) and G7 (**B**) infected Essex plants lacking *Rsv1* resistant loci, and treated with water and propiconazole (PPZ), a specific brassinosteroid (BR) biosynthesis Inhibitor (Hartwig et al., 2012). Lane numbers indicate days post SMV infection (dpi) from both local inoculated leaves (L) and un-inoculated systemic leaves (S). SMV CP was visualized using coat protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. Results represent three repeated times of the same experiment.

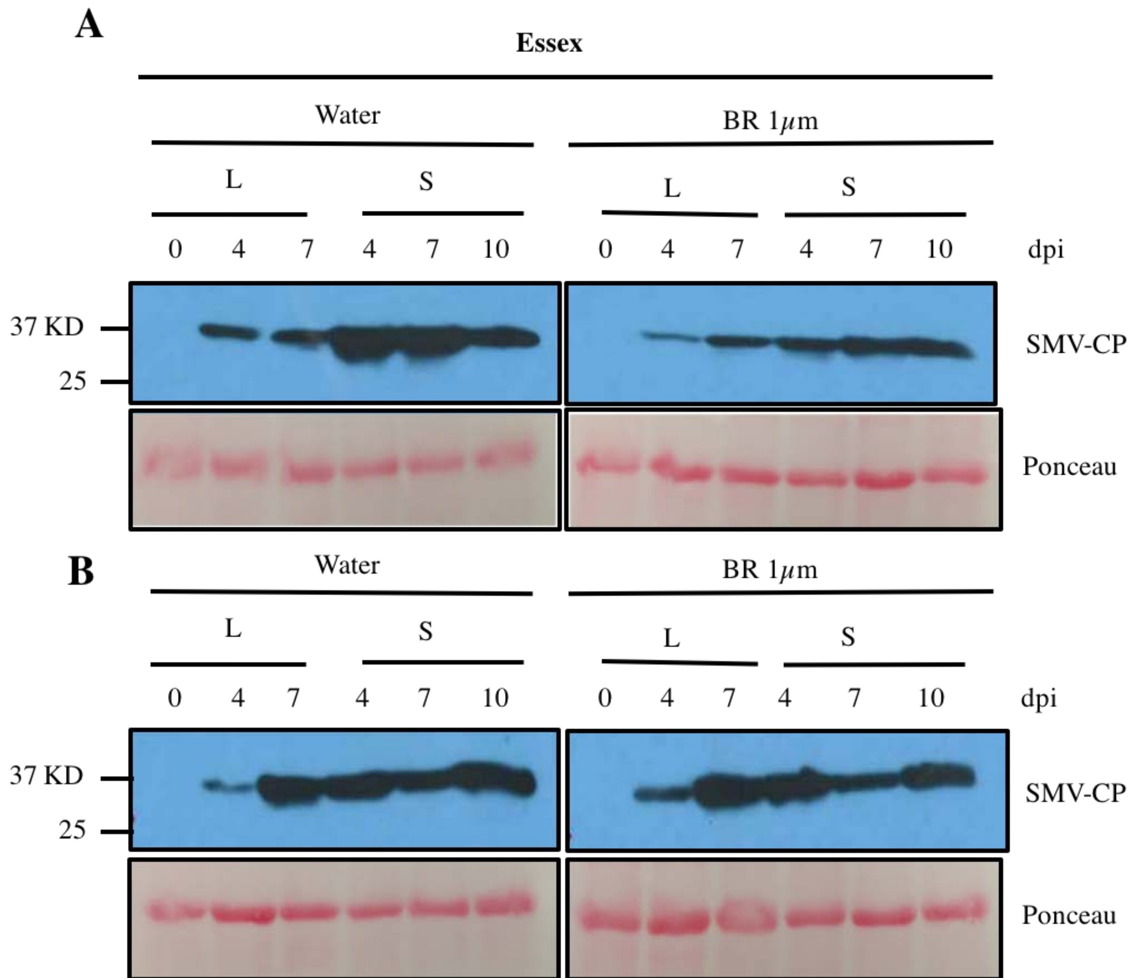

Fig. 4.23: Western blot analysis of protein extracts from SMV G5 (**A**) and G7 (**B**) infected Essex plants lacking *Rsv1* resistant loci, and treated with water and brassinoloide (BR). Lane numbers indicate days post SMV infection (dpi) from both local inoculated leaves (L) and un-inoculated systemic leaves (S). SMV CP was visualized using coat protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. Results represent 3 repeated times of the same experiment.

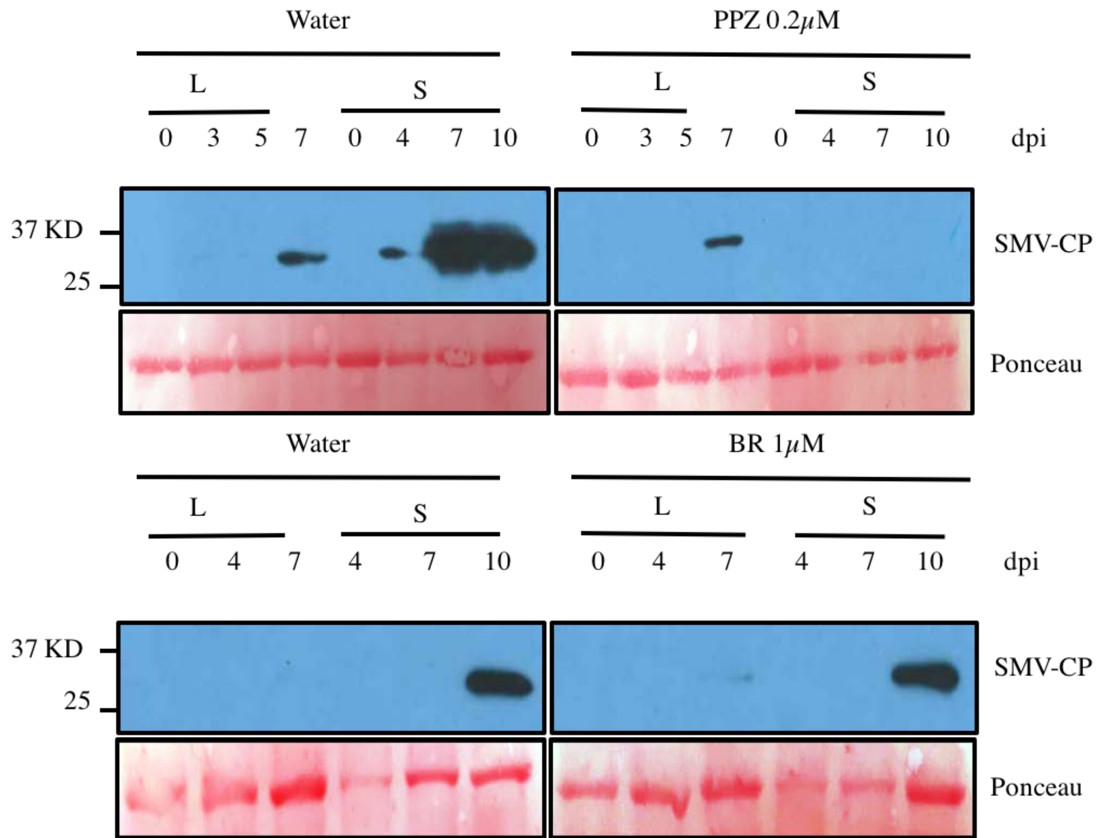

Fig. 4.24: Western blot analysis of protein extracts from SMV G7 infected plants carrying *Rsv1* resistant loci, and treated with water, brassinolide (BR) or propiconazole (PPZ), a specific brassinosteroid (BR) biosynthesis Inhibitor (Hartwig et al., 2012). Lane numbers indicate days post SMV infection (dpi) from both local inoculated leaves (L) and un-inoculated systemic leaves (S). SMV CP was visualized using coat protein-specific primary antibodies and HRP-conjugated specific secondary antibodies. Results represent 2-3 repeated times of the same experiment.

4.3 **DISCUSSION:**

Recently, *BAK1* showed very important role in several independent signaling pathways including BR response, PTI, and controlling cell death (Chinchilla et al., 2009). Here, I showed, for the first-time, that *BAK1* played a significant role in *Rsv1* mediated resistance against SMV. This is likely associated with the phosphorylation of the multifunction SMV effector protein (HC-Pro) in the presence of *BAK1*. Moreover, the amino acid residue T341 in HC-Pro protein, which regulates virus avirulence in *Rsv1* plants (Wen et al., 2013), is possibly was required for this phosphorylation.

Regulation of the different *GmBAK1* isoforms expression in both soybean susceptible (Essex) and resistance (Essex-*Rsv1*) cultivars after the infection of avirulent SMV G5 strain suggests different roles of each of them in the antiviral responses against SMV, or different behavior inside the plant cell during that infection. It also gave the first indication of the possible role of *GmBAK1* in *Rsv1*-SMV pathosystem. In support of this hypothesis, I found that knocking down the expression of *GmBAK1a* inhibited the *Rsv1* resistance against SMV. Indeed, this effect seems to be more effective to that loci than to the other *Rsv3* and *Rsv4* resistant ones. The result showed different phenotype between *Rsv1*, *Rsv3*, and *Rsv4* soybean cultivars against the avirulent SMV G5 strain, after knocking down the expression of *GmBAK1*. For example, viral coat proteins of SMV G5 can be detected in the systemic tissues of plants carrying *Rsv1* loci but not in those that carrying *Rsv3*, and *Rsv4* loci. Moreover, SMV RNA analysis proposed that viral replication seems to be exclusive to the local infected area in *Rsv1* background, however no significant differences were detected in the *Rsv3* or *Rsv4* backgrounds.

These results might indicate that *Rsv3* and *Rsv4* loci recruit other host factors to hinder the viral propagation and dissemination from local infected to systemic tissues. These factors seems to be working besides *BAK1* recognition pathway, and more likely they are not working in *Rsv1* background. Seo et al. (2014) proposed that failing of SMV dissemination from the local infected leaves to the distal tissues in *Rsv3* background was because the *Rsv3* loci could recognize the viral effector protein (CI) in the local leaves after its replication, and in turn this recognition triggered up-regulation of *GmPP2C3a* gene, a

subset protein of type 2C protein phosphatase family that works downstream abscisic acid (ABA) immune signaling pathway. They showed that this protein functioned as a positive regulator of the immune signaling, by stimulating callose, a plant β-1,3-glucan polymer, deposition in the plasmodesmata (PD). Callose deposition at PD hindered virus cell-to-cell movement and restricted virus accumulation to the initially infected cells.

The lethal necrotic phenotype of SMV G7 infection on the plants carrying *Rsv1* locus gave another evidence of the important role of *GmBAK1* in this pathosystem. I found that the *GmBAK1* silenced plants developed significantly less systemic HR detected as microscopic cell death in comparison to those that were infected with V control. Normally *BAK1* plays an important role in regulating cell death (CD) in many pathosystem, for example knockout of *bak1* in Arabidopsis mutant showed activated cell death in response to both bacterial (*Pseudomonase. syringae pv. tomato* DC3000) and fungal (*Botrytis cinerea)* infections (Halter et al., 2014; Kemmerling et al., 2007). In contrast to this finding, my data showed that *BAK1* negatively controlled the systemic cell death in case of *Rsv1*-mediated resistance, which suggests that *BAK1* played a dual role in plant immunity depending on the pathosystem it works with. Similarly, *BAK1* impeded CD in case of biotrophic fungal infection compared to other necrotrophic pathogens. Infection of *bak1* mutant Arabidopsis plants with *Hyaloperonospora parasitica (*a hemibiotrophic oomycete) did not promote CD compared to the wild plants (Col-0). Whereas, the same plants exhibited enhanced CD development in case of necrotrophic infection of *B. cinerea* (Kemmerling et al., 2007).

BR workes as inducer of disease resistance against a wide range of many pathogens in tobacco and rice (Nakashita et al., 2003). Tobacco plants treated with BR showed enhanced resistance against tobacco mosaic virus (TMV), the bacterial pathogen *Pseudomonas syringae pv. tabaci (Pst)*, and fungal pathogen *Oidium sp.* The same result was also observed in rice against the bacterial pathogen *Xanthomonas oryzae* pv. oryzae, and the fungal pathogens *Magnaporthe grisea* (Nakashita et al., 2003). On the other hand, BR is also known to inhibit many PTI responses. For example, activation of the transcription factor BZR1 by BR repressed promotors of many immune genes (Sun et al., 2010). Moreover, Arabidopsis plants treated with BR showed a remarkable reduction of

reactive oxygen species (ROS) triggered by flg22 or elf18 (Albrecht et al., 2012). My data showed that plants treated with BR developed the normal susceptibility to a compatible infection of SMV in both Essex and Essex-*Rsv1* backgrounds. In addition, PPZ treatment showed no effect in plants lacking *Rsv1* locus. Conversely, the plants carrying this locus showed enhanced resistance to the compatible SMV G7 infection after PPZ treatment. This result does not clarify whether BR is involved in soybean response to SMV. Further research will be required to investigate this.

It is known that large numbers of receptor like kinases (RLKs) and receptor like cytoplasmic kinases (RLCKs) modulate growth, development and innate immunity *in planta* by mediating diverse signaling pathways via their kinase domains (Shiu & Bleecker, 2001a). RLKs perceive different extrinsic and intrinsic molecules by their extracellular domains and form complexes with their corresponding RLCKs, which, in turn, relay the signaling via phosphorylation (Gómez-Gómez & Boller, 2000; Shiu & Bleecker, 2001a; Zipfel et al., 2006). Indeed, these pathways are induced upon interaction with other specific LRR-RLKs receptor ligands. For example, the LRR-RLKs flagellin receptor (FLS2) form complex with BAK1 upon perception of flagellin 22 (flg22), BAK1 directly phosphorylates the plasma membrane-associated RLCK Botrytis-Induced Kinase 1 (BIK1) that associates with FLS2/EFR. Phosphorylated BIK1 dissociates from FLS2 and positively regulates plant innate immunity (Heese et al., 2007; Lu et al., 2010; Roux et al., 2011; Zhang et al., 2010). Similarly, my data , for the first time, indicates that the SMV effector protein (HC-Pro) is phosphorylated in the presence of GmBAK1 and this requires the T341 residue which regulates virus avirulence in *Rsv1* plants (Wen et al., 2013). This phosphorylation of HC-Pro might affect its conformational structure. Thereby, it interferes with its virulence functions and might suppress the interaction with the proposed host factors. In support of this idea, the amino acids substitutions in HC-Pro between the avirulent and virulent SMV is sufficient to convert the avirulent strain to be virulent, and vice versa. These substitutions might cause changes in its conformational structures as well and enable or hinder interactions with the different host factors that interact with HC-Pro (Chowda-Reddy et al., 2011; Eggenberger et al., 2008; Wen et al., 2013). Alternatively, this phosphorylation could be recognized by *Rsv1* locus and trigger the immune response against SMV, in consistent with the guardee hypothesis that was proposed by Van Der

Biezen and Jones (1998).

This study highlighted a functional role of *GmBAK1* in the *Rsv1*-mediated resistance against SMV in soybean, which probably initiated by the phosphorylation of the multifunction SMV effector protein HC-Pro. Determining the phosphorylation status of virus delivered HC-Pro G5 in soybean would be key to proving this hypothesis. Notably, some effector proteins could induce phosphorylation of a host factor and trigger the corresponding *R*-mediated resistance. For example, Selote et al. (2013) showed the possible phosphorylation of the soybean RPM1 interacting protein4 like (GmRIN4b) in the presence of the *Pseudomonas syringae* effector protein (AvrB), and this was required to activate the corresponding Rpg1-b resistance protein. However, the direct phosphorylation of a pathogen effector protein by a host factor is a remarkably significant finding of this study. The underlying mechanisms need further investigation. This is not trivial because the *Rsv1* gene has not been cloned. Generating infectious clone of mutant virus also not trivial.

APPENDIX

LIST OF ABBREVIATIONS

Acronym/ abbreviation	Expansion
BiFC	Bi-molecular fluorescence complementation
BSA	Bovine serum albumin
DPI	Days post inoculation
KH_2PO_4	Potassium phosphate, monobasic
KOH	Potassium hydroxide
MOPS	3-(N-morpholino) propanesulfonic acid
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
R	Resistant
SSC	Sodium chloride, sodium citrate
TE	TRIS-EDTA
ABA	Abscisic acid
Avr	Avirulence gene
BAK1	BRI1-associated kinase 1
BPMV	Bean pod mottle virus
BR	Brassinosteroid
BRI1	Brassinosteroid-insensitive 1
$CaCl_2$	Calcium chroride
CC-NBS–LRR	Coiled coil-nucleotide binding site-leucine rich repeat
CD	Cell death
cDNA	Complimentary DNA
Co-IP	C o-immunoprecipitation
DAMP	Damage-associated molecular pattern
dATP	Deoxyribo adenosine triphosphate

LIST OF ABBREVIATIONS
continued

dCTP	Deoxyribo cytosine triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribo nucleic triphosphate
dpi	Days post inoculation
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EF-Tu	Elongation factor Tu
EGTA	Ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETI	Effector-triggered immunity
FLS2	Flagellin-sensing 2
g/mg/µg/ng	Gram/ milligram/ microgram/ nanogram
Gm	*Glycine max*
GmPP2C3a	subset protein of type 2C protein phosphatase
GTP	Guanosine triphosphate
h/min/sec	Hours/minutes /seco nds
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
K_2HPO_4	Potassium phosphate, dibasic
KCl	Potassium chloride
KD	Kinase domain
L/mL/µL	Liter/ milliliter/ microliter
LB	Luria-Bertani
LRR	Leucine rich repeat
M	Mock; plants without any infection
M/mM/µM	Molar/millimolar/ micromolar

LIST OF ABBREVIATIONS
continued

MAMP	Microbe associated molecular pattern
MAPK	Mitogen activated protein kinase
$MgCl_2$	Magnesium chloride
NaN_3	Sodium azide
NaOAc	Sodium acetate
NB	Nucleotide binding
^oC	Degrees centigrade
OD	Optical density
OE	Over expression
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Plasmodesmata
PPZ	Propiconazole
PR	Pathogenesis related gene
PRR	Pattern recognition receptor
Psg	*Pseudomonas syringae*
PTI	PAMP-trigger immunity
pv.	Pathovar
RLCKs	Receptor like cytoplasmic kinases
RLK	Receptor-like-kinase
RLPK	Receptor-like protein kinase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Salicylic acid

LIST OF ABBREVIATIONS
continued

SAR	systemic acquired resistance
SDS	Sodium dodecyl sulfate
SERK	Somatic embryogenesis receptor kinase
Sil	Silenced
TBE	Tris-borate/ EDTA electrophoresis buffer
TEV	Tobacco etch virus
TLR	Toll-like receptor
TM	Transmembrane domain
TMV	Tobacco mosaic virus
TRIS	Hydroxymethyl Aminomethane
TRSV	Tobacco ring spot virus
V plant	Empty BPMV vector-inoculated plant
VIGS	Virus-induced gene silencing
WT	Wild-type

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., Parker, J.E. 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. *Proceedings of the National Academy of Sciences*, **95**(17), 10306-10311.

Ala-Poikela, M., Goytia, E., Haikonen, T., Rajamäki, M.-L., Valkonen, J.P. 2011. Helper component proteinase of the genus Potyvirus is an interaction partner of translation initiation factors eIF (iso) 4E and eIF4E and contains a 4E binding motif. *Journal of virology*, **85**(13), 6784-6794.

Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., Rathjen, J.P., de Vries, S.C., Zipfel, C. 2012. Brassinosteroids inhibit pathogen-associated molecular pattern–triggered immune signaling independent of the receptor kinase BAK1. *Proceedings of the National Academy of Sciences*, **109**(1), 303-308.

Alcaide-Loridan, C., Jupin, I. 2012. Ubiquitin and plant viruses, let's play together! *Plant physiology*, **160**(1), 72-82.

Anandalakshmi, R., Marathe, R., Ge, X., Herr, J., Mau, C., Mallory, A., Pruss, G., Bowman, L., Vance, V.B. 2000. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science*, **290**(5489), 142-144.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V.B. 1998. A viral suppressor of gene silencing in plants. *Proceedings of the National Academy of Sciences*, **95**(22), 13079-13084.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., Sheen, J. 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, **415**(6875), 977-983.

Ashfield, T., Bocian, A., Held, D., Henk, A.D., Marek, L.F., Danesh, D., Peñuela, S., Meksem, K., Lightfoot, D.A., Young, N.D. 2003. Genetic and physical localization of the soybean Rpg1-b disease resistance gene reveals a complex locus containing several tightly linked families of NBS-LRR genes. *Molecular plant-microbe interactions*, **16**(9), 817-826.

Ashfield, T., Ong, L.E., Nobuta, K., Schneider, C.M., Innes, R.W. 2004. Convergent evolution of disease resistance gene specificity in two flowering plant families. *The Plant Cell*, **16**(2), 309-318.

Atreya, C., Atreya, P., Thornbury, D., Pirone, T. 1992. Site-directed mutations in the potyvirus HC-Pro gene affect helper component activity, virus accumulation, and symptom expression in infected tobacco plants. *Virology*, **191**(1), 106-111.

Atreya, C.D., Pirone, T.P. 1993. Mutational analysis of the helper component-proteinase gene of a potyvirus: effects of amino acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility. *Proceedings of the National Academy of Sciences*, **90**(24), 11919-11923.

Ballut, L., Drucker, M., Pugniere, M., Cambon, F., Blanc, S., Roquet, F., Candresse, T., Schmid, H.-P., Nicolas, P., Le Gall, O. 2005. HcPro, a multifunctional protein encoded by a plant RNA virus, targets the 20S proteasome and affects its enzymic activities. *Journal of General Virology*, **86**(9), 2595-2603.

Ballut, L., Petit, F., Mouzeyar, S., Le Gall, O., Candresse, T., Schmid, P., Nicolas, P., Badaoui, S. 2003. Biochemical identification of proteasome-associated endonuclease activity in sunflower. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, **1645**(1), 30-39.

Banerjee, D., Zhang, X., Bent, A.F. 2001. The leucine-rich repeat domain can determine effective interaction between RPS2 and other host factors in Arabidopsis RPS2-mediated disease resistance. *Genetics*, **158**(1), 439-450.

Baulcombe, D.C. 1999. Gene silencing: RNA makes RNA makes no protein. *Current Biology*, **9**(16), R599-R601.

Bazzini, A.A., Almasia, N.I., Manacorda, C.A., Mongelli, V.C., Conti, G., Maroniche, G.A., Rodriguez, M.C., Distéfano, A.J., Hopp, H.E., del Vas, M. 2009. Virus infection elevates transcriptional activity of miR164a promoter in plants. *BMC plant biology*, **9**(1), 1.

Bell, J.K., Mullen, G.E., Leifer, C.A., Mazzoni, A., Davies, D.R., Segal, D.M. 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends in immunology*, **24**(10), 528-533.

Bestwick, C.S., Bennett, M.H., Mansfield, J.W. 1995. Hrp mutant of Pseudomonas syringae pv phaseolicola induces cell wall alterations but not membrane damage leading to the hypersensitive reaction in lettuce. *Plant Physiology*, **108**(2), 503-516.

Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A., Innes, R.W. 1994. A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. *The Plant Cell*, **6**(7), 927-933.

Blanc, S., López-Moya, J.-J., Wang, R., García-Lampasona, S., Thornbury, D.W., Pirone, T.P. 1997. A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus. *Virology*, **231**(1), 141-147.

Blaum, B.S., Mazzotta, S., Nöldeke, E.R., Halter, T., Madlung, J., Kemmerling, B., Stehle, T. 2014. Structure of the pseudokinase domain of BIR2, a regulator of BAK1-mediated immune signaling in Arabidopsis. *Journal of structural biology*, **186**(1), 112-121.

Boller, T., Felix, G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology*, **60**, 379-406.

Bosher, J.M., Labouesse, M. 2000. RNA interference: genetic wand and genetic watchdog. *Nature Cell Biology*, **2**(2), E31-E36.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., Baulcombe, D.C. 1998. Retracted: Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. *The EMBO journal*, **17**(22), 6739-6746.

Brodersen, P., Voinnet, O. 2006. The diversity of RNA silencing pathways in plants. *TRENDS in Genetics*, **22**(5), 268-280.

Brown, I., Trethowan, J., Kerry, M., Mansfield, J., Bolwell, G.P. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains ofXanthomonas campestris andFrench bean mesophyll cells. *The Plant Journal*, **15**(3), 333-343.

Bruening, G. 1998. Plant gene silencing regularized. *Proceedings of the National Academy of Sciences*, **95**(23), 13349-13351.

Burgyán, J. 2008. Role of silencing suppressor proteins. *Plant Virology Protocols: From Viral Sequence to Protein Function*, 69-79.

Burrows, M.L., Boerboom, C., Gaska, J., Grau, C. 2005. The relationship between Aphis glycines and Soybean mosaic virus incidence in different pest management systems. *Plant disease*, **89**(9), 926-934.

Buss, G., Ma, G., Chen, P. 1995. The gene for resistance in soybean mosaic virus (SMV) in the Raiden cultivar is not independent of Rsv1. *Agronomy abstracts. ASA, Madison*.

Buss, G., Ma, G., Chen, P., Tolin, S. 1997. Registration of V94-5152 soybean germplasm resistant to soybean mosaic potyvirus. *Crop science*, **37**(6), 1987-1988.

Buss, G., Ma, G., Kristipati, S., Chen, P., Tolin, S. 1999. A new allele at the Rsv3 locus for resistance to soybean mosaic virus. *Proc. World Soybean Res. Conf., VI, Chicago, IL.* pp. 4-7.

Buzzell, R., Tu, J. 1984. Inheritance of soybean resistance to soybean mosaic virus. *Journal of Heredity*, **75**(1), 82-82.

Carrington, J.C., Herndon, K.L. 1992. Characterization of the potyviral HC-Pro autoproteolytic cleavage site. *Virology*, **187**(1), 308-315.

Catalanotto, C., Azzalin, G., Macino, G., Cogoni, C. 2000. Transcription: Gene silencing in worms and fungi. *Nature*, **404**(6775), 245-245.

Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E., Staskawicz, B.J. 1997. NDR1, a pathogen-induced component required for Arabidopsis disease resistance. *Science*, **278**(5345), 1963-1965.

Chandra-Shekara, A., Navarre, D., Kachroo, A., Kang, H.G., Klessig, D., Kachroo, P. 2004. Signaling requirements and role of salicylic acid in HRT-and rrt-mediated resistance to turnip crinkle virus in Arabidopsis. *The Plant Journal*, **40**(5), 647-659.

Chaparro-Garcia, A., Wilkinson, R.C., Gimenez-Ibanez, S., Findlay, K., Coffey, M.D., Zipfel, C., Rathjen, J.P., Kamoun, S., Schornack, S. 2011. The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen Phytophthora infestans in Nicotiana benthamiana. *PLoS One*, **6**(1), e16608.

Chen, H., Zhang, L., Yu, K., Wang, A. 2015. Pathogenesis of Soybean mosaic virus in soybean carrying Rsv1 gene is associated with miRNA and siRNA pathways, and breakdown of AGO1 homeostasis. *Virology*, **476**, 395-404.

Chen, P., Buss, G., Roane, C., Tolin, S. 1991. Allelism among genes for resistance to soybean mosaic virus in strain-differential soybean cultivars. *Crop science*, **31**(2), 305-309.

Chen, P., Buss, G., Roane, C., Tolin, S. 1994. Inheritance in soybean of resistant and necrotic reactions to soybean mosaic virus strains. *Crop science*, **34**(2), 414-422.

Chen, P., Buss, G., Tolin, S. 1993. Resistance to soybean mosaic virus conferred by two independent dominant genes in PI 486355. *Journal of Heredity*, **84**(1), 25-28.

Chen, P., Choi, C., Rao, G., Khurana, S., Lenardon, S. 2008. Soybean mosaic virus. *Characterization, diagnosis & management of plant viruses. Volume 1: industrial crops*, 389-422.

Cheng, Y.-Q., Liu, Z.-M., Xu, J., Zhou, T., Wang, M., Chen, Y.-T., Li, H.-F., Fan, Z.-F. 2008. HC-Pro protein of sugar cane mosaic virus interacts specifically with maize ferredoxin-5 in vitro and in planta. *Journal of general virology*, **89**(8), 2046-2054.

Chiasson, D., Ekengren, S.K., Martin, G.B., Dobney, S.L., Snedden, W.A. 2005. Calmodulin-like proteins from Arabidopsis and tomato are involved in host defense against Pseudomonas syringae pv. tomato. *Plant molecular biology*, **58**(6), 887-897.

Chinchilla, D., Shan, L., He, P., de Vries, S., Kemmerling, B. 2009. One for all: the receptor-associated kinase BAK1. *Trends in plant science*, **14**(10), 535-541.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D., Felix, G., Boller, T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, **448**(7152), 497-500.

CHIU, M.H., Chen, I., Baulcombe, D.C., TSAI, C.H. 2010. The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation through the proteasome pathway. *Molecular plant pathology*, **11**(5), 641-649.

Cho, E.-K., Chung, B.-J., Lee, S.-H. 1977. Studies on identification and classification of soybean virus diseases in Korea. II. Etiology of a necrotic disease of Glycine max. *Plant Disease Reporter*, **61**(4), 313-317.

Cho, E.-K., Goodman, R.M. 1979. Strains of soybean mosaic virus: classification based on virulence in resistant soybean cultivars. *Phytopathology*, **69**(5), 467-470.

Choi, I.-R., Stenger, D.C., French, R. 2000. Multiple interactions among proteins encoded by the mite-transmitted wheat streak mosaic tritimovirus. *Virology*, **267**(2), 185-198.

Chowda-Reddy, R., Sun, H., Hill, J.H., Poysa, V., Wang, A. 2011. Simultaneous mutations in multi-viral proteins are required for Soybean mosaic virus to gain virulence on soybean genotypes carrying different R genes. *PloS one*, **6**(11), e28342.

Chung, B.Y.-W., Miller, W.A., Atkins, J.F., Firth, A.E. 2008. An overlapping essential gene in the Potyviridae. *Proceedings of the National Academy of Sciences*, **105**(15), 5897-5902.

Cronin, S., Verchot, J., Haldeman-Cahill, R., Schaad, M.C., Carrington, J.C. 1995. Long-distance movement factor: a transport function of the potyvirus helper component proteinase. *The Plant Cell*, **7**(5), 549-559.

Dangl, J.L., Jones, J.D. 2001. Plant pathogens and integrated defence responses to infection. *nature*, **411**(6839), 826-833.

del Toro, F., Fernández, F.T., Tilsner, J., Wright, K.M., Tenllado, F., Chung, B.N., Praveen, S., Canto, T. 2014. Potato virus Y HCPro localization at distinct, dynamically related and environment-influenced structures in the cell cytoplasm. *Molecular Plant-Microbe Interactions*, **27**(12), 1331-1343.

Delauré, S.L., Van Hemelrijck, W., De Bolle, M.F., Cammue, B.P., De Coninck, B.M. 2008. Building up plant defenses by breaking down proteins. *Plant Science*, **174**(4), 375-385.

Devoto, A., Muskett, P.R., Shirasu, K. 2003. Role of ubiquitination in the regulation of plant defence against pathogens. *Current opinion in plant biology*, **6**(4), 307-311.

Ding, S.-W., Voinnet, O. 2007. Antiviral immunity directed by small RNAs. *Cell*, **130**(3), 413-426.

Dodds, P.N., Rathjen, J.P. 2010. Plant immunity: towards an integrated view of plant–pathogen interactions. *Nature Reviews Genetics*, **11**(8), 539-548.

Du, L., Ali, G.S., Simons, K.A., Hou, J., Yang, T., Reddy, A., Poovaiah, B. 2009. Ca2+/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature*, **457**(7233), 1154-1158.

Ebhardt, H.A., Thi, E.P., Wang, M.-B., Unrau, P.J. 2005. Extensive 3′ modification of plant small RNAs is modulated by helper component-proteinase expression. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(38), 13398-13403.

Eggenberger, A., Hajimorad, M., Hill, J. 2008. Gain of virulence on Rsv1-genotype soybean by an avirulent Soybean mosaic virus requires concurrent mutations in both P3 and HC-Pro. *Molecular plant-microbe interactions*, **21**(7), 931-936.

Esposito, D., Garvey, L.A., Chakiath, C.S. 2009. Gateway cloning for protein expression. *High Throughput Protein Expression and Purification: Methods and Protocols*, 31-54.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J., Parker, J.E. 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proceedings of the National Academy of Sciences*, **96**(6), 3292-3297.

Fang, Y., Spector, D.L. 2007. Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants. *Current Biology*, **17**(9), 818-823.

Fu, D.-Q., Ghabrial, S., Kachroo, A. 2009. GmRAR1 and GmSGT1 are required for basal, R gene-mediated and systemic acquired resistance in soybean. *Molecular plant-microbe interactions*, **22**(1), 86-95.

Gallie, D.R., Browning, K.S. 2001. eIF4G functionally differs from eIFiso4G in promoting internal initiation, cap-independent translation, and translation of structured mRNAs. *Journal of Biological Chemistry*, **276**(40), 36951-36960.

Gallois, J.-L., Charron, C., Sanchez, F., Pagny, G., Houvenaghel, M.-C., Moretti, A., Ponz, F., Revers, F., Caranta, C., German-Retana, S. 2010. Single amino acid changes in the turnip mosaic virus viral genome-linked protein (VPg) confer virulence towards Arabidopsis thaliana mutants knocked out for eukaryotic initiation factors eIF (iso) 4E and eIF (iso) 4G. *Journal of general virology*, **91**(1), 288-293.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y.T., Chen, S., Li, X. 2009. Regulation of cell death and innate immunity by two receptor-like kinases in Arabidopsis. *Cell host & microbe*, **6**(1), 34-44.

Gaur, R.K., Hohn, T., Sharma, P. 2013. *Plant Virus-host Interaction: Molecular Approaches and Viral Evolution*. Elsevier.

Gautier-Bert, K., Murol, B., Jarrousse, A.-S., Ballut, L., Badaoui, S., Petit, F., Schmid, H.-P. 2003. Substrate affinity and substrate specificity of proteasomes with RNase activity. *Molecular biology reports*, **30**(1), 1-7.

Giesler, L.J., Ghabrial, S.A., Hunt, T.E., Hill, J.H. 2002. Bean pod mottle virus: a threat to US soybean production. *Plant Disease*, **86**(12), 1280-1289.

Gómez-Gómez, L., Boller, T. 2000. FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell*, **5**(6), 1003-1011.

Goritschnig, S., Zhang, Y., Li, X. 2007. The ubiquitin pathway is required for innate immunity in Arabidopsis. *The Plant Journal*, **49**(3), 540-551.

Govier, D., Kassanis, B., Pirone, T. 1977. Partial purification and characterization of the potato virus Y helper component. *Virology*, **78**(1), 306-314.

Gunduz, I., Buss, G., Ma, G., Chen, P., Tolin, S. 2001. Genetic Analysis of Resistance to in OX670 and Harosoy Soybean. *Crop science*, **41**(6), 1785-1791.

Gunduz, I., Buss, G.R., Chen, P., Tolin, S.A. 2004. Genetic and phenotypic analysis of Soybean mosaic virus resistance in PI 88788 soybean. *Phytopathology*, **94**(7), 687-692.

Guo, D., Merits, A., Saarma, M. 1999. Self-association and mapping of interaction domains of helper component-proteinase of potato A potyvirus. *Journal of General Virology*, **80**(5), 1127-1131.

Guo, D., Rajamäki, M.-L., Saarma, M., Valkonen, J.P. 2001. Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *Journal of general virology*, **82**(4), 935-939.

Guo, D., Spetz, C., Saarma, M., Valkonen, J.P. 2003. Two potato proteins, including a novel RING finger protein (HIP1), interact with the potyviral multifunctional protein HCpro. *Molecular plant-microbe interactions*, **16**(5), 405-410.

Guo, H., Song, X., Xie, C., Huo, Y., Zhang, F., Chen, X., Geng, Y., Fang, R. 2013. Rice yellow stunt rhabdovirus protein 6 suppresses systemic RNA silencing by blocking RDR6-mediated secondary siRNA synthesis. *Molecular Plant-Microbe Interactions*, **26**(8), 927-936.

Haas, G., Azevedo, J., Moissiard, G., Geldreich, A., Himber, C., Bureau, M., Fukuhara, T., Keller, M., Voinnet, O. 2008. Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *The EMBO Journal*, **27**(15), 2102-2112.

Hajimorad, M., Eggenberger, A., Hill, J. 2008. Adaptation of Soybean mosaic virus avirulent chimeras containing P3 sequences from virulent strains to Rsv1-genotype soybeans is mediated by mutations in HC-Pro. *Molecular plant-microbe interactions*, **21**(7), 937-946.

Hajimorad, M., Eggenberger, A., Hill, J. 2005. Loss and gain of elicitor function of Soybean mosaic virus G7 provoking Rsv1-mediated lethal systemic hypersensitive response maps to P3. *Journal of virology*, **79**(2), 1215-1222.

Hajimorad, M., Eggenberger, A., Hill, J. 2006. Strain-specific P3 of Soybean mosaic virus elicits Rsv1-mediated extreme resistance, but absence of P3 elicitor function alone is insufficient for virulence on Rsv1-genotype soybean. *Virology*, **345**(1), 156-166.

Hajimorad, M., Wen, R.-H., Eggenberger, A., Hill, J., Maroof, M.S. 2011. Experimental adaptation of an RNA virus mimics natural evolution. *Journal of virology*, **85**(6), 2557-2564.

Halter, T., Imkampe, J., Mazzotta, S., Wierzba, M., Postel, S., Bücherl, C., Kiefer, C., Stahl, M., Chinchilla, D., Wang, X. 2014. The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. *Current Biology*, **24**(2), 134-143.

Hammond, S.M. 2005. Dicing and slicing. *FEBS letters*, **579**(26), 5822-5829.

Han, M.-H., Goud, S., Song, L., Fedoroff, N. 2004. The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proceedings of the National Academy of Sciences of the United States of America*, **101**(4), 1093-1098.

Hartwig, T., Corvalan, C., Best, N.B., Budka, J.S., Zhu, J.-Y., Choe, S., Schulz, B. 2012. Propiconazole is a specific and accessible brassinosteroid (BR) biosynthesis inhibitor for Arabidopsis and maize. *PLoS One*, **7**(5), e36625.

Havelda, Z., Várallyay, É., Válóczi, A., Burgyán, J. 2008. Plant virus infection-induced persistent host gene downregulation in systemically infected leaves. *The Plant Journal*, **55**(2), 278-288.

Hayes, A., Jeong, S., Gore, M., Yu, Y., Buss, G., Tolin, S., Maroof, M.S. 2004. Recombination within a nucleotide-binding-site/leucine-rich-repeat gene cluster produces new variants conditioning resistance to soybean mosaic virus in soybeans. *Genetics*, **166**(1), 493-503.

He, Z., Wang, Z.-Y., Li, J., Zhu, Q., Lamb, C., Ronald, P., Chory, J. 2000. Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science*, **288**(5475), 2360-2363.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., Rathjen, J.P. 2007. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proceedings of the National Academy of Sciences*, **104**(29), 12217-12222.

Hema, M., Sreenivasulu, P., Patil, B.L., Kumar, P.L., Reddy, D.V. 2013. Tropical Food Legumes: Virus Diseases of Economic Importance and Their Control. *Advances in virus research*, 431.

Hill, J.H. 2003. Soybean. in: *Virus and virus-like diseases of major crops in developing countries*, Springer, pp. 377-395.

Hill, J.H., Whitham, S.A. 2014. Control of virus diseases in soybeans. *Adv. Virus Res*, **90**, 355-390.

Horwich, M.D., Li, C., Matranga, C., Vagin, V., Farley, G., Wang, P., Zamore, P.D. 2007. The Drosophila RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Current Biology*, **17**(14), 1265-1272.

Huet, H., Gal-On, A., Meir, E., Lecoq, H., Raccah, B. 1994. Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility. *The Journal of general virology*, **75**, 1407-1414.

Huffaker, A., Ryan, C.A. 2007. Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. *Proceedings of the National Academy of Sciences*, **104**(25), 10732-10736.

Hunst, P., Tolin, S. 1982. Isolation and comparison of two strains of soybean mosaic virus. *Phytopathology*, **72**(7), 710-713.

Jamous, R.M., Boonrod, K., Fuellgrabe, M.W., Ali-Shtayeh, M.S., Krczal, G., Wassenegger, M. 2011. The helper component-proteinase of the Zucchini yellow mosaic virus inhibits the Hua Enhancer 1 methyltransferase activity in vitro. *Journal of general virology*, **92**(9), 2222-2226.

Jayaram, C., Hill, J.H., Miller, W.A. 1992. Complete nucleotide sequences of two soybean mosaic virus strains differentiated by response of soybean containing the Rsv resistance gene. *Journal of general virology*, **73**(8), 2067-2077.

Jin, Y., Ma, D., Dong, J., Li, D., Deng, C., Jin, J., Wang, T. 2007. The HC-pro protein of potato virus Y interacts with NtMinD of tobacco. *Molecular Plant-Microbe Interactions*, **20**(12), 1505-1511.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., Glazebrook, J. 1999. Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proceedings of the National Academy of Sciences*, **96**(23), 13583-13588.

John, K.M., Natarajan, S., Luthria, D.L. 2016. Metabolite changes in nine different soybean varieties grown under field and greenhouse conditions. *Food Chemistry*, **211**, 347-355.

Jones, J.D., Dangl, J.L. 2006. The plant immune system. *Nature*, **444**(7117), 323-329.

Jordá, L., Sopeña-Torres, S., Escudero, V., Nuñez-Corcuera, B., Delgado-Cerezo, M., Torii, K.U., Molina, A. 2016. ERECTA and BAK1 Receptor Like Kinases Interact to Regulate Immune Responses in Arabidopsis. *Frontiers in Plant Science*, **7**.

Kachroo, A., Ghabrial, S. 2012. Virus-induced gene silencing in soybean. *Antiviral Resistance in Plants: Methods and Protocols*, 287-297.

Kasschau, K.D., Carrington, J.C. 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell*, **95**(4), 461-470.

Kasschau, K.D., Cronin, S., Carrington, J.C. 1997. Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component–proteinase. *Virology*, **228**(2), 251-262.

Kegler, H., Meyer, U. 1987. Characterization and evaluation of quantitative virus resistance in plants:(Review). *Archives of Phytopathology & Plant Protection*, **23**(5), 343-348.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Müssig, C. 2007. The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. *Current Biology*, **17**(13), 1116-1122.

Kennedy, B., Cooper, R. 1967. Association of virus infection with mottling of Soybean seed coats. *Phytopathology*, **57**(1), 35-37.

Kiihl, R.A., Hartwig, E. 1979. Inheritance of reaction to soybean mosaic virus in soybeans. *Crop Science*, **19**(3), 372-375.

Kim, H.S., Delaney, T.P. 2002. Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *The Plant Cell*, **14**(7), 1469-1482.

King, A.M. 2011. *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier.

Kinoshita, T., Caño-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S., Chory, J. 2005. Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature*, **433**(7022), 167-171.

Kirino, Y., Mourelatos, Z. 2007. 2′-O-methyl modification in mouse piRNAs and its methylase. *Nucleic acids symposium series*. Oxford Univ Press. pp. 417-418.

Kolte, S. 1984. *Diseases of annual edible oilseed crops. Volume I: Peanut diseases*. CRC Press, Inc.

Kørner, C.J., Klauser, D., Niehl, A., Domínguez-Ferreras, A., Chinchilla, D., Boller, T., Heinlein, M., Hann, D.R. 2013. The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. *Molecular plant-microbe interactions*, **26**(11), 1271-1280.

Kotchoni, S.O., Gachomo, E.W. 2006. The reactive oxygen species network pathways: an essential prerequisite for perception of pathogen attack and the acquired disease resistance in plants. *Journal of biosciences*, **31**(3), 389-404.

Krol, E., Mentzel, T., Chinchilla, D., Boller, T., Felix, G., Kemmerling, B., Postel, S., Arents, M., Jeworutzki, E., Al-Rasheid, K.A. 2010. Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. *Journal of Biological Chemistry*, **285**(18), 13471-13479.

Kurihara, Y., Takashi, Y., Watanabe, Y. 2006. The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. *Rna*, **12**(2), 206-212.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.P., Dolja, V.V., Calvino, L.F., López-Moya, J.J., Burgyán, J. 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *The EMBO journal*, **25**(12), 2768-2780.

Li, J., Chory, J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell*, **90**(5), 929-938.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., Walker, J.C. 2002. BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell*, **110**(2), 213-222.

Lim, H.S., Ko, T.S., Hobbs, H.A., Lambert, K.N., Yu, J.M., McCoppin, N.K., Korban, S.S., Hartman, G.L., Domier, L.L. 2007. Soybean mosaic virus helper component-protease alters leaf morphology and reduces seed production in transgenic soybean plants. *Phytopathology*, **97**(3), 366-372.

Lim, S. 1985. Resistance to soybean mosaic virus in soybeans. *Phytopathology*, **75**(2), 199-201.

Lin, W., Li, B., Lu, D., Chen, S., Zhu, N., He, P., Shan, L. 2014. Tyrosine phosphorylation of protein kinase complex BAK1/BIK1 mediates Arabidopsis innate immunity. *Proceedings of the National Academy of Sciences*, **111**(9), 3632-3637.

Llave, C., Kasschau, K.D., Carrington, J.C. 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences*, **97**(24), 13401-13406.

Lobbes, D., Rallapalli, G., Schmidt, D.D., Martin, C., Clarke, J. 2006. SERRATE: a new player on the plant microRNA scene. *EMBO reports*, **7**(10), 1052-1058.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., He, P. 2010. A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proceedings of the National Academy of Sciences*, **107**(1), 496-501.

Ma, G. 1995. Genetic analysis of soybean reactions to soybean mosaic virus.

Ma, G., Buss, G., Tolin, S. 1994. Inheritance of lethal necrosis to soybean mosaic virus in PI507389 Soybean. *1994 Agronomy Abstracts. ASA, Madison, WI*, 106.

Ma, G., Chen, P., Buss, G., Tolin, S. 1995. Genetic characteristics of two genes for resistance to soybean mosaic virus in PI486355 soybean. *Theoretical and Applied Genetics*, **91**(6-7), 907-914.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., Dangl, J.L. 2003. Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell*, **112**(3), 379-389.

Maia, I.G., Bernardi, F. 1996. Nucleic acid-binding properties of a bacterially expressed potato virus Y helper component-proteinase. *Journal of General Virology*, **77**(5), 869-877.

Maia, I.G., Haenni, A.-L., Bernardi, F. 1996. Potyviral HC-Pro: a multifunctional protein. *Journal of General Virology*, **77**(7), 1335-1341.

Mallory, A.C., Bouché, N. 2008. MicroRNA-directed regulation: to cleave or not to cleave. *Trends in plant science*, **13**(7), 359-367.

Martin, G.B., Bogdanove, A.J., Sessa, G. 2003. Understanding the functions of plant disease resistance proteins. *Annual review of plant biology*, **54**(1), 23-61.

Martin, K., Kopperud, K., Chakrabarty, R., Banerjee, R., Brooks, R., Goodin, M.M. 2009. Transient expression in Nicotiana benthamiana fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. *The Plant Journal*, **59**(1), 150-162.

Matzke, M., Matzke, A.J., Kooter, J.M. 2001. RNA: guiding gene silencing. *Science*, **293**(5532), 1080-1083.

Mérai, Z., Kerényi, Z., Kertész, S., Magna, M., Lakatos, L., Silhavy, D. 2006. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *Journal of Virology*, **80**(12), 5747-5756.

Merits, A., Guo, D., Järvekülg, L., Saarma, M. 1999. Biochemical and genetic evidence for interactions between potato A potyvirus-encoded proteins P1 and P3 and proteins of the putative replication complex. *Virology*, **263**(1), 15-22.

Molnár, A., Csorba, T., Lakatos, L., Várallyay, É., Lacomme, C., Burgyán, J. 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *Journal of Virology*, **79**(12), 7812-7818.

Morel, J.-B., Dangl, J.L. 1997. The hypersensitive response and the induction of cell death in plants. *Cell death and differentiation*, **4**(8), 671-683.

Moury, B., Caromel, B., Johansen, E., Simon, V., Chauvin, L., Jacquot, E., Kerlan, C., Lefebvre, V. 2011. The helper component proteinase cistron of Potato virus Y induces hypersensitivity and resistance in potato genotypes carrying dominant resistance genes on chromosome IV. *Molecular plant-microbe interactions*, **24**(7), 787-797.

Mural, R.V., Liu, Y., Rosebrock, T.R., Brady, J.J., Hamera, S., Connor, R.A., Martin, G.B., Zeng, L. 2013. The Tomato Fni3 Lysine-63–Specific Ubiquitin-Conjugating Enzyme and Suv Ubiquitin E2 Variant Positively Regulate Plant Immunity. *The Plant Cell*, **25**(9), 3615-3631.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., Yoshida, S. 2003. Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *The Plant Journal*, **33**(5), 887-898.

Nam, K.H., Li, J. 2002. BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell*, **110**(2), 203-212.

Nimchuk, Z., Eulgem, T., Holt Iii, B.F., Dangl, J.L. 2003. Recognition and response in the plant immune system. *Annual review of genetics*, **37**(1), 579-609.

Nomura, H., Komori, T., Kobori, M., Nakahira, Y., Shiina, T. 2008. Evidence for chloroplast control of external Ca2+-induced cytosolic Ca2+ transients and stomatal closure. *The Plant Journal*, **53**(6), 988-998.

Nomura, H., Komori, T., Uemura, S., Kanda, Y., Shimotani, K., Nakai, K., Furuichi, T., Takebayashi, K., Sugimoto, T., Sano, S. 2012. Chloroplast-mediated activation of plant immune signalling in Arabidopsis. *Nature Communications*, **3**, 926.

Oh, M.-H., Wang, X., Wu, X., Zhao, Y., Clouse, S.D., Huber, S.C. 2010. Autophosphorylation of Tyr-610 in the receptor kinase BAK1 plays a role in brassinosteroid signaling and basal defense gene expression. *Proceedings of the National Academy of Sciences*, **107**(41), 17827-17832.

Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D., Bron, P. 2003. Structural characterization of HC-Pro, a plant virus multifunctional protein. *Journal of Biological Chemistry*, **278**(26), 23753-23761.

Pruss, G., Ge, X., Shi, X.M., Carrington, J.C., Vance, V.B. 1997. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *The Plant Cell*, **9**(6), 859-868.

Pumplin, N., Voinnet, O. 2013. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nature Reviews Microbiology*, **11**(11), 745-760.

Ramachandran, V., Chen, X. 2008. Small RNA metabolism in Arabidopsis. *Trends in plant science*, **13**(7), 368-374.

Redondo, E., Krause-Sakate, R., Yang, S.-J., Lot, H., Le Gall, O., Candresse, T. 2001. Lettuce mosaic virus pathogenicity determinants in susceptible and tolerant lettuce cultivars map to different regions of the viral genome. *Molecular plant-microbe interactions*, **14**(6), 804-810.

Riechmann, J.L., Lain, S., García, J.A. 1992. Highlights and prospects of potyvirus molecular biology. *Journal of General Virology*, **73**(1), 1-16.

Rojas, M.R., Zerbini, F.M., Allison, R.F., Gilbertson, R.L., Lucas, W.J. 1997. Capsid protein and helper component-proteinase function as potyvirus cell-to-cell movement proteins. *Virology*, **237**(2), 283-295.

Roudet-Tavert, G., German-Retana, S., Delaunay, T., Delécolle, B., Candresse, T., Le Gall, O. 2002. Interaction between potyvirus helper component-proteinase and capsid protein in infected plants. *Journal of general virology*, **83**(7), 1765-1770.

Roudet-Tavert, G., Michon, T., Walter, J., Delaunay, T., Redondo, E., Le Gall, O. 2007. Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro. *Journal of general virology*, **88**(3), 1029-1033.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., Malinovsky, F.G., Tör, M., de Vries, S., Zipfel, C. 2011. The Arabidopsis leucine-rich repeat receptor–like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *The Plant Cell*, **23**(6), 2440-2455.

Sáenz, P., Salvador, B., Simón-Mateo, C., Kasschau, K.D., Carrington, J.C., García, J.A. 2002. Host-specific involvement of the HC protein in the long-distance movement of potyviruses. *Journal of virology*, **76**(4), 1922-1931.

Saurin, A.J., Borden, K.L., Boddy, M.N., Freemont, P.S. 1996. Does this have a familiar RING? *Trends in biochemical sciences*, **21**(6), 208-214.

Schmidt, E., Guzzo, F., Toonen, M., De Vries, S. 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development*, **124**(10), 2049-2062.

Schulze, B., Mentzel, T., Jehle, A.K., Mueller, K., Beeler, S., Boller, T., Felix, G., Chinchilla, D. 2010. Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *Journal of Biological Chemistry*, **285**(13), 9444-9451.

Schulze-Lefert, P. 2004. Knocking on the heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall. *Current opinion in plant biology*, **7**(4), 377-383.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., Zipfel, C. 2011. Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS Genet*, **7**(4), e1002046.

Selote, D., Robin, G.P., Kachroo, A. 2013. GmRIN4 protein family members function nonredundantly in soybean race-specific resistance against Pseudomonas syringae. *New Phytologist*, **197**(4), 1225-1235.

Selote, D., Shine, M., Robin, G.P., Kachroo, A. 2014. Soybean NDR1-like proteins bind pathogen effectors and regulate resistance signaling. *New Phytologist*, **202**(2), 485-498.

Seo, J.-K., Kwon, S.-J., Cho, W.K., Choi, H.-S., Kim, K.-H. 2014. Type 2C protein phosphatase is a key regulator of antiviral extreme resistance limiting virus spread. *Scientific reports*, **4**.

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nürnberger, T., Martin, G.B., Sheen, J. 2008. Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell host & microbe*, **4**(1), 17-27.

Shi, X.M., Miller, H., Verchot, J., Carrington, J.C., Vance, V.B. 1997. Mutations in the region encoding the central domain of helper component-proteinase (HC-Pro) eliminate potato virus X/potyviral synergism. *Virology*, **231**(1), 35-42.

Shiboleth, Y.M., Haronsky, E., Leibman, D., Arazi, T., Wassenegger, M., Whitham, S.A., Gaba, V., Gal-On, A. 2007. The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. *Journal of virology*, **81**(23), 13135-13148.

Shirsekar, G., Dai, L., Hu, Y., Wang, X., Zeng, L., Wang, G.-L. 2010. Role of ubiquitination in plant innate immunity and pathogen virulence. *Journal of Plant Biology*, **53**(1), 10-18.

Shiu, S.-H., Bleecker, A.B. 2001a. Plant receptor-like kinase gene family: diversity, function, and signaling. *Sci stke*, **113**(re22), 1-13.

Shiu, S.-H., Bleecker, A.B. 2001b. Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proceedings of the National Academy of Sciences*, **98**(19), 10763-10768.

Shukla, D.D., Frcnkel, M., Ward, C.W. 1991. Structure and function of the potyvirus genome with special reference to the coat protein coding region. *Canadian Journal of Plant Pathology*, **13**(2), 178-191.

Smalle, J., Vierstra, R.D. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.*, **55**, 555-590.

Suh, S.J., Bowman, B.C., Jeong, N., Yang, K., Kastl, C., Tolin, S.A., Maroof, M., Jeong, S.-C. 2011. The Rsv3 locus conferring resistance to soybean mosaic virus is associated with a cluster of coiled-coil nucleotide-binding leucine-rich repeat genes. *The Plant Genome*, **4**(1), 55-64.

Sun, Y., Fan, X.-Y., Cao, D.-M., Tang, W., He, K., Zhu, J.-Y., He, J.-X., Bai, M.-Y., Zhu, S., Oh, E. 2010. Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. *Developmental cell*, **19**(5), 765-777.

Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A., Cornelissen, B.J. 2002. The tomato R gene products I-2 and MI-1 are functional ATP binding proteins with ATPase activity. *The Plant Cell*, **14**(11), 2929-2939.

Thornbury, D., Patterson, C., Dessens, J., Pirone, T. 1990. Comparative sequence of the helper component (HC) region of potato virus Y and a HC-defective strain, potato virus C. *Virology*, **178**(2), 573-578.

Thornbury, D.W., Hellmann, G.M., Rhoads, R.E., Pirone, T.P. 1985. Purification and characterization of potyvirus helper component. *Virology*, **144**(1), 260-267.

Tolin, S.A., Lacy, G.H. 2004. Viral, bacterial, and phytoplasmal diseases of soybean. *Soybeans: Improvement, Production, and Uses*(soybeansimprove), 765-819.

Trujillo, M., Shirasu, K. 2010. Ubiquitination in plant immunity. *Current opinion in plant biology*, **13**(4), 402-408.

Tu, J., Buzzell, R. 1987. Stem-tip necrosis: a hypersensitive, temperature dependent dominant gene reaction of soybean to infection by soybean mosaic virus. *Canadian journal of plant science*, **67**(3), 661-665.

Urcuqui-Inchima, S., Haenni, A.-L., Bernardi, F. 2001. Potyvirus proteins: a wealth of functions. *Virus research*, **74**(1), 157-175.

Urcuqui-Inchima, S., Maia, I.G., Arruda, P., Haenni, A.-L., Bernardi, F. 2000. Deletion mapping of the potyviral helper component-proteinase reveals two regions involved in RNA binding. *Virology*, **268**(1), 104-111.

Urcuqui-Inchima, S., Maia, I.G., Drugeon, G., Haenni, A.-L., Bernardi, F. 1999. Effect of mutations within the Cys-rich region of potyvirus helper component-proteinase on self-interaction. *Journal of general virology*, **80**(11), 2809-2812.

Van Der Biezen, E.A., Jones, J.D. 1998. Plant disease-resistance proteins and the gene-for-gene concept. *Trends in biochemical sciences*, **23**(12), 454-456.

Várallyay, É., Havelda, Z. 2013. Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level. *Molecular plant pathology*, **14**(6), 567-575.

Várallyay, É., Válóczi, A., Ágyi, Á., Burgyán, J., Havelda, Z. 2010. Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation. *The EMBO journal*, **29**(20), 3507-3519.

Vaucheret, H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes & Development*, **20**(7), 759-771.

Vaucheret, H., Mallory, A.C., Bartel, D.P. 2006. AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Molecular cell*, **22**(1), 129-136.

Vidal, S., Cabrera, H., Andersson, R.A., Fredriksson, A., Valkonen, J.P. 2002. Potato gene Y-1 is an N gene homolog that confers cell death upon infection with potato virus Y. *Molecular plant-microbe interactions*, **15**(7), 717-727.

Vierstra, R.D. 2003. The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends in plant science*, **8**(3), 135-142.

Voinnet, O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell*, **136**(4), 669-687.

Wang, J., Shine, M., Gao, Q.-M., Navarre, D., Jiang, W., Liu, C., Chen, Q., Hu, G., Kachroo, A. 2014. Enhanced Disease Susceptibility1 mediates pathogen resistance and virulence function of a bacterial effector in soybean. *Plant physiology*, **165**(3), 1269-1284.

Wang, L., Tsuda, K., Sato, M., Cohen, J.D., Katagiri, F., Glazebrook, J. 2009. Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against Pseudomonas syringae. *PLoS Pathog*, **5**(2), e1000301.

Wang, R., Kritzman, A., Hershman, D., Ghabrial, S. 2006. Aphis glycines as a vector of persistently and nonpersistently transmitted viruses and potential risks for soybean and other crops. *Plant Disease*, **90**(7), 920-926.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., Clouse, S.D. 2008. Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Developmental cell*, **15**(2), 220-235.

Waterhouse, P.M., Wang, M.-B., Lough, T. 2001. Gene silencing as an adaptive defence against viruses. *Nature*, **411**(6839), 834-842.

Welchman, R.L., Gordon, C., Mayer, R.J. 2005. Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature reviews Molecular cell biology*, **6**(8), 599-609.

Wen, R.-H., Khatabi, B., Ashfield, T., Maroof, M.S., Hajimorad, M. 2013. The HC-Pro and P3 cistrons of an avirulent Soybean mosaic virus are recognized by different resistance genes at the complex Rsv1 locus. *Molecular Plant-Microbe Interactions*, **26**(2), 203-215.

WEN, R.H., Maroof, M.S., Hajimorad, M. 2011. Amino acid changes in P3, and not the overlapping pipo-encoded protein, determine virulence of Soybean mosaic virus on functionally immune Rsv1-genotype soybean. *Molecular plant pathology*, **12**(8), 799-807.

Whitham, S., Dinesh-Kumar, S., Choi, D., Hehl, R., Corr, C., Baker, B. 1994. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell*, **78**(6), 1101-1115.

Wiermer, M., Feys, B.J., Parker, J.E. 2005. Plant immunity: the EDS1 regulatory node. *Current opinion in plant biology*, **8**(4), 383-389.

Wrather, J., Koenning, S. 2011. Soybean disease loss estimates for the United States, 1996–2010. Delta Research Center: agriculture experiment station. University of Missouri, College of agriculture, food and natural resources. *Food and Natural Resources*.

Wrather, J.A., Anderson, T., Arsyad, D., Gai, J., Ploper, L., Porta-Puglia, A., Ram, H., Yorinori, J. 1997. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. *Plant disease*, **81**(1), 107-110.

Yambao, M.L.M., Masuta, C., Nakahara, K., Uyeda, I. 2003. The central and C-terminal domains of VPg of Clover yellow vein virus are important for VPg–HCPro and VPg–VPg interactions. *Journal of general virology*, **84**(10), 2861-2869.

Yang, Z., Ebright, Y.W., Yu, B., Chen, X. 2006. HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2′ OH of the 3′ terminal nucleotide. *Nucleic acids research*, **34**(2), 667-675.

Yin, X., Wang, J., Cheng, H., Wang, X., Yu, D. 2013. Detection and evolutionary analysis of soybean miRNAs responsive to soybean mosaic virus. *Planta*, **237**(5), 1213-1225.

Yu, B., Chapman, E.J., Yang, Z., Carrington, J.C., Chen, X. 2006. Transgenically expressed viral RNA silencing suppressors interfere with microRNA methylation in Arabidopsis. *FEBS letters*, **580**(13), 3117-3120.

Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R.W., Steward, R., Chen, X. 2005. Methylation as a crucial step in plant microRNA biogenesis. *Science*, **307**(5711), 932-935.

Zhang, C., Ghabrial, S.A. 2006a. Development of Bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. *Virology*, **344**(2), 401-411.

Zhang, C., Ghabrial, S.A. 2006b. Development of< i> Bean pod mottle virus</i>-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. *Virology*, **344**(2), 401-411.

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S. 2010. Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a Pseudomonas syringae effector. *Cell host & microbe*, **7**(4), 290-301.

Zhou, G.-A., Chang, R.-Z., Qiu, L.-J. 2010. Overexpression of soybean ubiquitin-conjugating enzyme gene GmUBC2 confers enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in Arabidopsis. *Plant molecular biology*, **72**(4-5), 357-367.

Zhu, S., Jeong, R.-D., Venugopal, S.C., Lapchyk, L., Navarre, D., Kachroo, A., Kachroo, P. 2011. SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. *PLoS Pathog*, **7**(11), e1002318.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., Felix, G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell*, **125**(4), 749-760.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., Boller, T. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature*, **428**(6984), 764-767.

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