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농학박사학위논문

콩 모자이크 바이러스 감염 및 복제에 대한 저항성 관련 엽록체 단백질의 특성 규명

Characterization of resistance-related chloroplast proteins of soybean against soybean mosaic virus infection and replication

2023년 8월

서울대학교 대학원
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Characterization of resistance-related chloroplast proteins of soybean against soybean mosaic virus infection and replication

A dissertation submitted in partial
fulfillment of the requirement for
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By

JOHN BWALYA

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of resistance-related chloroplast proteins of soybean against soybean mosaic virus infection and replication

UNDER THE DIRECTION OF DR. KOOK-HYUNG KIM

SUBMITTED TO THE FACULTY OF GRADUATE SCHOOL OF

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Characterization of resistance-related chloroplast proteins of soybean against soybean mosaic virus infection and replication

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GENERAL ABSTRACT

Accumulating evidence attests that chloroplast-related genes are involved in plant–virus interactions. However, the involvement of photosynthesis-related genes in plant immunity is largely unexplored. Analysis of RNA-Seq data from the soybean cultivar L29, which carries the Rsv3 resistance gene, showed that several chloroplast-related genes were strongly induced in response to infection with an avirulent strain of soybean mosaic virus (SMV), G5H, but were weakly induced in response to a virulent strain, G7H.

For further analysis, we selected the PSaC gene from the photosystem I and the ATP-synthase α-subunit (ATPsyn-α) gene whose encoded protein is part of the ATP-synthase complex. Overexpression of

either gene within the G7H genome reduced virus levels in the susceptible cultivar Lee74 (rsv3-null). This result was confirmed by transiently expressing both genes in Nicotiana benthamiana followed by G7H infection. Both proteins localized in the chloroplast envelope as well as in the nucleus and cytoplasm. Because the chloroplast is the initial biosynthesis site of defence-related hormones, we determined whether hormone-related genes are involved in the ATPsyn-α- and PSaC-mediated defense. Interestingly, genes involved in the biosynthesis of several hormones were up-regulated in plants infected with SMV-G7H expressing ATPsyn-α. However, only jasmonic and salicylic acid biosynthesis genes were up-regulated following infection with the SMV-G7H expressing PSaC. Both chimeras induced the expression of several antiviral RNA silencing genes, which indicate that such resistance may be partially achieved through the RNA silencing pathway.

PSaC and ATPsyn-α proteins interacted with NIb and NIa-Pro of SMV respectively. These interactions were confirmed by co-immunoprecipitation (Co-IP). Overexpression of the C-terminal either from PSaC or ATPsyn-α in the SMV-G7H genome reduced viral accumulation and systemic infection on susceptible cultivar. Our findings suggest that PSaC and ATPsyn-α genes modulate resistance to SMV infection by affecting the function of NIb and NIa-Pro in SMV replication and movement. These findings highlight the role of photosynthesis-related genes in regulating resistance to viruses.

Keyword: Soybean mosaic virus, Glycine max, Nicotiana benthamiana, Viral replication complex, Chloroplast-virus interaction, Viral replication

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CHAPTER I.

The Crucial Role of Chloroplast-Related Proteins in Viral Genome Replication and Host Defense against Positive-Sense Single-Stranded RNA Viruses

This chapter was published in Plant Pathology Journal

John Bwalya and Kook-Hyung Kim (2023). Plant Pathology Journal, 39: 28-38

ABSTRACT

Plant viruses are responsible for worldwide production losses of numerous economically important crops. The most common plant RNA viruses are positive sense single-stranded RNA viruses. These viruses have small genomes that encode a limited number of proteins. The viruses depend on their host's machinery for the replication of their RNA genome, assembly, movement, and attraction to the vectors for dispersal. Recently researchers have reported that chloroplast proteins are crucial for replicating (+)ss plant RNA viruses. Some chloroplast proteins, help viruses fulfill their infection cycle in plants. In contrast, other chloroplast proteins such as play active roles in plant defense against viruses. This is also consistent with the idea that reactive oxygen species, salicylic acid, jasmonic acid, and abscisic acid are produced in chloroplast. However, knowledge of molecular mechanisms and functions underlying these chloroplast host factors during the virus infection is still scarce and remains largely unknown. Our review briefly summarizes the latest knowledge regarding the possible role of chloroplast in plant virus replication, emphasizing chloroplast-related proteins. We have highlighted current advances regarding chloroplast-related proteins' role in replicating plant (+)ss RNA viruses.

Key words: chloroplast; chloroplast-virus interactions; plant defense; viral replication complex; virus replication.

INTRODUCTION

Viruses cause major crop losses worldwide and thus are a threat to sustainable and productive agriculture. New plant viruses are being discovered and continue to pose a clear danger to our food systems globally (Hilaire et al., 2022; Jones and Naidu, 2019; Rubio et al., 2020; Whitfield et al., 2015). Once positive-sense single-stranded RNA viruses [(+)ss RNA viruses] are inside the cell, the viral genome serves as a template for the production of a large number of progeny viruses (den Boon et al., 2010; Nagy and Pogany, 2008b), and RNA replication is done by viral proteins and host plant proteins in chloroplast membranes of the infected cell. Many viruses invade specific host chloroplast membranes in the process of viral genome replication (Hyodo and Okuno, 2016; Nagy et al., 2012; Sanfaçon, 2005; Xu and Nagy, 2014). Therefore, chloroplasts play central roles in replicating several plant virus species and biosynthesis of most plant hormones, making chloroplast factors crucial for plant defense response.

The chloroplast is a vital organelle of plant cells carrying out photosynthesis. In addition to the outer and inner membranes, mature chloroplasts have an internal membrane network of thylakoids, where the light energy is converted into chemical energy stored in ATP (Fig. 1) (Li et al., 2016). Many researchers have published quality data using confocal microscopes, and they have captured stunning and excellent images (Bhat et al., 2013; Kaido et al., 2014; Thivierge et al., 2008) which show that

majority of the plant (+)ss RNA viruses replicate, assemble, and mature in chloroplast components called viral replication complexes (VRCs), including plum pox virus (PPV) (Martin et al., 1995), tobacco etch virus (Gadh and Hari, 1986), turnip mosaic virus (TuMV) (Kitajima and Costa, 1973), maize draft mosaic virus (Mayhew and Ford, 1974), and tobacco mosaic virus (TMV) (Bhat et al., 2013). Moreover, chloroplast-related proteins are reportedly involved in defense against plant viruses because chloroplasts are sites where defense-related hormones are synthesized (Bhattacharyya and Chakraborty, 2018; Bobik and Burch-Smith, 2015; Kozuleva et al., 2011; Nambara and Marion-Poll, 2005; Padmanabhan and Dinesh-Kumar, 2010; Seyfferth and Tsuda, 2014; Stael et al., 2015; Torres et al., 2006; Wasternack and Hause, 2013; Wildermuth et al., 2001; Yang et al., 2021). Recently, the chloroplast membrane-associated protein pSaC in photosystem I (PSI) and the ATP-synthase α-subunit whose chloroplast DNA encoded protein is part of the ATP-synthase complex induced resistance in soybean mosaic virus (SMV) belonging to the *Potyvirus* genus (Bwalya et al., 2022).

I. Chloroplasts and Chloroplast-Related Proteins Facilitate the Viral Replication Cycle of Plant (+)ss RNA Viruses

The viral replication process of (+)ss RNA viruses starts from the switch from translation to replication, including a selection of template RNA (Nagy and Pogany, 2006; Nishikiori et al., 2006; Panavas et al., 2005). The viral and host components required for replication are targeted to chloroplast (subcellular) membranes, where the VRCs are assembled. VRCs synthesize the negative-sense (−) and (+) RNAs and release (+) RNA progenies (Fig. 1).

For plant (+)ss RNA viruses, host translation machinery uses the viral genome as an mRNA to produce replicating proteins and other viral proteins. Host and viral proteins have been well-documented to regulate the switch from translation to replication (Budziszewska and Obrępalska-Stęplowska, 2018; Nagy and Pogany, 2008a). However, most host proteins are more likely to participate in the recruitment step. For example, the recruitment of brome mosaic virus RNAs for replication was affected by Lsm1p, Pat1p, and Dhh1p (Díez et al., 2000; Noueiry and Ahlquist, 2003). When the (+)ss RNA viruses come into the cell, the RNAs come out of the virions and are released into the cytoplasm. The genomes of (+)ss RNA viruses act as a template for translation to produce viral replication proteins, including RNA-dependent RNA polymerase (RdRp), using host machinery leading to a series of interactions between host translation factors and RNA replication (Dreher and Miller, 2006; Simon and Miller, 2013). The viral and host

proteins are bound in a discriminatory manner to the (+)ss RNA template and target subcellular membranes. Translation and selection of the viral (+)ss RNA for replication takes place in the cytoplasm, whereas replication of (+)ss RNA viruses occurs on the surface of various intracellular membranes, including chloroplast membranes (Ahlquist, 2002; Ahlquist et al., 2003; Burgyan et al., 1996; den Boon et al., 2010; Rubino and Russo, 1998; Salonen et al., 2005; Widyasari et al., 2020). Intracellular targeting of the RdRp and viral (+)ss RNA to the replication site occurs in a favorable microenvironment to assemble VRCs. VRC formation at the replication site occurs through viral replicase, virus-encoded accessory proteins, and host factors recruited (Jin et al., 2017; Panavas et al., 2005; Salonen et al., 2005). The process of VRC formation is intimately associated with viral translation, intracellular movement, and intercellular movement. In early models of cell-to-cell movement, the viral MP alone was thought to be responsible for intracellular and intercellular movement. Proper coordination of these processes is required for efficient viral infection. Therefore, viruses must properly build VRCs to avoid or minimize disruption of a coordinated balance, in which host factors play a role in the assembly of viral replicase and regulate its function (Hafrén et al., 2010; Jungfleisch et al., 2015; Kaido et al., 2014).

The critical step is the synthesis of viral RNA (De Graaff et al., 1993). The VRC synthesizes complementary (−) RNA using the original genomic (+)

RNA as a template. The (−) RNA synthesizes more (+) RNAs. Newly synthesized (+) RNAs are then released from VRC to undergo rounds of translation and replication or move to adjacent cells or package into virions (Fig. 1). Nowadays, many studies have shown the association of the VRC with the outer chloroplast membrane (De Graaff et al., 1993; Moriceau et al., 2017; Nishikiori et al., 2006; Thivierge et al., 2008). Table 1 summarizes chloroplast-related proteins in the viral replication process. It was reported a long time ago that VRCs of alfalfa mosaic virus (AMV), the genus *Alfamovirus*, are associated with the chloroplast outer membrane (De Graaff et al., 1993). Turnip yellow mosaic virus (TYMV; a (+)ss RNA virus that shares replication features with other alphavirus-like supergroups) replication also occurs in close association with the chloroplast outer envelope membranes (Hatta et al., 1973; Koonin and Dolja, 1993; Prod'homme et al., 2001). The TYMV 140K protein was previously shown to be responsible for the recruitment of the polymerase to VRCs and targeting the VRCs to the outer chloroplast membrane where viral replication occurs (Jakubiec et al., 2004; Prod'homme et al., 2003). Moriceau et al. (2017) investigated determinants for the in vivo chloroplast targeting of the TYMV 140K replication protein. They identified the two amphipathic helices αA and αB within 140K/98K that constitute the determinants for chloroplast targeting of the TYMV VRCs. However, detailed delivery mechanisms remain to be determined. Furthermore, published data for 3D electron tomography of barley stripe mosaic virus

revealed replication factories and remodeling of the chloroplast outer membranes, characterized by clustering outer membrane-invaginated spherules in inner membrane-derived packets (Budziszewska and Obrępalska-Stęplowska, 2018). Our recent study also showed that chloroplast-related proteins pSaC and ATPsyn-α were localized in the chloroplast envelope (Bwalya et al., 2022). Nonetheless, helicases and chloroplast factors participate in each step of RNA synthesis. One such example of (+)ss RNA viruses infecting plant species is the eukaryotic translation elongation factor 1A (eEF1A), which was found to be a permanent resident of the tombusvirus replicase complex and eEF1A promotes the synthesis of (−) RNA by replicase complex (Li et al., 2009). The elevated abundance of eEF1A in the cells and eEF1A interaction with viral (+)RNAs, including the 3′ untranslated region of TYMV (Dreher, 1999), TMV, and TuMV, might facilitate the recruitment of eEF1A into VRC (Nishikiori et al., 2006; Thivierge et al., 2008). In TuMV infection, 6K2 vesicles were transported to the chloroplast and accumulated at the chloroplast membrane, where they induce the formation of chloroplast-bound elongated tubular structures and ultimately form chloroplast aggregation (Wei et al., 2013). Plant viruses co-opt host proteins such as methyltransferase and chaperone for viral replication (Budziszewska and Obrępalska-Stęplowska, 2018), which may perform similar tasks in viral replication. During viral infection, an RNA helicase protein, ISE2 (increased size exclusion limit 2), was highly expressed and accumulated in the

chloroplast affecting viral replication and cell-to-cell communication (Ganusova et al., 2017).

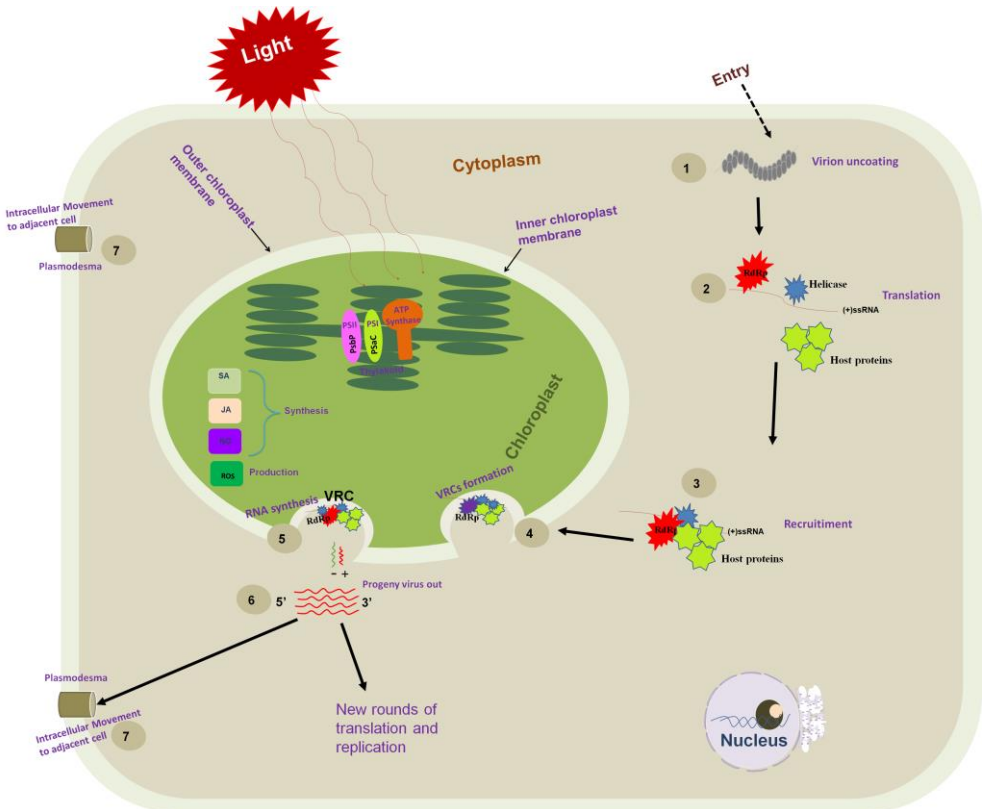

Figure 1. Schematic representation of necessary steps for positive-sense single-stranded RNA [(+)ss RNA] virus genome replication.

(1) Following the entry into host cells, viral genomic RNAs are released from virions into the cytoplasm, and (2) viral RNA [(+)ss RNA] translates RNA-dependent RNA polymerase (RdRp) at the early stage of infection and recruits additional factors (such as methyltransferase and chaperone). (3) The resulting viral replication proteins target themselves to recruit the host translation machinery for the successful production of viral replication proteins. (4) The recruited viral genomic (+) RNAs and host proteins are then trafficked to chloroplast membranes, where they assemble viral replication complexes (VRCs) on the host chloroplast membrane. VRCs are shown by an invagination of the plant chloroplast membrane containing the viral protein (blue shape), the viral RdRP (red shape), host proteins (green shape), and viral RNA (red line). The VRC synthesizes a complementary negative-strand RNA (green line) using the original (+)RNA as a template. The (−) RNA is then used as a template to synthesize many new (+) RNAs (red lines). Progeny viruses are released from VRCs, undergo additional translation and replication, or move to adjacent cells.

Table 1. Chloroplast factors and their cellular localization during virus replication

Plant virus	Chloroplast factor	Subcellular localization	References
Soybean mosaic virus(SMV)	GmPAP2.1	Chloplast	Widyasari et al. (2021)
	Rieske Fe/S	Chloplast membrane	Shi et al.(2007)
	GmATPsy-α	Chloplast membrane, cytopplasm and nucleuous	Bwalya et al. (2021)
	GmPSaC		
Bamboo mosaic virus (BaMV)	NbLTP1	Chloroplast	Shi et al. (2007)
	cPGK	Chloroplast cytoplasm,	Cheng et al. (2013)
Tobacco vein-mottling virus (TVMV)	PSI-K	Chloplast membrane	Jimenez et al. 2006
Tobacco vein banding mosaic virus (TVBMV)	NbRPL1	Chloroplast	Cheng et al. (2021)
Alfalfa mosaic virus (AMV)	PsbP	Cytoplasm	Balasubramaniam et al. (2014)
Cucumber mosaic virus Y strain	ChlI mRNA	Cytoplasm	Shimura et al. (2011)
Cucumber mosaic virus (CMV)	Tsi1- interacting protein 1	Cytoplasm	Huh et al. (2011)
Potato mop-top virus (PMTV)	Chloroplast lipid	Chloroplast	Cowan et al. (2012)
Potato virus X (PVX)	Plastocyanin	Chloroplast	Qiao et al. (2009)
Alternanthera mosaic virus (AltMV)	PsbO	Chloplast membrane	Jang et al. (2013)

II. Chloroplast-Related Proteins Interact with Viral Components during Virus Replication

The accumulating evidence highlights that chloroplasts and chloroplast-related proteins can interact with viral components to favor the replication and movement of (+)ss RNA viruses (Bobik and Burch-Smith, 2015; Zhao et al., 2016, 2019) (Table 2, Fig. 2). In soybean (*Glycine max*), purple acid phosphatase (GmPAP2.1) conferred salicylic acid (SA)-dependent resistance to a susceptible cultivar by interacting with the SMV P1 protein in the chloroplasts, transient knockdown of endogenous SA-related genes resulted in systemic infection by SMV strain G5H (Widyasari

et al., 2022). In *N. benthamiana*, overexpression of chloroplast nicotinamide adenine dinucleotide dehydrogenase-like complex M subunit gene (*NdhM*) inhibited TuMV accumulation, and the localization of NbNdhM is altered by its interaction with TuMV VPg in a way that promoted virus infection (Zhai et al., 2021). Cylindrical inclusion (CI) protein from PPV interacted with the PSI PSI-K protein (a product of the gene psaK) of *N. benthamiana*. Transient coexpression of PPV CI in *N. benthamiana* leaves decreased the level of PSI-K while silencing of *psaK* enhanced PPV accumulation (Jiménez et al., 2006). A chloroplast ribosomal protein large subunit 1 (NbRPL1), localized to the chloroplasts via its transit peptide, interacted with tobacco vein banding mosaic virus (TVBMV) nuclear inclusion protein b (NIb) and enhanced TVBMV infection. Silencing of NbRPL1 expression reduced TVBMV replication in *N. benthamiana* (Cheng et al., 2021). The photosystem II (PSII) oxygen-evolving complex protein (PsbP) interacted with the AMV capsid protein, and its overexpression reduced virus replication (Balasubramaniam et al., 2014). The helicase domain of the TMV replicase interacted with the psbO-encoded 33-kDa protein, a component of the oxygen-evolving complex. TMV infection depleted the *psbO* gene and the thylakoid's entire PSII core complex. Silencing of the *psbO* gene increased TMV replication (Abbink et al., 2002; Balasubramaniam et al., 2014; Lehto et al., 2003). In maize (*Zea mays*), the multifunctional helper component-proteinase protein of sugar cane mosaic virus (SCMV) interacted with chloroplast precursor of ferredoxin-5 (FdV),

and SCMV infection significantly downregulated the expression level of *FdV* mRNA in maize plants (Cheng et al., 2008). SMV P1 protein interacted with Rieske Fe/S protein in tobacco, retarded the Fe/S transport to the chloroplast (Shi et al., 2007). Many more interactions between chloroplast and viral proteins are listed in Table 2.

Table 2. Summary of viral protein-chloroplast factor interactions

Plant virus	Virus component	Chloroplast factor	Reference
soybean Mosaic Virus(SMV)	P1	GmPAP2.1	Widyasari et al., 2021
		Rieske Fe/S	Shi et al., 2007
	–	GmATPsy-α	Bwalya et al., 2021
		GmPSaC	Bwalya et al., 2021
Plum pox virus (PPV)	CI	PSI-K	Jimenez et al., 2006
Tobacco vein-mottling virus (TVMV)		PSI-K	Jimenez et al., 2006
		PsaK	Caplan et al., 2008
Tobacco mosaic virus (TMV)	RNA replicase	PsbO	Abbink et al., 2002
	126K replicase	NRIP 1	Caplan et al., 2008
	126K/183K replicase	ATP synthase-γ subunit (AtpC)	Bhat et al., 2013
	126K/183K replicase	Rubisco activase (RCA)	Bhat et al., 2013
Tomato mosaic virus	MP	RbcS	Zhao et al., 2013
Potato virus Y	HC-Pro	MinD	Jin et al., 2007
Alfalfa mosaic virus	CP	PsbP	Balasubramaniam et al., 2014
Sugarcane mosaic virus	HC-Pro	Ferredoxin	Cheng et al., 2008
Turnip mosaic virus	VPg	NADH	Zhai et al., 2021
	P3	Rubisco	Lin et al., 2011
Alternanthera mosaic	TGB3	PsbO	Jang et al., 2013

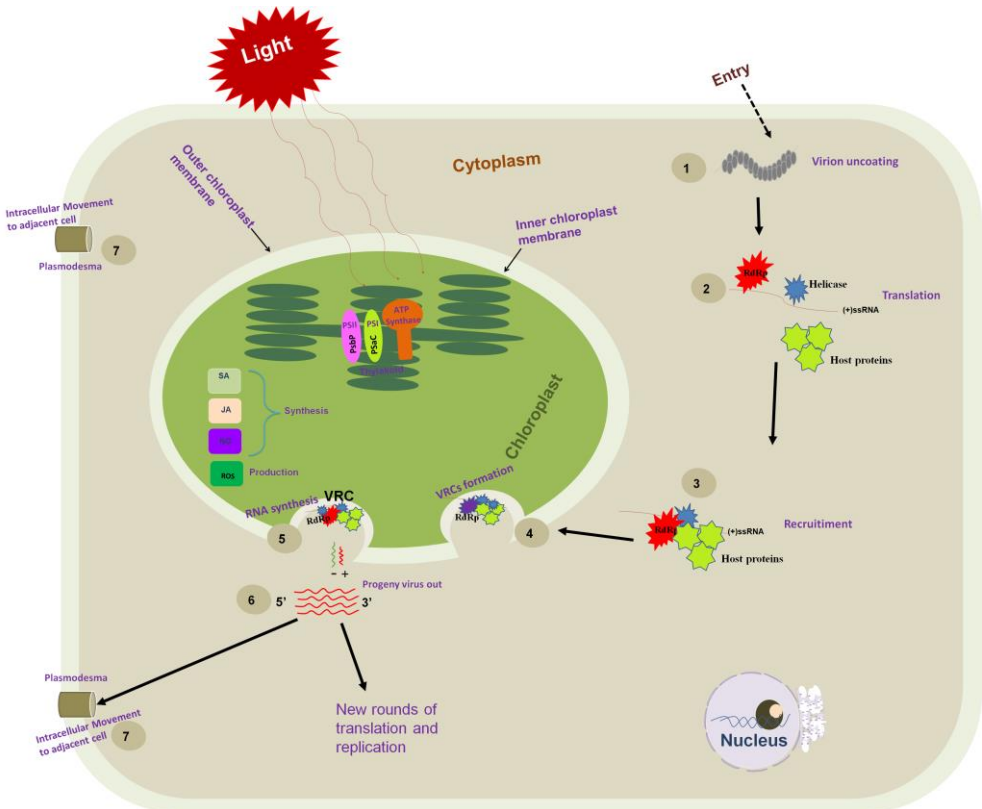

Figure 2. Schematic representation of the important events during chloroplast host factors and potyvirus interactions in a plant cell.

After entry into host plant cells, a potyvirus virion undergoes the disassembly of viral particles and releases the viral genome. Viral genomic RNA is then used as the template for translation to produce viral polyproteins (11 viral proteins indicated by grey squares). The 6K2 remodels the subcellular membranes to form the viral replication complexe (VRC)-containing vesicles for potyvirus genome replication. The 6K2-induced vesicles may subsequently target chloroplasts for robust viral replication. The NIb is recruited to the VRC, likely via its interaction with the VPg domain of 6K2–VPg-Pro. Then, NIb recruits many host factors, such as eEF1A, PSaC, and ATP-synthase α-subunit. In the figure, each viral protein is represented by a grey color-coded square in a location where they play a crucial role. A light blue color-coded semi-circle indicates chloroplast-related host proteins identified for virus infection. They are depicted in sites where they interact with viral protein or play a crucial role.

III. Chloroplast Plays a Vital Role in Plant Antiviral Defense

The chloroplast is one of the crucial organelles that provides energy and carbon through photosynthesis. However, the plant defense mechanism tends to decrease the photosynthetic rate and other anabolic processes to reduce the organic carbon supply to pathogens (Serrano et al., 2016). Other than photosynthesis, chloroplast plays significant roles in plant defense. It provides plant defense against viruses because of the production of secondary metabolites, including calcium, reactive oxygen species (ROS), and biosynthesis of several defense-related hormones like SA, jasmonic acid (JA), and abscisic acid (ABA) that have an important connection with plant immunity (Bobik and Burch-Smith, 2015; Kozuleva et al., 2011; Nambara and Marion-Poll, 2005; Padmanabhan and Dinesh-Kumar, 2010; Seyfferth and Tsuda, 2014; Stael et al., 2015; Torres et al., 2006; Wasternack and Hause, 2013; Wildermuth et al., 2001).

Most plant virus-induced SA is synthesized through the isochorismate pathway in chloroplast and plays crucial roles in plant defense against viruses, and is essential for local and systemic acquired resistance (Alazem and Lin, 2015; Boatwright and Pajerowska-Mukhtar, 2013; Seyfferth and Tsuda, 2014; Zhao et al., 2016). For example, overexpression of GmPAP2.1, a chloroplast-localized protein, resulted in the upregulation of the SA pathway. Overexpression of GmPAP2.1 showed resistance to SMV infection, while transient knockdown of endogenous SA-

related genes caused severe systemic symptoms by SMV (Widyasari et al., 2022). Another chloroplast-localized protein, named calcium-sensing receptor, acts upstream of SA accumulation and connects chloroplasts to cytoplasmic-nuclear immune responses in Arabidopsis thaliana (Nomura et al., 2012). JA is synthesized from linolenic acid by the octadecanoid pathway, and biosynthesis starts with the conversion of linolenic acid to 12-oxo-phytodienoic acid in the chloroplast membranes (Turner et al., 2002). JA also plays a crucial role in plant-virus interaction. For example, in silencing the JA perception gene, COI1 (coronatine insensitive 1) accelerates the development of symptoms caused by the co-infection of potato virus X (PVX) and potato virus Y. It increases the level of viral titers at the early stages of infection (García-Marcos et al., 2013). Pathways of ABA are also involved in plant resistance to viruses (Alazem and Lin, 2015; Alazem et al., 2017, 2018, 2019; Nomura et al., 2012; Zhang et al., 2012; Zhao et al., 2016). Previously published reports showed that ABA enhanced the expression of the antiviral RNA silencing genes in soybean and A. thaliana and that the enhanced expression confers partial resistance against SMV, bamboo mosaic virus, and PVX (Alazem et al., 2017, 2019).

The reaction centers of PSI and PSII in chloroplast thylakoids are the primary generation site of ROS. ROS is a sign of activated antiviral defense (Bwalya et al., 2022; Calil and Fontes, 2017; Wu et al., 2017). The reaction centers of PSI and PSII in chloroplast thylakoids produce ROS and the

photosynthetic electron transport chain. Superoxide anion (O^{2-}) is the primary reduced product of O_2 photoreduction, and its disproportionation produces H_2O_2 in chloroplast thylakoids membranes (Asada, 2006; Mühlenbock et al., 2008; Zhao et al., 2016). ROS and calcium bursts in chloroplasts activate signaling cascades that regulate the expression of defense-related genes; therefore, ROS and calcium bursts act as chloroplast-to-nucleus retrograde signals when plants recognize the early step of virus infection (Bhattacharyya and Chakraborty, 2018; Medina-Puche et al., 2020; Nomura et al., 2012; Zhao et al., 2016). The burst of intracellular ROS can be detected during virus infection in incompatible and compatible interactions (Allan et al., 2001; Hakmaoui et al., 2012). Chloroplast-sourced ROS are essential for hypersensitive response induced by the incompatible defensive response (Torres et al., 2006; Zurbriggen et al., 2010).

CONCLUSION AND FUTURE PERSPECTIVES

Chloroplasts have been recognized as a common target by many plant viruses. Hence chloroplast-virus interaction is an epicenter of plant-virus interplays. Viruses may directly modify chloroplast membranes to assemble their replication complex for viral genome replication. Based on previously published reports (Balasubramaniam et al., 2014; Bhat et al., 2013; Bhattacharyya and Chakraborty, 2018; Budziszewska and Obrępalska-Stęplowska, 2018; Bwalya et al., 2022; Cheng et al., 2021;

Jiménez et al., 2006; Nagy et al., 2012; Serrano et al., 2016; Wei et al., 2013; Widyasari et al., 2022; Yang et al., 2021).

We can conclude that some chloroplast-related proteins could function in virus replication, while some are involved in inhibiting viruses. Our review has summarized a few chloroplast-related proteins identified by researchers and their possible roles in virus infection. However, more chloroplast-related proteins must be determined to understand the proteins involved in host defense accurately. If the desired chloroplast-related gene functions are observed from host plants, they might be used to genetically engineer other plants to express these gene products after isolation and cloning. For example, scientists have boosted a carbon-craving enzyme called RuBisCO to turbocharge photosynthesis in corn (Salesse-Smith et al., 2018). The discovery promises to be a critical step in improving agricultural efficiency and yield. Moreover, more reports showed that overexpression of photosynthesis genes could enhance virus resistance in maize and soybean plants (Bwalya et al., 2022; Wang et al., 2018; Widyasari et al., 2022).

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CHAPTER II

Photosynthesis-related genes induce resistance against soybean mosaic virus: Evidence for involvement of the RNA silencing pathway

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ABSTRACT

Increasing lines of evidence indicate that chloroplast-related genes are involved in plant–virus interactions. However, the involvement of photosynthesis-related genes in plant immunity is largely unexplored. Analysis of RNA-Seq data from the soybean cultivar L29, showed that several chloroplast-related genes were strongly induced in response to infection with an avirulent strain of soybean mosaic virus (SMV), G5H, but were weakly induced in response to a virulent strain, G7H. For further analysis, we selected the PSaC gene and the ATP-synthase α-subunit (ATPsyn-α) gene. Overexpression of either gene within the G7H genome reduced virus levels in the susceptible cultivar Lee74 (rsv3-null). This result was confirmed by transiently expressing both genes in *Nicotiana benthamiana* followed by G7H infection. Both proteins localized in the chloroplast envelope as well as in the nucleus and cytoplasm. Because the chloroplast is the initial biosynthesis site of defence-related hormones, we determined whether hormone-related genes are involved in the ATPsyn-α- and PSaC-mediated defence. Interestingly, genes involved in the biosynthesis of several hormones were up-regulated in plants infected with SMV-G7H expressing ATPsyn-α. Both chimeras induced the expression of several antiviral RNA silencing genes, which indicate that such resistance may be partially achieved through the RNA silencing pathway. These findings highlight the role of photosynthesis-related genes in regulating resistance to viruses.

Key words: ATPsyn-α, photosynthesis, plant hormones, plant–virus interactions, PSaC, RNA silencing, soybean, soybean mosaic virus

INTRODUCTION

Symptoms of viral infection in plants usually include a change in the green pigmentation such as mottling, mosaic, chlorosis, and yellowing. Most of these symptoms indicate changes in photosynthetic activity in the infected plants (Liu et al., 2020; Scholthof et al., 2011). It has long been known that viral infection leads to reduced photosynthesis and major changes in chloroplast ultrastructure (Bhattacharyya & Chakraborty, 2018; Lehto et al., 2003). The roles of chloroplasts in virus replication, virus movement, and plant defence have only recently been investigated (Azim & Burch-Smith, 2020; Bhattacharyya & Chakraborty, 2018; Ganusova et al., 2020; Zhao et al., 2016).

Photosynthesis includes two major stages: a light-dependent stage and a light-independent stage. In the light-dependent stage, photosystem I (PSI), cytochrome, photosystem II (PSII), and ATPase synthesis sequentially contribute to the production of NADPH and then ATP, which are used in the light-independent stage to produce sugar through the Calvin cycle (Moejes et al., 2017; Nevo et al., 2012; Yu et al., 2020). Virus interference with chloroplasts in general, and with photosynthesis in particular, can occur on different levels. Because the chloroplast is the site for the biosynthesis of several defence-related hormones and helps control plasmodesmata (PD) permeability, some viruses reduce host defences by targeting the chloroplast with specific viral proteins (Alazem & Lin, 2015,

2020; Ganusova et al., 2020). The P25 protein of potato virus X (PVX), for example, interferes with the function of ferredoxin 1 (FD1), an important protein involved in electron transfer between PSII and PSI, resulting in reduced levels of the defence-related hormones abscisic acid (ABA) and salicylic acid (SA) (Yang et al., 2020). This reduction decreases callose accumulation at PD, and consequently increases PD permeability and PVX spread in the host plant (Yang et al., 2020). Because the chloroplast is also the site for the replication of several RNA viruses, viral effectors are expected to recruit specific chloroplast proteins into their viral replication complex (Budziszewska & Obrepalska-Steplowska, 2018; Cheng et al., 2013; Ganusova et al., 2020; Zhang et al., 2017). Bamboo mosaic virus (BaMV), for example, recruits the chloroplast phosphoglycerate kinase (chl-PGK) protein, that is, the viral RNA genome binds to chl-PGK and transports it to the chloroplast (Cheng et al., 2013). Once in the chloroplast, BaMV recruits further chloroplast proteins into the viral replication complex to complete the infection cycle (Huang et al., 2017). In another example, infection with rice stripe virus (RSV) dramatically changes the proteome profiles of the *Nicotiana benthamiana* protoplast and chloroplast, resulting in a significant decrease in the number of nuclear-encoded chloroplast-localized proteins; the decrease is caused by RSV interference with three host factors (K4CSN4, K4CR23, and K4BXN9) that are involved in protein delivery to the chloroplast (Zhao et al., 2019).

It follows that viral interference with the functions of chloroplast proteins explains why photosynthesis is reduced in susceptible plants (i.e., in compatible interactions). In contrast, some resistant plants show increased expression of photosynthesis-related genes. For example, expression of photosynthesis-related genes in soybean cultivar L29 (which carries the resistance [*R*]-gene *Rsv3*) was increased in response to infection by the avirulent G5H strain but not in response to the virulent G7H strain of soybean mosaic (SMV) (Alazem et al., 2018).

Soybean mosaic virus is a member of the genus Potyvirus and has a single-stranded, positive-sense RNA genome that encodes 11 viral proteins and is about 10 kb in length (Hajimorad et al., 2018; Liu et al., 2016). SMV has many strains distributed worldwide and, depending on the phenotypic responses of various soybean cultivars, these strains have been classified into seven distinct strains in the United States (G1 to G7) and into 21 strains in China (SC1 to SC21) (Hajimorad et al., 2018). Genetic resistance to SMV is mainly achieved through different strain-specific NLR-type R-genes such as the Rsv and the Rsc groups (Widyasari et al., 2020). There are several other non-NLR host factors that have been found to be critical for resistance, either because they are key components in the signalling pathway downstream of the R-gene or because they are part of a plant system that degrades viral RNA or protein (i.e., antiviral RNA silencing and double-

stranded RNA ribonuclease) (Ishibashi et al., 2019; Liu et al., 2014; Widyasari et al., 2020).

Here, we investigated the roles of two photosynthesis-related proteins, PSaC and ATPsyn-α, in the resistance to SMV in soybean cultivar L29, which is resistant to G5H but not to G7H. Both proteins were strongly up-regulated in cultivar L29 in response to G5H, whereas the response to G7H infection was rather weak. Constitutive expression of PSaC, a member of PSI, and ATPsyn-α, a component in the ATPase synthase complex, increased resistance to SMV-G7H infection in Lee74 (a susceptible rsv-null soybean cultivar) and in *N. benthamiana* plants. Genes involved in the antiviral RNA pathways were up-regulated in the plants transiently expressing *PSaC* or *ATPsyn-α*, which may account for the resistance phenotype induced by both genes.

MATERIAL AND METHOD

I. Construction of the SMV vector expressing ATPsyn-α and PSaC genes

The CDS of *ATPsyn-α* and *PSaC* genes were amplified and cloned from several soybean cultivars and were then cloned into a TA vector (pGEM-T Easy; Promega). The clones were confirmed by sequencing with gene-specific primers (Table1). The CDS of both genes from L29 plants were then cloned into the pSMV-G7H::eGFP infectious clone to generate pSMV-G7H::eGFP::ATPsyn-α and pSMV-G7H::eGFP::PSaC as previously described (Seo, Lee, Choi, et al., 2009).

II. Plant materials, growth conditions, and virus infections

The following five soybean cultivars were used in this study: Lee74, L29, V94, Somyoungkong (SMK), and William 82 (W82). Soybean and N. benthamiana plants were grown in growth chambers at 25°C with 70% relative humidity and a 16/8 h photoperiod. To prepare infectious sap, the first unifoliate leaf from Lee74 plants was inoculated with 10 µg per leaf of the infectious clones pSMV-G7H::eGFP, pSMV-G7H::eGFP::PSaC, and pSMV-G7H::eGFP::ATPsyn-α as previously described (Seo et al., 2009). About 15 dpi, a pool of SL from three plants was mixed and divided into 0.1-g portions as a source of virus inoculum. After each 0.1-g portion was ground into powder in liquid nitrogen, it was mixed with 1 ml of phosphate buffer. The mixture was placed on ice for 10 min and was then centrifuged

for 10 min at 4°C and 13,580 × g. A 50-µl volume of the supernatant was rub-inoculated onto each leaflet of the trifoliate leaf on each soybean plant, or on two leaves on each *N. benthamiana* plant. Samples were collected from three plants (a total of nine leaves for soybean and six leaves for *N. benthamiana*) at 5 and 10 dpi for further analyses.

To investigate the effects of PSaC and ATPsyn-α on the accumulation of SMV-G7H::eGFP in Lee74 plants, plasmids of the infectious clones pSMV-G7H::eGFP, pSMV-G7H::eGFP::PSaC, and pSMV-G7H::eGFP::ATPsyn-α were directly rub-inoculated on Lee74 plants using 10 µg of plasmid per leaf. The ILs and SLs were collected at 7 and 14 dpi, respectively, for RNA and protein extraction.

Table 1. Primers used in this study

	Primer	Fw	Rv	Purpose	Accession No.
PSaC	Glyma18g32250 FW+MluI	gcacgcgtATGTCACATTCAGTAAAGATTTA	gcacgcgtATAAGCTAGACCCATGCTTTGAGT	Clonine into pSNU-SMV-G7H	Glyma18g32250
ATPsyn-α	Glyma12g36106 FW+MluI	gcacgcgtATGGTAACCATTCGTGCAGA	gcacgcgtATTTTTTTCTACCTGTTCCTGT		Glyma12g36106
ATPsyn-α	Glyma12gFW+XbaI / Glyma12gRV+BamHI	gctctagaATGGTAACCATTCGTGCAGA	gcggatccATTTTTTTCTACCTGTTCCTGT	Cloning into pBin-3HA-mCherry	
PSaC	Glyma18gFW+XbaI / Glyma18gRV+BamHI	gctctagaATGTCACATTCAGTAAAGATTTA	gcggatccATAAGCTAGACCCATGCTTTGAGT		
PSaC	Fw-BamHI / Rv-XhoI	CGCTCGAGGCCCAACGGATGTATTAGAAAT	GCGGATCCTAGACCCATGCTTTGAGTTGTT	VIGS	
ATPsyn-α	Fw-BamHI / Rv-XhoI	CGCTCGAGAGAGCCGCTAAATTAAGTTCTCA	GCGGATCCAATCGTTGACCTCTTGCTAATTG		
ATPsyn-α	Glyma12g36106	CCGTGAACGCATTGAGCAAT	ATACGAGCAATACCGTCGCC	**RTqPCR**	
PSaC	Glyma18g32250	ACTCAATGTGTCCGAGCCTG	CAGTCCTCTATTCTTGGGGCA		
	JAR1	GAGCAAGTTTGGGAGGAGCT	CCAGCTCAGGGTCAGGTTTT		Glyma.07G057900
	Lox2	CCACGGCCTCAAGCTAATCA	CGTCGGCGTAGTAGAGGTTC		Glyma.11G130300
	GmDREB1A-1	TTCCTTGGACACATCCTCGC	CGTCTCCCGGAACTTCTTCC		Glyma.09g147200
	GmDREB1A-2	CTAACTTTGGCTTCAGCGGC	AGGAAGAGGAGAGCTCAGGG		Glyma.16g199000
	ICS1	ATGGCAATGGGCAC	ATGGTATTGGAGGAAGTATAT		Glyma.03G070600
	PAD4	CAATCCATCTCTTCATCTGTGTC	CATTATGTCATGCTTTGACACCA		Glyma.13g04540
	ABA1	CGCATCCGTCCATCTGATGT	TCCCTGCAAAGCTAGTGTGC		Glyma.17G174500
	ABA2	CATGGTTGATGGAGGCTTCAC	ACCCTTGTACTAGACATCAGGA		Glyma.11G151400
	RDR1a	TCCAAGTTACTGGGGTTGCT	ACGCAACCCACTGAAACTGT		Glyma.02G086100
	RDR2a	GGTAACGTGCAAAACCGTGC	ACGCGGTTGGCAACTAGTTT		Glyma.05g02000
	RDR6a	CAGTTGATTACCTAGCTCGC	GAGCAAGCTCTCAATGGAAT		Glyma.04G067300
	DCL2a	GGCGGTGCTCATAAGGACAC	ACCCTTGTGCACAGTACACA		Glyma.09G025400
	DCL4a	GAGGGACCAGACCACCTGAA	CCATAATGCACCCTCTGCCG		Glyma.13G156500
	GFP	GACGACGGCAACTACAAGAC	TCCTTGAAGTCGATGCCCTT		*_*
	Actin11	ATCTTGACTGAGCGTGGTTATTCC	GCTGGTCCTGGCTGTCTCC		Glyma.18G290800.1
	Source for soybean genes:	https://phytozome.jgi.doe.gov/			

III. Silencing *ATPsyn-α* and *PSaC* in soybean plants

The BPMV silencing vector was used to silence *ATPsyn-α* and *PSaC* genes in Lee74 plants. In brief, fragments of 173 bp from *PSaC* CDS, and 347 bp from *ATPsyn-α* CDS were cloned in the antisense direction in the multiple cloning site of RNA2 of BPMV, as described previously (Zhang et al., 2010). Ten micrograms of BPMV plasmids (RNA1 and RNA2) were rub-inoculated onto the first unifoliate leaves of Lee74 plants, and the silencing efficiency was tested at 14 dpi in the second trifoliate leaf. The same leaf was sap-inoculated with G7H::eGFP as described in section 4.2. Samples were collected from the SL 10 days after G7H::eGFP infection for further analyses.

IV. RNA extraction and RT-qPCR

Total RNA was extracted using TRIzol (Sigma) following the manufacturer's instructions. A 1-μg quantity of total RNA was used for cDNA synthesis using the GoScript kit (Promega). RT-qPCR was carried out with SYBR Green (Promega) to measure the relative expression of target genes using the ΔΔCt method. Actin11 was used as an internal control, and the primers used in this study are listed in Table S1. One-sided Student's t tests ($p < 0.05$) were used to determine whether the expression level of each gene in each line was up-regulated or down-regulated relative to the mock-treated plants.

V. Statistical analysis

RT-qPCR was carried out in three biological replicates, and each biological replicate was repeated in three technical replicates. In Figures 1 and 3–8, values were compared to that of the mock-treated, uninfected plants (the bar on the left) with one-sided Student's t tests; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively. Error bars in the charts are means of standard deviation of three biological replicates.

VI. Western protein blot

Total protein was extracted from 0.1 g of tissue collected from a pool of IL and SL from three plants, as described previously (Alazem et al., 2018). Constructs expressing GFP were detected by western blot using polyclonal anti-GFP antibody, and those expressing HA were detected using monoclonal anti-HA antibody (Sigma); Ponceau S staining was used on the loading control.

VII. Phylogenetic analysis

Amino acid sequences of ATPsyn-α and PSaC for Glycine max, *N. benthamiana*, *S. lycopersicum*, and *A. thaliana* were obtained from the soybean database (DB) (Soybase), the Sol Genomics Network, and the Tair DB (Brown et al., 2021; Fernandez-Pozo et al., 2015). The phylogenetic trees were generated using MEGA 7.0 software and by applying the neighbour-joining method (Kumar et al., 2016).

VIII. Gene description, function, and pathways

Information about gene annotations and functions was obtained from the Soybase DB assembly 4, v. 1 (https://www.soybase.org/). The Phytozome soybean DB was used when the Soybase DB did not have gene annotation information. Both DBs predicted the pathways of the genes from the following DBs: Pfam v. 33.1, release 2019/08 (http://pfam.xfam.org/) (El-Gebali et al., 2019), Tair DB (https://www.arabidopsis.org/), and KEGG Pathway (https://www.genome.jp/kegg/pathway.html).

VIII. Subcellular localization of ATPsyn-α and PSaC proteins

ATPsyn-α and PSaC were cloned into the binary vector pBin61-3HA-mCherry (Alazem et al., 2020). Agrobacterium infiltration was carried out on N. benthamiana plants using Agrobacterium tumefaciens C58C1 at OD600 = 0.5, with the aid of 2b, the viral suppressor of RNA silencing (pPZP-2b), to enhance the expression of both genes. Infection with pSMV-G7H::eGFP was carried out 1 day after agroinfiltration using 50 µl of infectious sap extract/leaf. Samples were collected at 3 dpi for confocal microscopy, and at 5 dpi for protein and RNA analysis. The chloroplast marker protein gene AtEMB1301 was cloned into pBin-eGFP and used as a marker for the localization of ATPsyn-α and PSaC proteins.

IX. Visualization of GFP expression and localization of the target proteins in plants

GFP fluorescence of the IL and SL was examined with UV light and with a digital camera (D700; Nikon) with a green filter. A Leica confocal microscope was used to determine the subcellular localization of AtEMB1303, ATPsyn-α, and PSaC with a 40× lens (HC PL APO CS2 40×/1.10 WATER), and the detectors HyD (421–467 nm) and PMT (654–711 nm), with bidirectional scanning at a speed of 400 Hz and a resolution of 2048 × 2048. Leica application suite X package was used to analyse images.

RESULTS

I. Chloroplast-related genes are induced in the resistant cultivar L29 in response to SMV-G5H infection

The soybean cultivar L29 carries the R-gene *Rsv3*, which confers resistance against the SMV avirulent strain G5H but is ineffective against the virulent strain G7H (Seo et al.., 2009). We previously obtained RNA-Seq data from L29 plants infected with strains G5H and G7H (Alazem et al., 2018). The data showed that, in the incompatible interaction (resistance against G5H), a large number of differentially regulated genes were photosynthesis-related (Alazem et al., 2018).

To examine this list more closely, we searched for the top up-regulated genes (fold change >1) that were induced only in response to G5H infection at any time point (Figure 1a). Most of these genes have different functions related to photosynthesis/chloroplasts (Table 2). While the expression of most of these genes was induced in response to G5H, the expression of several was temporarily and slightly increased in response to G7H at 8 h postinfection (hpi) but then decreased at 24 and 54 hpi (Figure 1a). This suggests a possible relationship between their suppression and G7H virulence. We selected two genes, Glyma.18G155300.1 and Glyma.12G232000.1, which were strongly down-regulated in response to G7H but up-regulated in response to G5H (Figure 1a), for further analysis.

In the soybean DB (Soybase) assembly 4 v. 1, Glyma.18G155300.1 and Glyma.12G232000.1 were reported to encode the PSaC subunit of the PSI subunit (*PSaC*) and the ATP-synthase α-subunit (*ATPsyn-α*), respectively (Table 1) (Brown et al., 2021; Grant et al., 2010).

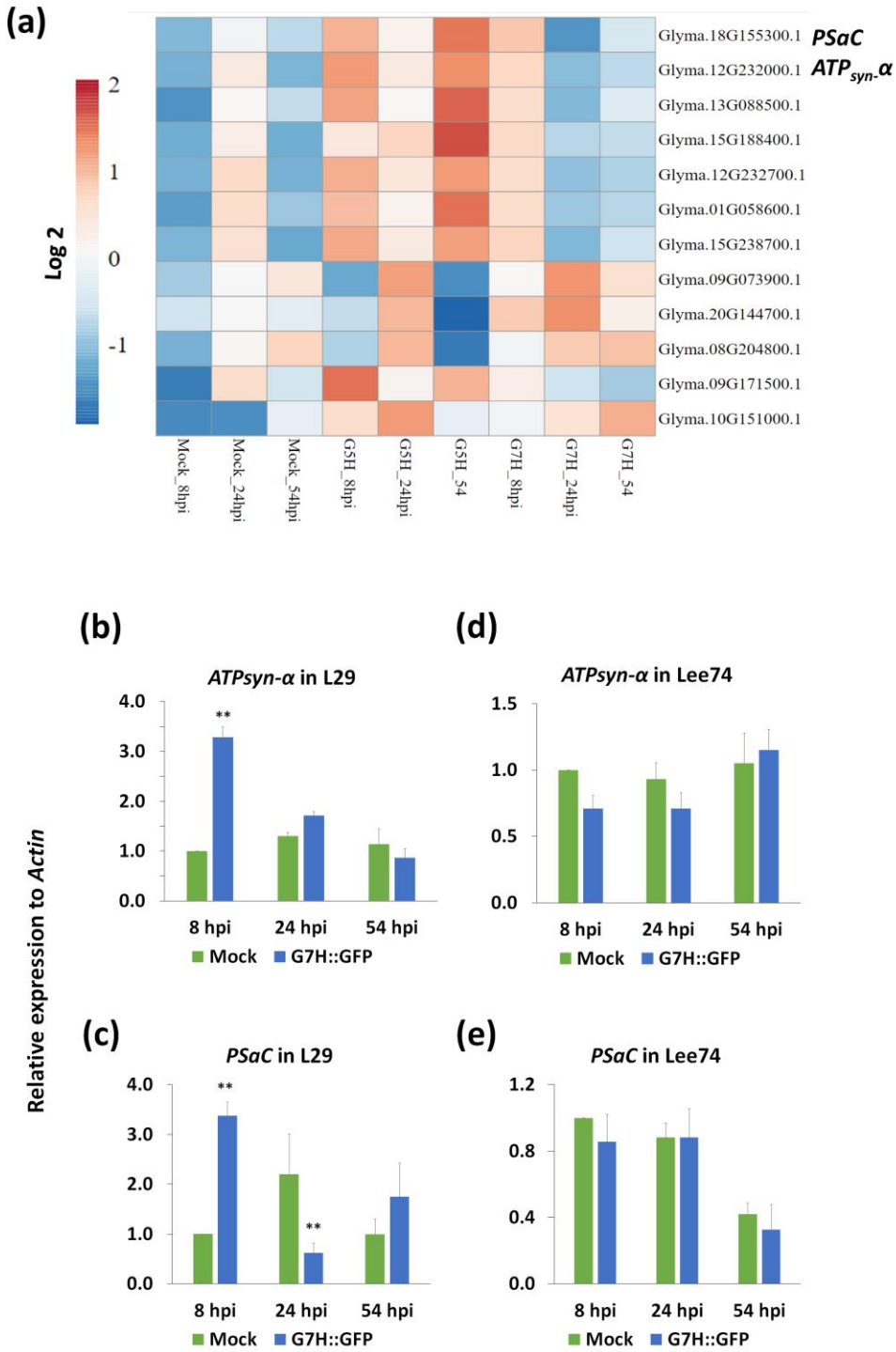

Figure 1. Expression of photosynthesis-related genes in response to soybean mosaic virus (SMV) infection.

(a) Heat-map of photosynthesis-related genes regulated by infection with the avirulent strain G5H or the virulent strain G7H of SMV. Expression of ATPsyn-α (b) and PSaC (c) in L29 plants (which carry the Rsv3 resistance gene) at 8, 24, and 54 h postinfection (hpi) by G7H::eGFP. Expression of ATPsyn-α (d) and PSaC (e) in Lee74 plants (rsv-null) at 8, 24, and 54 hpi by G7H::eGFP. Actin11 was used as the internal control. In (b–e), values are means + SD of three biological replicates. Values were compared to that of the corresponding mock-treated plants (the bar on the left) with one-sided Student's t tests; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively

TABLE 2. Functional analysis and gene ontology of the photosynthesis-related genes regulated by SMV infection

Gene ID	Annotation	Predicted Localization based on Arabidopsis ortholog	Database ID	Pathway	Arabidopsis relative ortholog	Blast results against Arabidopsis ortholog
Glyma.18G155300.1	PsaC subunit of photosystem I subunit	chloroplast, nucleus	PF12838	Photosystem I / 4Fe-4S dicluster domain	ATCG01060.1	Identities = 223/243 (88%), Gaps = 0/246 (0%)
Glyma.12G232000.1	ATP synthase subunit alpha (ATPsyn-α)	chloroplast, cytosol, mitocondria, plasma membrane	K02887	Ribosome	ATCG00120.1	Identities = 1345/1514 (89%), Gaps = 0/1514 (0%)
Glyma.13G088500.1	4Fe-4S binding domain / Photosystem I	chloroplast, nucleus	PF00037	Photosystem I	ATCG01060.1	Identities = 132/143 (92%), Gaps = 0/143 (0%)
Glyma.15G188400.1	Photosystem II reaction centre N protein (psbN)	chloroplast, nucleus	PF02468	Photosystem II reaction center protein N	ATCG00700.1	Identities = 121/132 (92%), Gaps = 0/132 (0%)
Glyma.12G232700.1	photosystem II cytochrome b559 subunit alpha (psbE)	chloroplast	K02711	Photosystem II PsbJ protein (psbJ)	ATCG00580.1	Identities = 220/238 (92%), Gaps = 0/238 (0%)
Glyma.01G058600.1	cytochrome b6 (petB)	chloroplast	K02704	photosystem II CP47 chlorophyll apoprotein	ATCG00720.1	Identities = 305/337 (91%), Gaps = 0/337 (0%)
Glyma.15G238700.1	photosystem II cytochrome b559 subunit alpha (psbE)	chloroplast	K02707	photosystem II cytochrome b559 subunit alpha	ATCG00580.1	Identities = 228/252 (90%), Gaps = 0/252 (0%)

Glyma.09G073900.1	photosystem II subunit X	chloroplast	PF06596	Photosystem II	AT2G06520.1	Identities = 152/226 (67%), Gaps = 9/226 (4%)
Glyma.20G144700.1	photosystem I subunit D-2	chloroplast, cytosol	PF02531	Photosystem I	AT4G02770.1	Identities 365/466 (78%), Gaps = 3/466 (1%)
Glyma.08G204800.1	photosystem I subunit H2	chloroplast, nucleus	PF03244	Photosystem I	AT1G52230.1	Identities = 327/438 (75%), Gaps = 3/438 (1%)
Glyma.09G171500.1	ATP synthase subunit alpha / defense response to bacterium	chloroplast, cytosol, mitocondria, plasma membrane	PF02874	chloroplast ATP synthase complex	ATCG00120.1	Identities = 312/357 (87%), Gaps = 0/357 (0%)
Glyma.10G151000.1	large subunit ribosomal protein L22e	nucleus	K02891	Ribosome / Coronavirus disease - COVID-19	AT1G56220.1	Identities = 218/288 (76%), Gaps = 17/288 (6%)
Glyma.05G119100.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	plasma membrane	PF14368	Probable lipid transfer	AT5G64080.1	Identities = 186/268 (69%), Gaps = 0/268 (0%)

To confirm the RNA-Seq data, we used reverse transcription quantitative PCR (RT-qPCR) to measure the expression of both genes in L29 plants infected with G7H. Expression of *GmATPsyn-α* significantly increased at 8 hpi but then declined at 24 and 54 hpi to levels comparable to that in mock treatments (Figure 1b). *GmPSaC* increased only at 8 hpi, then decreased to a level lower than that of the mock treatment at 24 hpi (Figure 1c). We then analysed the expression of these genes in Lee74 plants, a susceptible rsv-null soybean cultivar. Interestingly, the expression pattern of both genes did not differ with G7H infection compared with mock treatment at any time point, except for a slight decrease of *GmATPsyn-α* at 8 hpi (Figure 1d,e). This suggests that although the interaction is compatible with L29, the Rsv3 gene might be involved in the early induction of both genes in L29 plants but that G7H was able to suppress the responses as the infection progressed.

Sequence analysis revealed that GmPSaC is a small protein composed of 81 amino acid residues and has two copies of the ferredoxin-like 4Fe-4S binding site in the specific Fer4-7 domain located between amino acids 10 and 61 (Figure 2a). PSaC is an essential member of PSI (iron- sulphur protein PSaC) in the chloroplast and functions in the fast electron transfer to ferrodoxin through the Fer4-7 domain (Fischer et al., 1998; Kubota-Kawai et al., 2018). The other protein, GmATPsyn-α, encodes the ATPase α subunit of 510 amino acids from the ATP synthase α/β family

with three domains, including the β-barrel domain positioned between amino acids 29 and 93, the nucleotide-binding domain positioned between amino acids 150 and 365, and the C-terminal domain positioned between amino acids 372 and 496 (Figure 2b). The enzyme complexes catalyse the conversion of ADP to ATP using proton motive force, confer redox regulatory properties, and are located in the thylakoid membrane of the chloroplast (Table 2) (Hahn et al., 2018; Hisabori et al., 2013).

Analysis of amino acid sequences from five soybean cultivars with different resistance backgrounds (L29, *Rsv3*; William 82 (W82), rsv-null; Lee74, *rsv*-null; Somyoungkong (SMK), rsv-null; and V94, *Rsv4*) showed that the sequence for GmATPsyn-α is identical in all five cultivars (Figure S1a). However, the sequence of GmPSaC in W82 differed in six amino acids relative to the other cultivars (Figure S1b). Phylogenetic analysis clustered GmPSaC close to orthologs from *Arabidopsis thaliana* and *N. benthamiana*, and only the ortholog from *Solanum lycopersicum* was genetically distant from the others (Figure 2c). Analysis revealed much closer relatedness for most orthologs except for At.GmATPsyn-α, which clustered far from the others (Figure 2d). Hereafter, the genes Glyma.18G155300.1 and Glyma.12G232000.1 will be referred to as GmPSaC and GmATPsyn-α, respectively.

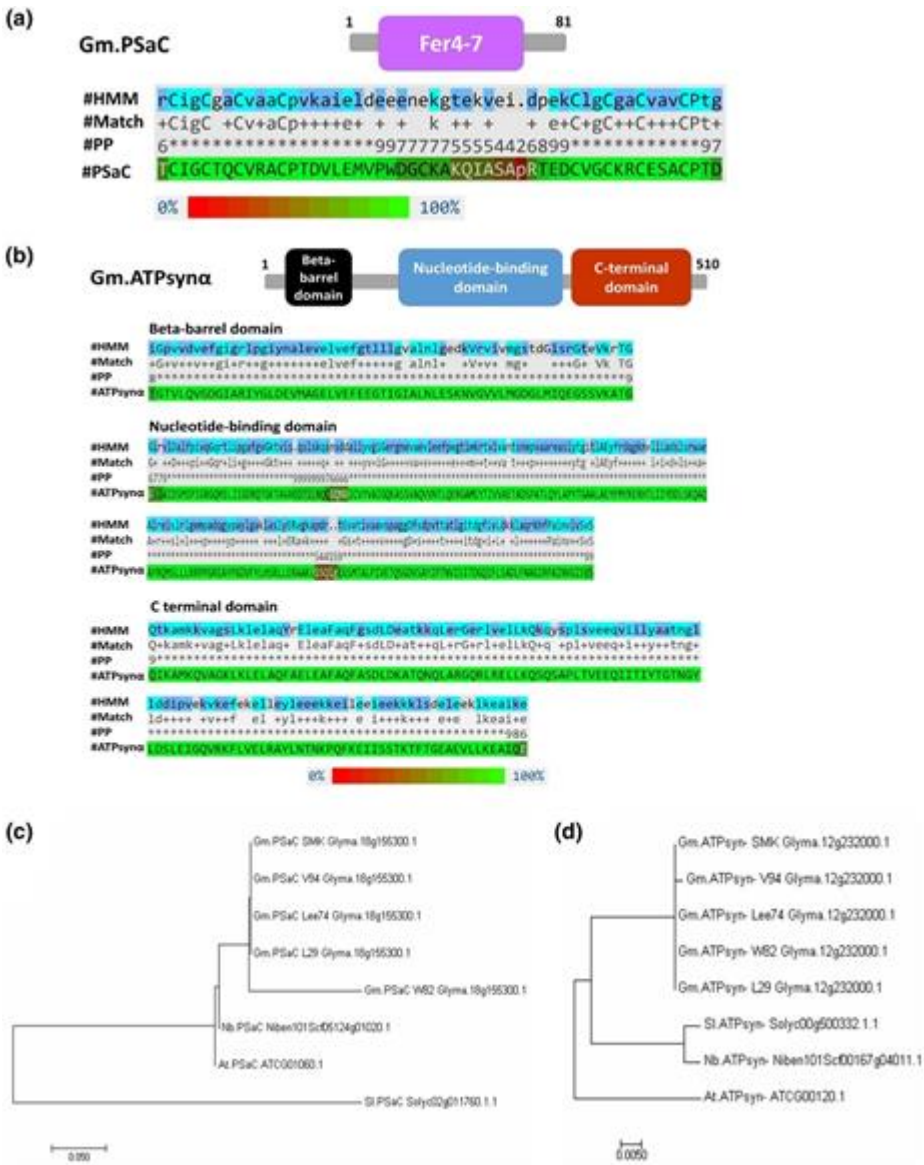

Figure 2. Domains and phylogenetic analyses of GmPSaC and GmATPsyn-α.

(a)Conserved domain in GmPSaC. Protein sequence of GmPSaC BLASTed against the Pfam database showed that GmPSaC belongs to the Fer4-7 superfamily. Alignment result between the hidden Markov model (HMM) and GmPSaC (positions 10–61, E-value 1.55e−07). (b) Conserved domains in GmATPsyn-α. Protein sequence of GmATPsyn-α BLASTed against the Pfam database showed that GmATPsyn-α belongs to

the ATP synthase α/β family and has three domains. Alignment between HMM and GmATPsyn-α showed that the protein has three domains: the 1-β-barrel domain (positions 29–93, E-value 3.66e−16), the 2-nucleotide-binding domain (positions 150–365, E-value 5.21e−114), and the 3-C terminal domain (positions 372–496, E-value 3.34e−59). #HMM is the hidden Markov model, and #Match indicates the match between the query sequence and the HMM. #PP indicates the posterior probability (or degree of confidence) in each individual aligned residue. The coloured PSaC or ATPsyn-α sequences indicate the posterior probability according to the scale from 0% to 100% at the bottom. Analysis was carried out in the Pfam database v. 33.1. (c, d) Phylogenetic analysis (nucleic acid sequences) of GmPSaC (c) and GmATPsyn-α (d) with homologs from five soybean cultivars: William 82 (W82), Lee74, Somyoungkong (SMK), V94, and L29, as well as orthologs from Arabidopsis thaliana, Nicotiana benthamiana, and Solanum lycopersicum. The phylogeny was generated using the neighbour-joining method with MEGA 7 software. Numbers represent relative phylogenetic distance.

II. *GmPSaC* and *GmATPsyn-α* genes are induced in cultivars with different resistance backgrounds

The finding that *GmPSaC* and *GmATPsyn-α* are temporarily induced in L29, which is immune to G5H via the *Rsv3* gene but is susceptible to G7H, prompted us to determine the expression of both genes in other cultivars with different resistance backgrounds. For this, three *rsv*-null cultivars (Lee74, W82, and SMK), one *Rsv4* cultivar (V94), and one Rsv3 cultivar (L29) were assessed for their susceptibility to G7H. Infection by G7H (which expresses green fluorescent protein, GFP) induced visual symptoms in the systemically infected leaves (SL) of all cultivars except V94 at 10 days postinoculation (dpi) (Figure 3a). Confirming this, a protein blot revealed that GFP from G7H was undetectable in cultivar V94 but accumulated to different levels in the other cultivars, with Lee74 being the most susceptible to infection (Figure 3b). Reactive oxygen species (ROS), which is a sign of activated antiviral defence (Calil & Fontes, 2017; Wu et al., 2017), was not detected in any of the tested cultivars regardless of the resistance levels exhibited in response to G7H infection (Figure 3c). The expression level of *GmPSaC* and *GmATPsyn-α* was then measured in the inoculated leaves (IL) of the five infected cultivars at 5 dpi. Interestingly, only the resistant cultivar V94 showed a significant increase in the expression of both genes; the other cultivars did not exhibit significant changes in the expression except for a c.50% increase in *ATPsyn-α* in SMK

plants, which accumulated less G7H than the other susceptible cultivars (Figure 3d,e). These findings indicate that tolerance/resistance to G7H infection might be related to the function of both genes in soybean plants, and that the presence of an anti-SMV *R*-gene may enhance their regulation in response to SMV infection.

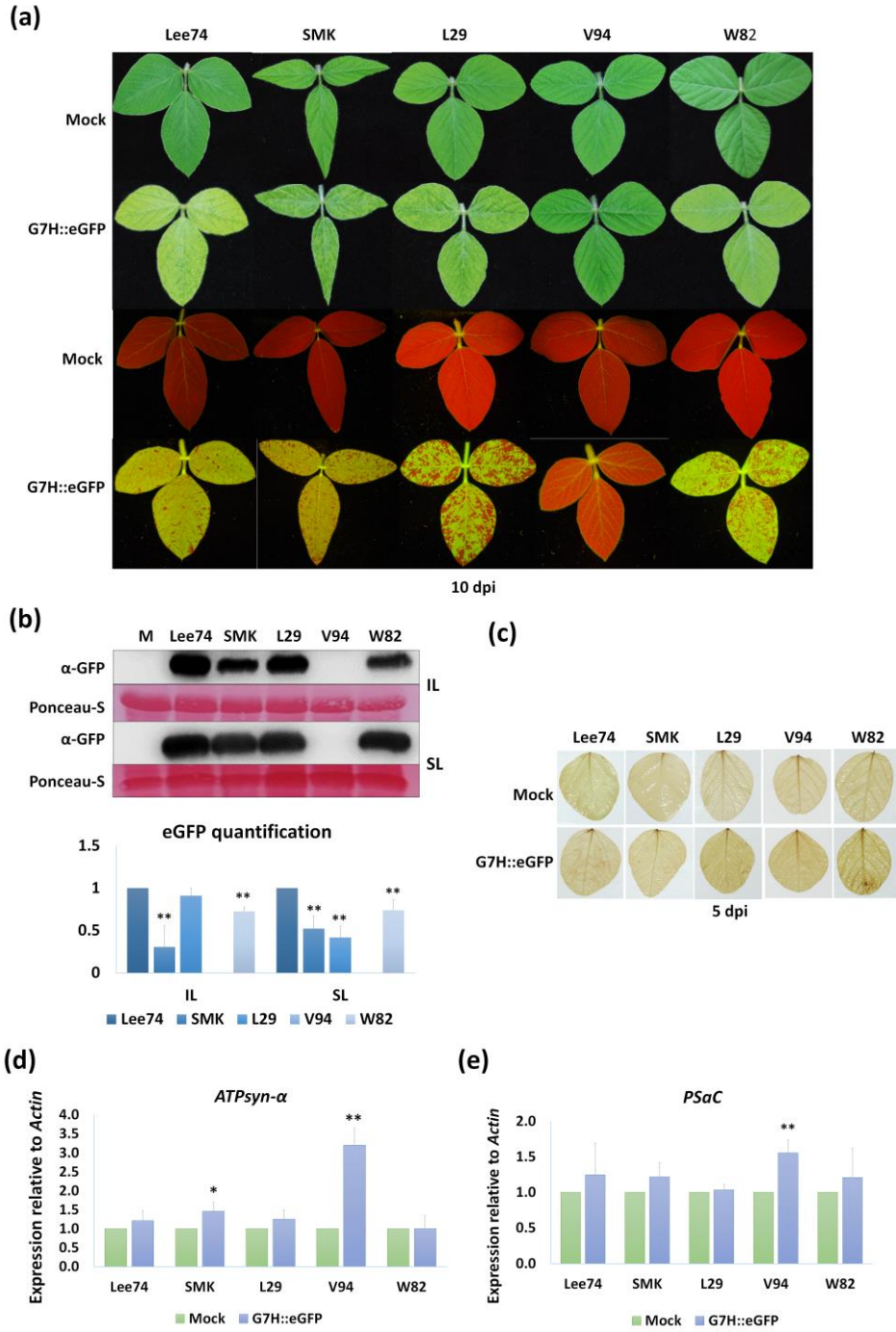

Figure 3. Soybean susceptibility to infection by SMV-G7H.

(a) Visual symptoms on the following five soybean cultivars infected with pSMV-

G7H::eGFP: Lee74, Somyoungking (SMK), L29, V94, and William 82 (W82). (b) Western protein blot for green fluorescent protein (GFP) levels (upper panel) in soybean cultivars infected with pSMV-G7H::eGFP and their quantified levels (lower panel). Inoculated leaves (IL) were assayed at 5 days postinoculation (dpi) and systemically infected leaves (SL) were assayed at 10 dpi. M is mock from uninfected Lee74 plants, which were used as a negative control. Ponceau S staining of RuBisCO was used on the loading control. The blot is a representative of three biological replicates with similar results. (c) Accumulation of reactive oxygen species (ROS) in soybean cultivars as indicated by 3,3′-diaminobenzidine staining at 5 dpi of pSMV-G7H::eGFP. (d, e) Relative expression levels of ATPsyn-α (d) and PSaC (e) in the five soybean cultivars infected with pSMV-G7H::eGFP at 5 dpi. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in the legend of Figure 1; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively.

III. Overexpression of ATPsyn-α and PSaC induces resistance against G7H in the susceptible cultivar Lee74

To determine the effect of ATPsyn-α and PSaC on resistance to G7H, the coding sequence (CDS) of each gene was cloned from L29 plants into the G7H genome to create pSMV-G7H::eGFP::ATPsyn-α and pSMV-G7H::eGFP::PSaC constructs (Figure 4a). As a member of the *Potyvirus* genus, SMV uses the host's cellular translation machinery to translate its RNA into one single polyprotein, which undergoes self-cleavage to produce 11 different viral proteins (Hajimorad et al., 2018). We previously took advantage of this characteristic by *inserting Green fluorescent protein* (GFP) as a reporter gene within the SMV-infectious clone pSMV-G7H::eGFP (Seo et al., 2014). Here, we inserted both genes downstream of the GFP within the G7H genome (Figure 4a). The rsv-null cultivar Lee74 was rub-inoculated at the unifoliate stage with plasmids of both constructs (the seedlings were about 12 days old) and the accumulation level was measured in IL and SL at 7 and 14 dpi, respectively. While Lee74 developed strong GFP fluorescence in the SL following infection with pSMV-G7H::eGFP, GFP fluorescence was weak in the case of pSMV-G7H::eGFP::PSaC and undetectable in the case of pSMV-G7H::eGFP::ATPsyn-α (Figure 4b). A western protein blot confirmed this observation, that is, GFP protein accumulation was lower for pSMV-G7H::eGFP::PSaC than for pSMV-G7H::eGFP, and was undetectable for pSMV-G7H::eGFP::ATPsyn-α in both

IL and SL (Figure 4c). Expression levels of eGFP RNA were also confirmed by RT-qPCR for both constructs, that is, expression was significantly lower in the chimeras than in the pSMV-G7H::eGFP control, and was lowest in pSMV-G7H::eGFP::ATPsyn-α (Figure 4d). These findings indicate that both genes contribute to resistance against G7H in soybean plants, although to different degrees.

The effect of ATPsyn-α and PSaC was also assayed on resistance to the avirulent strain G5H. Lee74 plants were infected with G5H::eGFP, G5H::eGFP::ATPsyn-α or G5H::eGFP::PSaC infectious clones (Figure S2a). Plants developed strong GFP patches following the infection with G5H::eGFP. However, GFP fluorescence was less in G5H::ATPsyn-α or G5H::PSaC constructs than in the G5H::eGFP control (Figure S2b), and the western protein blot showed very low accumulation of eGFP in plants infected with G5H::eGFP::ATPsyn-α or G5H::eGFP::PSaC compared to those infected with G5H::eGFP (Figure S2c). This result indicates that both genes induce a common defence mechanism against SMV virulent and avirulent strains.

To confirm that both inserts translated into proteins, we first checked for the presence of both genes in the pSMV-G7H::eGFP genome from RNA extracted from the soybean SL using primers targeting the flanking regions of the insert site. Indeed, both inserts were detected in the pSMV-G7H::eGFP genome (Figure S3a), and sequencing of PCR products showed

that both insets remained intact throughout the replication and movement of pSMV-G7H::eGFP. Next, an HA-tag was fused to each insert to generate G7H::eGFP::PSaC::HA and G7H::eGFP::ATPsyn-α::HA clones. A western protein blot showed that both genes were translated into proteins and that they were not lost or missed in the translation of the SMV polyprotein in the SL. In addition, the expression of these genes in pSMV-G7H::eGFP might trigger their silencing in plants. To examine this, RT-qPCR with primers annealing to the 3′ untranslated regions of both genes showed that endogenous transcripts of both genes were not affected by the constitutive expression via pSMV-G7H::eGFP (Figure S4a,b). To determine whether this resistance is connected to ROS, 3,3′-diaminobenzidine staining on the IL 7 dpi showed no ROS in response to G7H::eGFP or the constructs expressing either gene (Figure 4e). This indicated that ROS may not be part of the resistance induced by PSaC or ATPsyn-α.

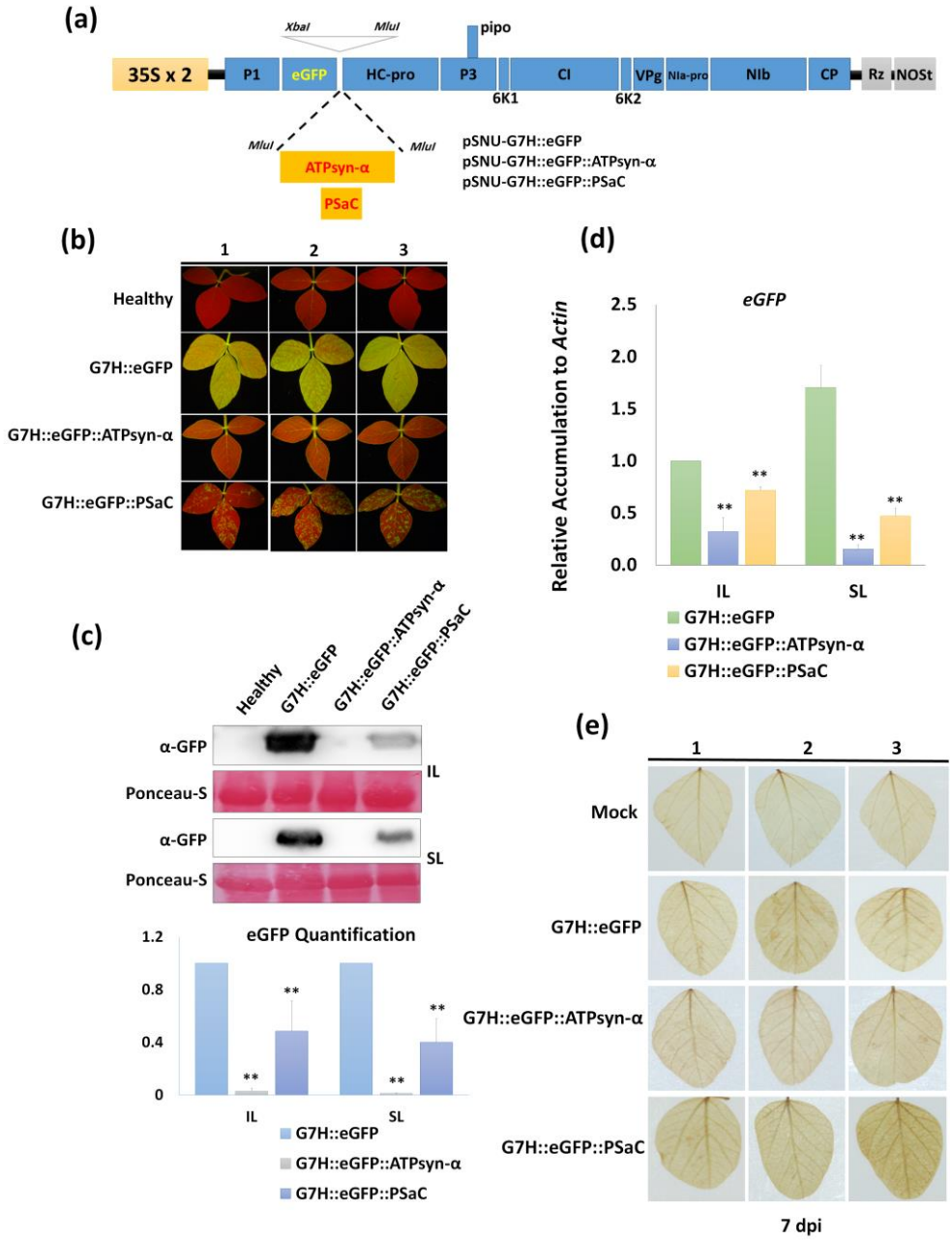

Figure 4. Effect of overexpressing ATPsyn-α and PSaC on resistance against G7H in the susceptible cultivar Lee74.

(a) Schematic drawing of pSMV-G7H::eGFP construct with the insertion site for ATPsyn-α and PSaC downstream of the GFP coding sequence; the expression of the construct is driven by two copies of the CaMV 35S promoter (35S × 2) and is terminated by an NOS terminator (NOSt). Rz is a cis-cleaving ribozyme sequence. (b) Green fluorescent protein

(GFP) visual levels in the systemically infected leaves (SL) of Lee74 plants. The first unifoliate leaves of 12-day-old seedlings were infected with pSMV-G7H::eGFP, pSMV-G7H::eGFP::ATPsyn-α, or pSMV-G7H::eGFP::PSaC. Fourteen days later, the SL from three plants (1, 2, and 3) were photographed under UV light. (c) Western protein blot for GFP levels (upper panel) in the inoculated leaves (IL) and the SL, and their quantified levels (lower panel). Ponceau S staining of RuBisCO was used on the loading control. (d) Relative expression levels of GFP RNA in IL and SL of Lee74 infected with pSMV-G7H::eGFP constructs. Actin11 was used as the internal control. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in the legend of Figure 1; ** indicates a significant difference at $p < 0.01$. (e) Detection of reactive oxygen species in Lee74 as indicated by 3,3′-dimainobenzidine staining at 7 days postinoculation (dpi).

IV. Knockdown of ATPsyn-α and PSaC increased Lee74 susceptibility against G7H infection

To confirm the role of both genes in resistance against G7H, virus-induced gene silencing was employed using the silencing vector bean pod mottle virus (BPMV). Knocking down either gene significantly reduced its expression by c.60% compared with the empty vector of BPMV (BPMV-EV) (Figure 5a). No visual symptoms were developed on the knocked-down plants other than the typical BPMV mottling symptoms observed at 12 dpi (Figure 5b). Lee74 plants were then infected with G7H::eGFP, which developed a strong GFP signal in the SL at 10 dpi in ATPsyn-α-silenced plants, but was of similar intensity to that of PSaC-silenced plants (Figure 5c). RT-qPCR and western blot for eGFP confirmed that G7H::eGFP accumulated more in the *ATPsyn-α* knocked-down plants, and that G7H accumulation level was similar between BPMV-EV and BPMV-PSaC plants (Figure 5d,e). These data indicated that silencing *ATPsyn-α* has a strong influence on plant susceptibility to G7H infection, unlike that of *PSaC*, which was similar to the control BPMV-EV treatment.

To determine whether the silencing process may affect off-target transcripts, a BLAST search using both genes was made in the Soybase database in a search for paralogs. Only *ATPsyn-α* had two close paralogs: *Glyma*.16G115300.1 (which encodes a chloroplast ATP synthase subunit α) and *Glyma*.05G092300.1 (which encodes a mitochondrial ATP synthase

subunit α). However, the designated fragment for silencing shares low similarity with the two paralogs (Figure S5). Expression levels of either gene were not affected by the silencing of ATPsyn-α (Figure S6a,b), which indicates that silencing probably did not affect off-target transcripts.

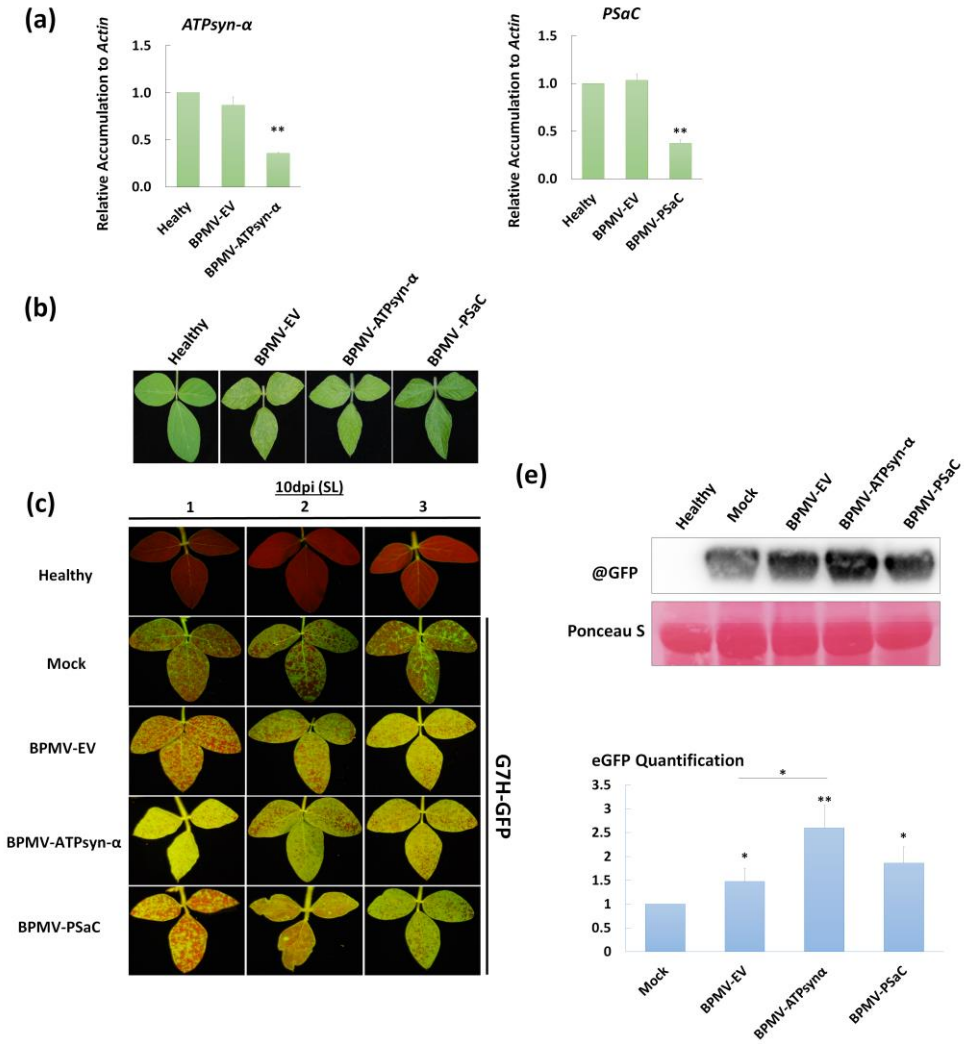

Figure 5. Effect of silencing GmATPsyn-α and GmPSaC on soybean susceptibility to SMV-G7H infection.

Lee74 plants were silenced in GmATPsyn-α and GmPSaC using BPMV silencing vector. (a) Relative expression levels of ATPsyn-α (left) and PSaC (right) in the upper systemic leaves of Lee74 plants 14 days after BPMV infection in the empty vector (BPMV-EV), ATPsyn-α-silenced (BPMV-ATPsyn-α), and PSaC-silenced plants (BPMV-PSaC). Healthy plants were used as negative control. Actin was used as internal control. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in the legend of Figure 1; ** indicates a significant difference at $p < 0.01$. (b) Mottling symptoms developed in silenced Lee74 plants compared to BPMV-EV control or healthy plants. (c) Green fluorescent protein (GFP) fluorescence from the upper systemic leaves of

silenced plants infected with pSMV-G7H::eGFP at 10 days postinoculation (dpi). Mock plants were treated with phosphate buffer as a control for BPMV infection. (d) Relative expression levels of eGFP in the Lee74 systemically infected leaves with pSMV-G7H::eGFP at 10 dpi. Healthy plants were used as negative control. Actin was used as internal control. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in the legend of Figure 1; significant difference at $*p < 0.05$, $**p < 0.01$. (e) Protein blot of GFP levels (upper panel) in the Lee74 systemically infected leaves with pSMV-G7H::e GFP at 10 dpi, and their quantified levels (lower panel). Ponceau S staining of RuBisCO was used on the loading control. The blot is a representative of three biological replicates with similar results

V. Localization of ATPsyn-α and PSaC in N. benthamiana and their effects on N. benthamiana resistance against SMV-G7H

To investigate the localization of ATPsyn-α and PSaC, we expressed both genes in the binary vector pBin61-HA-mCherry (Alazem et al., 2020). We used the chloroplast-localized protein from Arabidopsis, EMB1303, fused with eGFP as a marker protein (Huang et al., 2009). AtEMB1303 localized in the chloroplast membrane, and the GFP signal was also detected in the extended stromules (Figure 6a). Both PSaC and ATPsyn-α localized in the chloroplast envelope, the nucleus, and the cytoplasm (Figure 6b,c). We next examined the effect of the transient expression of both genes on G7H accumulation in N. benthamiana. Although N. benthamiana is not a preferred host for SMV, the virus can accumulate to detectable levels in this host. Interestingly, both soybean genes reduced the accumulation of SMV-G7H in N. benthamiana plants, indicating that the resistance mechanism regulated by these genes could be similar in the two hosts and independent of the Rsv3-mediated resistance (Figure 6d).

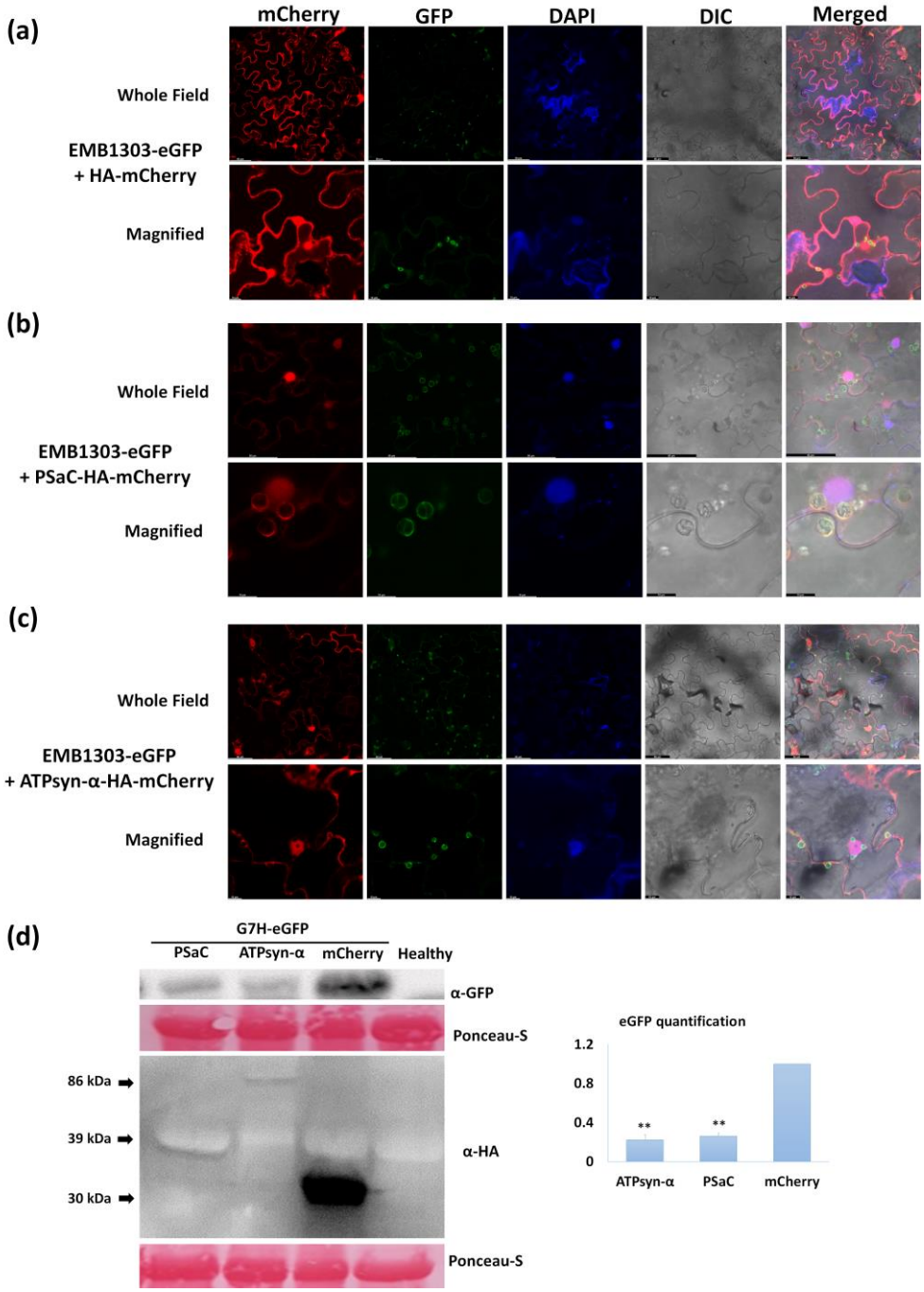

Figure 6. Localization and effects of GmATPsyn-α and GmPSaC on resistance to SMV-G7H in Nicotiana benthamiana leaves.

(a) Localization of the chloroplast-marker protein AtEMB1303-eGFP with pBin-3HA-mCherry as a control. (b) Co-localization of EMB1303-eGFP and PSaC-HA-mCherry. (c)

Co-localization of EMB1303-eGFP and ATPsyn-α-3HA-mCherry. N. benthamiana plants were agroinfiltrated with pBin-eGFP-AtEMB1303 (chloroplast-marker protein) with pBin-3HA-mCherry constructs carrying GmATPsyn-α or GmPSaC, and pPZP-2b, which carries the CMV suppressor of RNA-silencing protein gene (2b) to enhance the transient expression. DAPI was used to stain nuclei. Leaves were photographed 3 days after agroinfiltration. Scale bars measure 50 µm for the whole field and 10 µm for the magnified field. (d) Effect of transient overexpression of PSaC and ATPsyn-α on resistance to SMV-G7H in N. benthamiana plants. The same agrobacterial cultures used for the localization test were used without pPZP2b for the SMV-G7H::eGFP infection. One day after agroinfiltration, N. benthamiana leaves were sap-infected with SMV-G7H::eGFP prepared from infected soybean plants. Samples were collected at 5 days postinoculation, and western protein blots were hybridized with anti-GFP to detect eGFP from SMV-G7H, and anti-HA to detect GmPSaC (39 kDa), GmATPsyn-α (80 kDa), and the empty vector 3HA-mCherry (30 kDa). eGFP levels were quantified using ImageJ (right panel). Ponceau S staining of RuBisCO was used as the internal control, and the blots are representatives of three biological replicates. ** indicates a significant difference at $p < 0.01$

VI. Involvement of defence-related hormones in ATPsyn-α and PSaC- mediated resistance

The chloroplast plays a critical role in plant immunity because it is a major site for the production of several plant hormones such as SA, ABA, jasmonic acid (JA), and ethylene (ET) (Alazem & Lin, 2015; Bhattacharyya & Chakraborty, 2018; Zhao et al., 2016). To investigate whether ATPsyn-α and PSaC have any effect on the signalling pathway of defence-related hormones, the expression levels of key genes in the signalling pathways of SA, ABA, JA, and ET were measured in Lee74 plants infected with pSMV-G7H::eGFP, pSMV-G7H::eGFP::PSaC, and pSMV-G7H::eGFP::ATPsyn-α. SMV-G7H::eGFP infection of Lee74 plants decreased the expression of *ICS1* in the SA pathway and of ABA1 in the ABA pathway (Figure 7a,e). However, the following genes belonging to different pathways were increased in response to G7H infection: *PAD4* in the SA pathway (Figure 7b), *JAR1* in the JA pathway (Figure 7c), ABA2 in the ABA pathway (Figure 7f), and *DREB1A-1* and *DREB1A-2* in the ET pathway (Figure 8g,h). This indicated that SMV-G7H infection disrupts the hormone balance in the infected plant by inducing several antagonistic hormone signalling pathways.

The expression levels of *ICS1* and *PAD4* in the SA biosynthesis pathway were significantly higher in the SL of plants infected with pSMV-G7H::eGFP::PSaC and pSMV-G7H::eGFP::ATPsyn-α than in plants infected with pSMV-G7H::eGFP. Such an increase was only recorded for

PAD4 in the SL of plants infected with both constructs (Figure 7a,b). Similarly, the expression levels of the JA-related genes *JAR1* and *Lox2* were significantly higher in both IL and SL of plants infected with both constructs than in plants infected with pSMV-G7H::eGFP (Figure 7c,d). However, only the IL of pSMV-G7H::eGFP::ATPsyn-α infected plants exhibited increased levels of *ABA1* and *ABA2* from the ABA biosynthesis pathway (Figure 7e,f). Compared to its expression in response to pSMV-G7H::eGFP infection, expression of the ET-related transcription factor (TF) *GmDREB1A-1* increased only in response to infection by SMV-G7H::eGFP::ATPsyn-α in the IL (Figure 7g). The other ET TF *GmDREB1A-2* was not affected by infection of either constructs compared to SMV-G7H::eGFP infection (Figure 7h). These data indicate that ATPsyn-α has a strong effect on the expression of SA-, JA-, and ABA-related genes, although they function antagonistically under abiotic stress conditions, and that PSaC increased the expression of the SA- and JA-related genes.

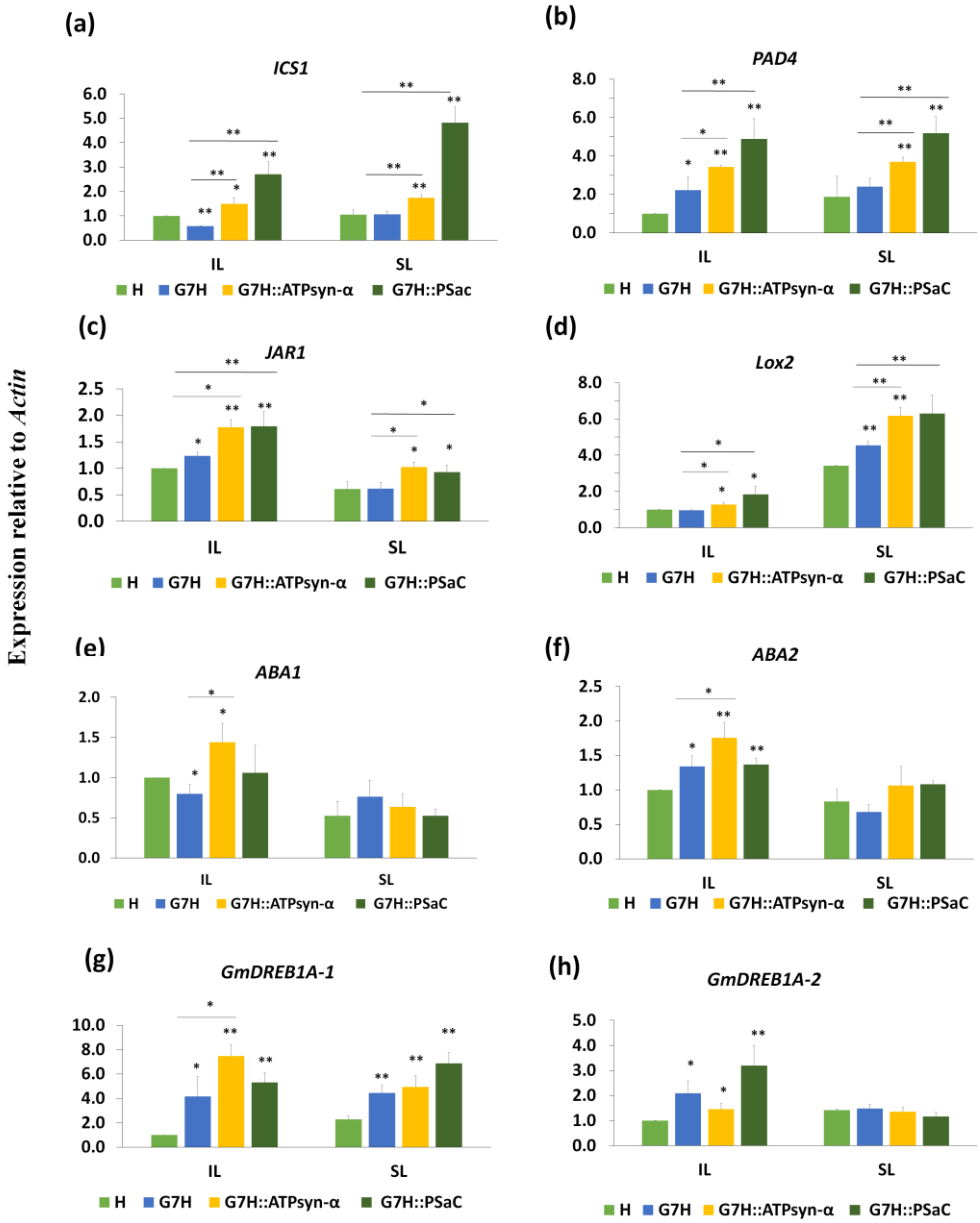

Figure 7. Expression levels of key genes of defence-related hormones in Lee74 plants in response to SMV-G7H expressing ATPsyn-α and PSaC genes.

Relative expression levels in Lee74 plants of salicylic acid-related genes *ICS1* (a) and *PAD4* (b); jasmonic acid-related genes *JAR1* (c) and *Lox2* (d), abscisic acid biosynthesis genes ABA1 (e) and ABA2 (f), and ethylene-related genes *GmDREB1A-1* (g) and *GmDREB1A-2* (h). The unifoliate leaves of Lee74 plants were inoculated with pSMV-

G7H::eGFP expressing ATPsyn-α or PSaC genes (pSMV-G7H::eGFP::ATPsyn-α or pSMV-G7H::eGFP::PSaC, respectively); the inoculated leaves (IL) and systemically infected leaves (SL) were collected at 7 and 14 days post inoculation, respectively. Actin11 was used as the internal control. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in Figure 1; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively. An additional t test was carried out to compare expressions in the pSMV-G7H::eGFP::ATPsyn-α and pSMV-G7H::eGFP::PSaC treatments to that in pSMV-G7H::eGFP.

VII. Antiviral RNA silencing genes are regulated in ATPsyn-α and PSaC- mediated resistance

Because SA and ABA affect the expression of RNA silencing genes (Alazem & Lin, 2020) the expression levels of the key genes in this pathway were measured in response to infection by pSMV-G7H::eGFP, pSMV-G7H::eGFP::PSaC, or pSMV-G7H::eGFP::ATPsyn-α. The expression levels of the Dicer-like (*DCL*) genes *DCL2a* and *DCL4a*, and of the RNA-dependent RNA polymerase (*RDR*) genes *RDR1a*, *RDR2a*, and *RDR6a* were up-regulated in response to infection with either construct (Figure 8). Compared to infection of Lee74 plants with pSMV-G7H::eGFP, infection with pSMV-G7H:e:GFP::ATPsyn-α significantly increased the expression of *DCL4a*, *RDR2a*, and *RDR2a* in the IL (Figure 8b,d,e), and this effect was evident only for *RDR2a* and *RDR6a* in the SL (Figure 8d). In contrast, *DCL2a* and *RDR1a* were down-regulated or unchanged, respectively, in response to pSMV-G7H::eGFP::ATPsyn-α local infection (Figure 8a,c). The effect of pSMV-G7H::eGFP::PSaC infection was weaker than that of pSMV-G7H::eGFP::ATPsyn-α infection with only *RDR2a* induced locally and systemically (Figure 8d), and RDR6a induced systemically (Figure 8e).

We next determined if this effect on the RNA silencing genes was similar to that in N. benthamiana plants infected with G7H::eGFP expressing both gene. *NbDCL2*, *NbDCL4*, *NbRDR2*, and *NbRDR6* were significantly increased response to virus infection (Figure 9). However, the

expression was significantly higher for plants infected with G7H::eGFP::ATPsyn-α or G7H::eGFP::PSaC than those infected with G7H::eGFP for *NbDCL4*, *NbRDR2*, and *NbRDR6* genes (Figure 9b–d). These data indicate that the defence mechanisms affected by both genes are similar between soybean and *N. benthamiana* plants. Collectively, the antiviral RNA silencing genes may partially contribute to the ATPsyn-α- and PSaC-mediated resistance in soybean plants, and the influence of ATPsyn-α on RNA silencing genes is greater than that of PSaC.

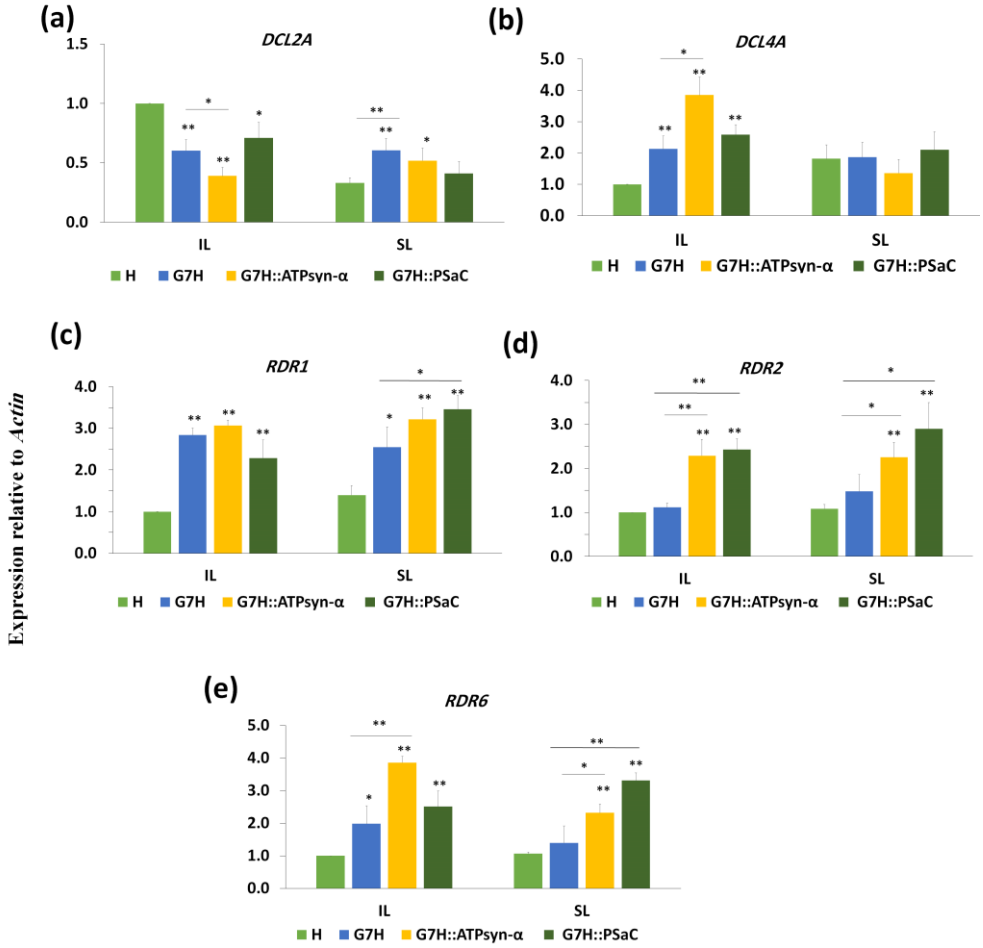

Figure 8. Expression levels of RNA silencing genes in Lee74 plants in response to SMV-G7H expressing ATPsyn-α and PSaC genes.

Relative expression of Dicer-like (DCL) 2a (a) and DCL4a (b), and of RNA-dependent RNA polymerase (RDR) 1a (c), RDR2a (d), and RDR6a (e) in Lee74 plants. Actin11 was used as the internal control. Values are means + SD of three biological replicates. Statistical analysis was carried out as described in Figure 1; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively. An additional t test was carried out to compare expression in the pSMV-G7H::eGFP::ATPsyn-α and pSMV-G7H::eGFP::PSaC treatment with that in the pSMV-G7H::e GFP treatment’

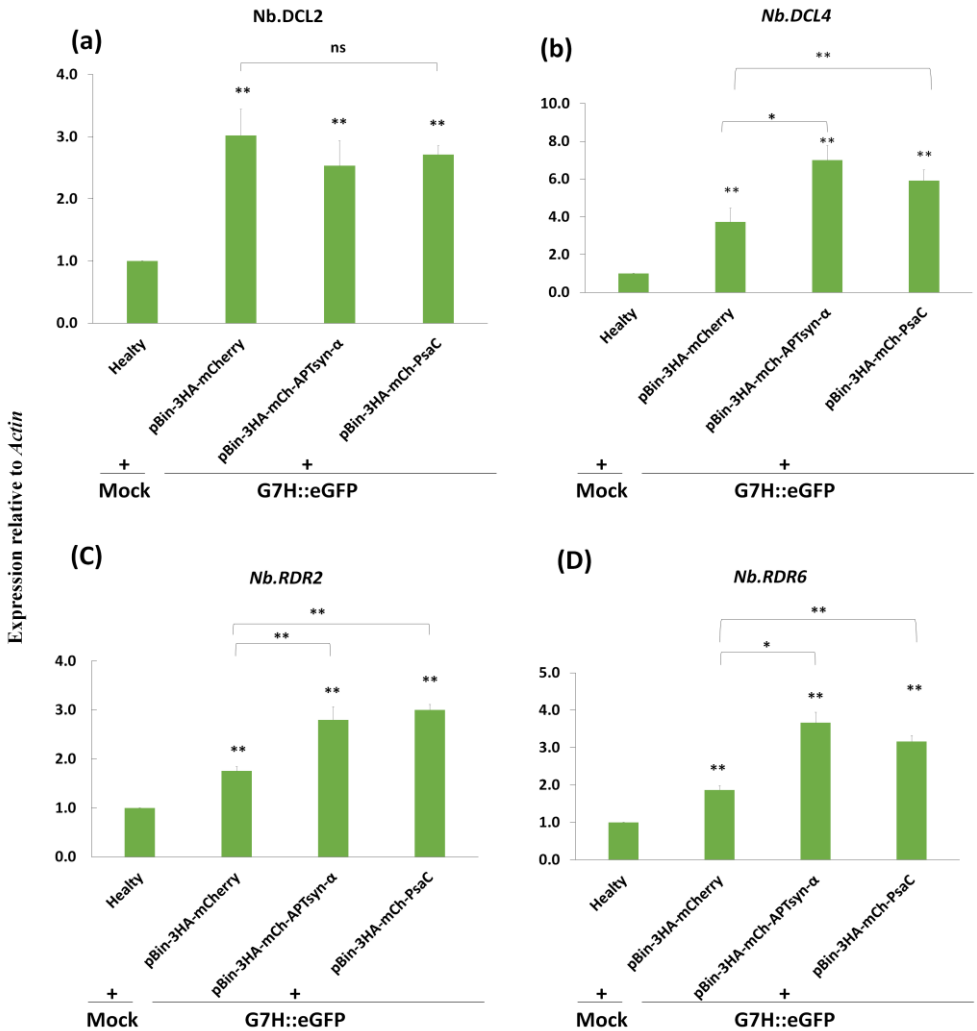

Figure 9. Expression levels of RNA silencing genes in *Nicotiana benthamiana* in response to infection with chimeras of pSMV-G7H::eGFP.

Relative expression levels of Dicer-like (*DCL*) 2a (a) *DCL4a* (b), RNA-dependent RNA polymerase (*RDR*) 2a (c), and *RDR6a* (d) in *N. benthamiana* plants infected with pSMV-G7H::eGFP, pSMV-G7H::eGFP::ATPsyn-α, or pSMV-G7H::eGFP::PSaC. *Actin* was used as internal control. Values are means + *SD* of three biological replicates. Statistical analysis was carried out as described in Figure 1; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively. An additional *t* test was carried out to compare expression in the pSMV-G7H::eGFP::ATPsyn-α and pSMV-G7H::eGFP::PSaC treatment with that in the pSMV-G7H::eGFP treatment

DISCUSSION

An increasing body of evidence connects plant virus replication and movement with the chloroplast. The effects of chloroplast genes on viruses are diverse and vary among virus groups. While some viruses recruit specific chloroplast proteins to their replication or movement complex, others reduce the expression of specific chloroplast genes to facilitate their replication and spread (Cheng et al., 2013; Ganusova et al., 2020; Jiang et al., 2020; Zhao et al., 2016, 2019). The current study provides evidence of positive roles of two photosynthesis-related genes, *GmPSaC* and *GmATPsyn-α*, in inducing resistance against SMV infection in the susceptible soybean cultivar Lee74. Previous studies reported a similar role for other ATPsyn subunits in resistance to other viruses. For example, infection with tobacco mosaic virus (TMV) reduced the expression levels of the *ATPsyn-γ* subunit, and when *ATPsyn-γ* was silenced in *N. benthamiana* plants, TMV accumulation and pathogenicity were greatly enhanced, indicating that *ATPsyn-γ* is involved in limiting the intracellular trafficking of TMV as well as in inducing defence signalling pathways (Bhat et al., 2013). Interestingly, an opposite effect was found for *ATP-syn-γ* in response to infection with PVX or tomato bushy stunt virus, that is, their spread was decreased in *ATP-syn-γ*-silenced plants (Bhat et al., 2013). In another example, infection with potato virus Y reduced the photosynthesis rate through the HC-Pro protein in *Nicotiana tabacum* plants; HC-Pro interacted

with the ATPsyn-β subunit but did not affect the enzymatic activity of ATP synthase, leading to a reduced ATP synthase content in HC-Pro-transgenic plants (Tu et al., 2015). In other words, we cannot generalize about the effects of ATPsyn subunits on host plant resistance to viruses; the influence on resistance can vary depending on the virus group.

ATPsyn-α and -β form the hydrophilic head (cF1) powered by the membrane-embedded-cF0 rotary motor in the ATP synthase complex. ATPsyn-α guides protons to and from the c-ring protonation site (Hahn et al., 2018). In general, ATP synthase is redox-regulated and controlled by the chloroplast thioredoxin system, which is connected with photosynthesis (Hisabori et al., 2013). Regulation of redox controls the accumulation of ROS and nitrogen species, both of which are important for resistance against several pathogens (Bentham et al., 2020; Frederickson Matika & Loake, 2014). Given the absence of necrotic lesions in soybean expressing PSaC or ATPsyn-α, however, it is unlikely that ROS is involved in ATPsyn-α- or PSaC-mediated-defence against SMV-G7H.

PSaC encodes a subunit in the PSI complex and functions in electron transfer and ferrodoxin docking on the stromal side of PSI (Rantala et al., 2020). Although studies on the role of PSaC in plant–virus interactions are lacking, a previous report indicated a positive role for another member of the PSI complex, PSaK, in resistance against plum pox virus (PPV) (Jimenez et al., 2006). Infection with PPV decreased PSaK expression in *N.*

benthamiana, and when PSaK was knocked down, PPV accumulation was enhanced. In addition, the cylindrical inclusion protein of PPV interacted with PSaK and possibly interfered with its function (Jimenez et al., 2006). Our data showed that, in response to SMV-G7H infection, expression of PSaC and ATPsyn-α increased in resistant soybean plants but did not decrease in susceptible plants (Figure 3d,e). That their overexpression reduced SMV-G7H accumulation (Figure 4b,d) suggests that both genes partially contributed to resistance against SMV. In line with this finding, silencing *ATPsyn-α*, but not *PSaC*, increased soybean susceptibility to SMV-G7H infection (Figure 5). This confirms the role of ATPsyn-α in resistance, but also suggests functional redundancy for genes might interrelate with PSaC, which could be members of the PSI.

The resistance conferred by ATPsyn-α is stronger than that conferred by PSaC in both *N. benthamiana* and soybean plants (Figure 4b,d). This could be attributed to the simultaneous induction of several genes in the defence signalling pathways of SA, JA, and ABA in response to pSMV-G7H::eGFP::ATPsyn-α, but for pSMV-G7H::eGFP::PSaC the response was limited to SA and JA (Figure 7). Pathways of all of these hormones are involved in soybean resistance to SMV (Alazem et al., 2018, 2019; Zhang et al., 2012). In fact, the connection between defence hormones and the antiviral RNA silencing pathway is well established (Alazem et al., 2019; Alazem & Lin, 2015, 2020). We previously showed that SA and ABA

enhance the expression of the antiviral RNA silencing genes in soybean and *A. thaliana*, and that the enhanced expression confers partial resistance against SMV, BaMV, and PVX (Alazem et al., 2017, 2019). Our current findings show that ATPsyn-α induced the expression of more genes (*DCL4a*, *RDR2a*, and *RDR6a*) in the antiviral RNA silencing pathway than PSaC, which only induced the expression of RDR2a and only in the IL (Figure 8). It is therefore likely that the stronger resistance triggered by ATPsyn-α than PSaC is due to the greater influence of ATPsyn-α on the antiviral RNA-silencing genes.

Because trafficking through PD is strongly regulated by light and the circadian clock (Brunkard & Zambryski, 2019; Ganusova et al., 2020), it is highly probable that chloroplast-related genes can adversely affect viruses in two ways, that is, the gene products may hinder cell-to-cell trafficking through PD and may also induce defence-related hormone signalling pathways. Our results provide evidence that induction of these photosynthesis genes induces hormone signalling pathways that eventually trigger antiviral RNA silencing pathways that partially contribute to local and systemic resistance to SMV (Figure 8). Whether SMV trafficking through PD is affected by photosynthesis genes requires further investigation. The effect of enhanced photosynthesis on plant resistance to viruses is incompletely understood and also warrants additional research.

We expected to detect ATPsyn-α and PSaC inside the chloroplast,

but, surprisingly, we found that they were localized in the chloroplast envelope. In addition, both proteins were localized in the cytoplasm and the nucleus (Figure 6b, c). We did not detect any degradation of either protein by western blot (Figure 6d), which indicates that both proteins can be distributed to the cytoplasm and the nucleus for further functions that remain to be examined.

In conclusion, strong photosynthesis can increase resistance against viruses. Additional research is needed to clarify how chloroplasts in general, and photosynthesis in particular, enhance resistance against plant viruses.

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CHAPTER III.

Chloroplast- related host proteins interact with NIb and NIa-Pro of soybeans mosaic virus and induce resistance in the susceptible cultivar

This chapter is in preparation for publication

ABSTRACT

To gain a deeper understanding of the molecular mechanisms involved in viral infection and the corresponding plant resistance responses, it is essential to investigate the interactions between viral and host proteins. In the case of viral infections in plants, a significant portion of the affected gene products are closely associated with chloroplasts and photosynthesis. However, the molecular mechanisms underlying the interplay between the virus and host chloroplast proteins during replication remain poorly understood. In our previous study, we have made an interesting discovery regarding the soybean mosaic virus (SMV) infection in resistant and susceptible soybean cultivars. We have found that the PSI subunit (*PSaC*) and ATP-synthase-subunit (*ATPsyn-α*) genes are upregulated in the resistant cultivar following SMV-G7H and SMV-G5H infections, compared to the susceptible cultivar. Overexpression of them within the SMV-G7H genome in the susceptible cultivar Lee74 (*rsv3*-null) reduced SMV accumulation while silencing the *PSaC* and *ATPsyn-α* genes promoted SMV accumulation. We also found that the PSaC and ATPsyn-α proteins are present in the chloroplast envelope, nucleus, and cytoplasm.

Building on these findings, we characterized protein-protein interactions between PSaC and ATPsyn-α with two viral proteins, NIb and NIa-Pro, respectively, of SMV. Through co-immunoprecipitation (Co-IP) experiments, we confirmed the interactions between these proteins.

Moreover, when the C-terminal region of either PSaC or ATPsyn-α was overexpressed in the SMV-G7H genome, we observed a reduction in viral accumulation and systemic infection in the susceptible cultivar. Based on these results, we propose that the *PSaC* and *ATPsyn-α* genes play a modulatory role in conferring resistance to SMV infection by influencing the function of NIb and NIa-Pro in SMV replication and movement. The identification of these photosynthesis-related genes as key players in the interplay between the virus and the host provides valuable insights for developing more targeted control strategies against SMV. Additionally, by utilizing these genes, it may be possible to genetically engineer plants with improved photosynthetic efficiency and enhanced resistance to SMV infection.

Keywords: soybeans, chloroplast-virus interplay, plant defense, soybean mosaic virus, viral replication

INTRODUCTION

Soybean mosaic virus (SMV), a member of the genus *Potyvirus*, infects soybeans and is spread by aphids, resulting in severe diseases and significant economic losses around the globe (Hajimorad et al., 2018). The Rsv and Rsc sets of strain-specific NLR-type R-genes, are primarily used to provide genetic resistance to SMV (Widyasari et al. 2020). While Rsc genes that offer resistance to the SC1 to SC22 strains recorded in China, Rsv genes that confer resistance to the G1 to G7 SMV strains discovered in the United States. The R gene may result in HR or ER responses depending on the strain and load of the virus. (Alazem et al., 2023; Widyasari et al., 2020). There are several other non-NLR host factors for example, GmPP2C3a, GmPAP2.1, PSaC, and ATPsyn-α that have been found to be crucial for resistance, either because they are key components in the signaling cascade that runs downstream of the *R*-gene or because they regulate immune responses, including plant hormones and RNAi pathways (Bwalya et al., 2022; Seo et al., 2014; Widyasari et al., 2022).

For SMV to infect and replicate on its host, complex molecular interactions between viral proteins and host proteins are required particularly for this kind of virus like other positive-sense single-stranded RNA viruses have a small genome therefore, the host machinery is

responsible for the replication of viral genomes (Bwalya and Kim, 2023; Hajimorad et al., 2018; Zhao et al., 2016, 2019).

Innumerable host factors of plant-virus interactions have been identified, and interestingly, large proportions of these host factors are chloroplast- and photosynthesis-related proteins (Bhattacharyya and Chakraborty, 2018; Bwalya and Kim, 2023; Yadav et al., 2019; Zhao et al., 2016, 2019). Although photosynthesis is the major function of the chloroplast, its roles clearly extend further than converting light energy into chemical energy. It is evident that plants require more energy from photosynthesis during interactions with pathogens since initiating defense responses requires the energy that photosynthesis provides (Hammerschmidt,1999; Swarbrick and Lefert, 2006). Chloroplast not only provides energy but also plays important roles in the production of reactive oxygen species (ROS), calcium (Ca2+), and several defense-related hormones like salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) that have significant connections to plant immunity (Bhattacharyya and Chakraborty, 2018; Bobik and Burch-Smith, 2015; Bwalya and Kim, 2023; Kozuleva et al., 2011; Nambara and Marion-Poll, 2005; Padmanabhan and Dinesh-Kumar, 2010; Seyfferth and Tsuda, 2014; Stael et al., 2015; Torres et al., 2006; Wasternack, 2007; Wasternack and Hause, 2013; Widyasari et al., 2022; Wildermuth et al., 2001; Yang et al., 2021). Although research on the molecular mechanisms underlying SMV infection

of plants has advanced, little is known about how SMV proteins interact with chloroplast-related proteins.

In our previous study (Bwalya et al., 2022), we observed strong upregulation of two chloroplast-related proteins, the PSI subunit (GmPSaC) and ATP-synthase-subunit (GmATPsyn-α), in cultivar L29 in response to SMV-G5H infection, but a weaker response to SMV-G7H. Overexpression of either GmPSaC or GmATPsyn-α in the SMV-G7H genome induced resistance against SMV infection in the susceptible soybean cultivar Lee74, and both proteins were found to localize in the chloroplast envelope, the nucleus, and the cytoplasm. Knockdown of either GmPSaC or GmATPsyn-α significantly reduced their expression, and pSMV-G7H::GFP-infected knockdown plants exhibited a strong GFP signal in the systemic leaves.

In this study, we used a yeast two-hybrid system to identify interactions between SMV viral proteins and soybean host proteins GmPSaC and GmATPsyn-α. Our results demonstrated that nuclear inclusion protein b (NIb) and nuclear inclusion protein a (NIa-Pro) interacted with GmPSaC and GmATPsyn-α, respectively, in the cytoplasm and nucleus, impairing the replication and movement of these viral proteins. We also showed that the C-terminal portion of GmPSaC or GmATPsyn-α is crucial for these interactions. Overexpression of the C-terminal portion of either protein in the SMV-G7H genome reduced viral accumulation and systemic infection.

MATERIALS AND METHODS

I. Plant materials, growth conditions, and virus infections

Soybean (Glycine max) and Nicotiana benthamiana plants were grown in growth chambers at 25 ◦C with 70% relative humidity and a 16/8h photoperiod. To investigate the effects of PSaC and ATPsyn-α mutants on the accumulation of SMV-G7H::GFP in Lee74 plants, plasmids of the infectious clones pSMV-G7H::GFP, pSMV-G7H::GFP::PSaC, pSMV-G7H::eGFP::PSaCΔN&ΔFer, pSMV-G7H::eGFP:: PSaCΔC&ΔN, pSMV-G7H::eGFP:: PSaCΔC&ΔFer4, pSMV-G7H::eGFP::PSaCΔN, pSMV-G7H::eGFP::PSaCΔC, pSMV-G7H::GFP::ATPsyn-α, pSMV-G7H::eGFP::ATPsyn-αΔN&NBD, pSMV-G7H::eGFP::ATPsyn-αΔN&ΔC, pSMV-G7H::eGFP::ATPsyn-αΔC, pSMV-G7H::eGFP::ATPsyn-αΔN, and pSMV-G7H::eGFP::ATPsyn-αΔC &NBD were directly rub-inoculated on Lee74 plants using 10 μg of plasmid per leaf. The upper systemic leaves (SLs) were collected at 14 days post-inoculation (dpi) for RNA and protein extraction

II. RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol (Sigma) following the manufacturer's instructions. A 1 μg quantity of total RNA was used for cDNA synthesis using the GoScript kit (Promega, USA). RT-qPCR was carried out with SYBR-Green (Promega, USA) to measure the relative

expression of target genes using the ΔΔCT method. *Actin11* was used as an internal control, and the primers used in this study are listed in Table S1. One-sided Student's *t*-tests ($p < 0.05$) were used to determine the expression level of RNA/eGFP. The experiment was conducted with at least 3 biological replicates.

III. Plasmid construction

Total RNA was isolated from leaf samples harvested from soybean plants using TRIzol (Sigma, USA) reagent. The RNA samples were used for cDNA synthesis using the GoSript kit (Promega, USA). The mutants were amplified and cloned were then cloned into a TA vector (pGEM-T Easy, Promega, USA). The clones were confirmed by sequencing with gene-specific primers (Table S1) and then cloned into the pSMV-G7H::GFP infectious clone to generate clones pSMV-G7H::GFP, pSMV-G7H::GFP::PSaC, pSMV-G7H::eGFP::PSaC$^{\Delta N\&\Delta Fer}$, pSMV-G7H::eGFP::PSaC$^{\Delta C\&\Delta N}$, pSMV-G7H::eGFP::PSaC$^{\Delta C\&\Delta Fer4}$, pSMV-G7H::eGFP::PSaC$^{\Delta N}$, pSMV-G7H::eGFP::PSaC$^{\Delta C}$, and then pSMV-G7H::GFP::ATPsyn-α, pSMV-G7H::eGFP::ATPsyn-α$^{\Delta N\&NBD}$, pSMV-G7H::eGFP:: ATPsyn-α$^{\Delta N\&\Delta C}$, pSMV-G7H::eGFP::ATPsyn-α$^{\Delta C}$, pSMV-G7H::eGFP::ATPsyn-α$^{\Delta N}$, and pSMV-G7H::eGFP::ATPsyn-α$^{\Delta C\ \&NBD}$ as previously described (Seo et al., 2009).

IV. Western blotting

Total proteins were extracted from 0.1 g of tissue collected from a pool of inoculated or systemically infected leaves from three plants as described previously (Bwalya et al., 2022). Constructs expressing GFP were detected by protein blot using a polyclonal anti-GFP antibody (Sigma, USA). The primary antibody was bound with the goat anti-rabbit secondary antibody (Bio-Rad, USA). Ponceau-S was used as a loading control.

V. Co-Immunoprecipitation (Co-IP) assay

For the Co-IP assay, total proteins from *N. benthamiana* leaves were collected three days after agroinfiltration. Total protein was extracted from 2g of leaves (a pool of 6 leaves from 3 plants) in extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 M MgCl2, 5 mM DTT, and 5% Nonidet P-40) with protease inhibitor cocktail (Roche, Swiss). Immunoprecipitation was carried out by incubating protein extracts with GFP-Trap beads for 3 hours at 4℃ on a gentle rotary shaking. The precipitations were washed four times with cold immunoprecipitation buffer at 4℃ and beads were suspended in 100 µL of extraction buffer, and 35 µL was proportionally mixed with 4× NuPAGE loading buffer (Thermo-Scientific, USA) and loaded onto a 12% acrylamide SDS-PAGE gel which was then analyzed by

immunoblot using anti-mCherry or anti-GFP antibodies as prescribed (Yang et al., 2014)

VI. Statistical analysis

RT-qPCR was carried out in at least three biological replicates, and each biological replicate was repeated in three technical replicates. In the figure panels with bar graphs, values were compared to that of the mock-treated, uninfected plants (the bar on the left) with one-sided Student's t-tests; * and ** indicate a significant difference at $P < 0.05$ and <0.01, respectively. Error bars in the charts are means of the standard deviation of three biological replicates.

VII. Subcellular localization

ATPsyn-α, PSaC, and chloroplast marker protein EMB1301 were previously cloned into the binary vector pBin61-3HA-mCherry (Bwalya et al., 2022). pBin61-eGFP was constructed by replacing 3HA-mCherry with eGFP, and NIa-Pro, NIb, or chloroplast marker protein EMB130. Agrobacterium infiltration was carried out on *N. benthamiana* plants using *Agrobacterium tumefaciens* strain GV3101 at OD_{600}=0.5. Samples were collected at 3 dpi for confocal microscopy and protein extraction. A Leica confocal microscope was used to determine the subcellular localization with a 40x lens and a scanning speed of 400 Hz. The ImageJ software evaluated co-localization between proteins using Pearson's correlation coefficient.

VIII. Yeast two-hybrid and X-α-Gal assays

For Y2H assays, ATPsyn-α, PSaC, and their mutants were cloned into pACT2 (AD), and SMV-G5H proteins were cloned into pAS2-1 (BD). Different constructs with the combination of BD and AD vectors were co-transformed into AH109 and grown on plates of SD medium lacking leucine and tryptophan (SD-Leu/-Trp) for 2 d at 30 °C. Single colonies were selected and grown on SD-Trp/-Leu broth medium until OD_{600} =0.5 and then transferred to either SD-Leu/-Trp, or SD-His/-Leu/-Trp, or SD-His/-Leu/-Trp/-Ade agar medium at serial dilutions of 10^0, 10^{-1}, 10^{-2}, and 10^{-3} for 2 d at 30 °C. All protein-protein interactions were confirmed by α-galactosidase activity using X-α-Gal reagent (Clontech) by streaking newly formed co-transformants on SD-His/-Leu/-Trp/-Ade coated with X-α-gal.

Table 1 Primers used in this study

Primer	Fw	Rv	Purpose	Accession No.
PSaC	gcacgcgtATGTCACATTCAGTAAAGATTTA	gcacgcgtATAAGCTAGACCCATGCTTTGAGT	Clonine into pSNU-SMV-G7H	Glyma. 12g36106
ATPsyn-α	gcacgcgtATGGTAACCATTCGTGCAGA	gcacgcgtATTTTTTTCTACCTGTTCCTGT		Glyma. 18g32250
ATPsyn-$\alpha^{\Delta C\ \&NBD}$	GCACGCGTACGGGTACCGTACTTCAAGTA	gcACGCGTTCCTGTTGCTTTTACTGAACTTCC		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N\&\Delta C}$	GCACGCGTGGACTTATTGCTATTGATTCGATG	GCACGCGTGGAAACGGAAATACCTACATTA		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta C}$	gcacgcgtATGTCACATTCAGTAAAGATTTA	GCACGCGTGGAAACGGAAATACCTACATTA		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N}$	GCACGCGTGGACTTATTGCTATTGATTCGATG	GCACGCGTTTCTTGAATAGCTTCCTTCAAAA		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N\&NBD}$	GCACGCGTGCGGCTCAAATTAAAGCCAT	GCACGCGTTTCTTGAATAGCTTCCTTCAAAA		Glyma. 12g36106
PSaC$^{\Delta C\ \&\Delta Fer4}$	CGACGCGTATGTCACATTCAGTAAAGATTT	GCACGCGTATCATAAATCTTTACTGAATGTG		Glyma. 18g32250
PSaC$^{\Delta C\&\Delta N}$	CGACGCGTACATGTATAGGATGTACTCAAT	CGATCTGTCGGACAGGCGGA		Glyma. 18g32250
PSaC$^{\Delta N\ \&\Delta Fer}$	CGACGCGTACATGTATAGGATGTACTCAAT	CGACGCGTAGCTAGACCCATGCTTTGAG		Glyma. 18g32250
PSaC$^{\Delta N}$	CGACGCGTACATGTATAGGATGTACTCAAT	CGACGCGTAGCTAGACCCATGCTTTGAG		Glyma. 18g32250
pPSaC$^{\Delta C}$	CGACGCGTATGTCACATTCAGTAAAGATTT	CGATCTGTCGGACAGGCGGA		Glyma. 18g32250
ATPsyn-α	gctctagaATGGTAACCATTCGTGCAGA	gcggatccATTTTTTTCTACCTGTTCCTGT	Cloning into pBin-3HA-mCherry	Glyma. 12g36106
PSaC	gctctagaATGTCACATTCAGTAAAGATTTA	gcggatccATAAGCTAGACCCATGCTTTGAGT		Glyma. 18g32250
ATPsyn-α	GCCATATGATGGTAACCATTCGTGCAGA	GCGTCGACATTTTTTTCTACCTGTTCCTGT	Cloning into pAS2-1	Glyma. 12g36106
PSaC	gcCATATGATGTCACATTCAGTAAAGATTTA	gCGTCGACATAAGCTAGACCCATGCTTTGAG		Glyma. 18g32250
ATPsyn-α	GCGAGCTCATTTTTTTCTACCTGTTCCT	GCGGATCCTCATGGTAACCATTCGTGCA	Cloning into pACT2	Glyma. 12g36106
PSaC	gcGAGCTCATAAGCTAGACCCATG	GCGGATCCTCATGTCACATTCAGTAAAG		Glyma. 18g32250
ATPsyn-$\alpha^{\Delta C\ \&NBD}$	GCGAGCTCCTGTTGCTTTTACTGA	CGGATCCTACGGGTACCGTAC		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N\&NBD}$	GAGCTCTTCTTGAATAGCTTCC	GGATCCTCCTCAAATTAAAGCC		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N\&\Delta C}$	gcGAGCTCTGGAAACGGAAATACC	CGGGATCCGGACTTATTGCTATT		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta C}$	GCGAGCTCCTGTTGCTTTTACTGA	CGGGATCCGGACTTATTGCTATT		Glyma. 12g36106
ATPsyn-$\alpha^{\Delta N}$	gcGAGCTCTGGAAACGGAAATACC	GGATCCTCCTCAAATTAAAGCC		Glyma. 12g36106
PSaC$^{\Delta C\&\Delta N}$	GCGAGCTCATATCTGTCGGACAGG	GCGGATCCTCTGTATAGGATGTACTC		Glyma. 18g32250
PSaC$^{\Delta C\ \&\Delta Fer4}$	GCGAGCTCATATGTATCATAAAT	GCGGATCCTCATGTCACATTCAGTAAAG		Glyma. 18g32250
PSaC$^{\Delta N\ \&\Delta Fer}$	GCGGATCCGCTAAACAAATAGCT	GCGAGCTCAGCTAGACCCA		Glyma. 18g32250
PSaC$^{\Delta N}$	GCGAGCTCATATCTGTCGGACAGG	GCGAGCTCAGCTAGACCCA		Glyma. 18g32250
pPSaC$^{\Delta C}$	GCGAGCTCATATGTATCATAAAT	GCGGATCCTCTGTATAGGATGTACTC		Glyma. 18g32250
GFP	GACGACGGCAACTACAAGAC	TCCTTGAAGTCGATGCCCTT	RTqPCR	
Actin11	ATCTTGACTGAGCGTGGTTATTCC	GCTGGTCCTGGCTGTCTCC		
Source for soybean genes: https://phytozome.jgi.doe.gov/				

RESULTS

I. Soybean proteins ATPsyn-α and PSaC interact with NIa and NIb, respectively

The yeast two-hybrid assay results suggest that GmPSaC interacts with SMV NIb and GmATPsyn-α interacts with SMV NIa-Pro. We observed strong interaction between GmPSaC and Nib by the growth of yeast colonies on media deficient in Leu -Trip -His -Ade and the blue color on X-α-Gal -containing media (Figure 1A). Similarly, the strong interaction between GmATPsyn-α and NIa-Pro was confirmed by the growth of yeast colonies on media deficient in -Leu-Trip-His-Ade and then blue color was observed on X-α-Gal -containing media (Figure 1B). Co-IP assay with GFP-trap beads confirmed these interactions by showing the immunoprecipitation of GmPSaC and GmATPsyn-α with NIb and NIa-Pro, respectively (Figure 1C and D). These findings suggest that GmPSaC and GmATPsyn-α may modulate resistance to SMV infection by affecting the function of NIb and NIa-Pro in SMV replication and movement.

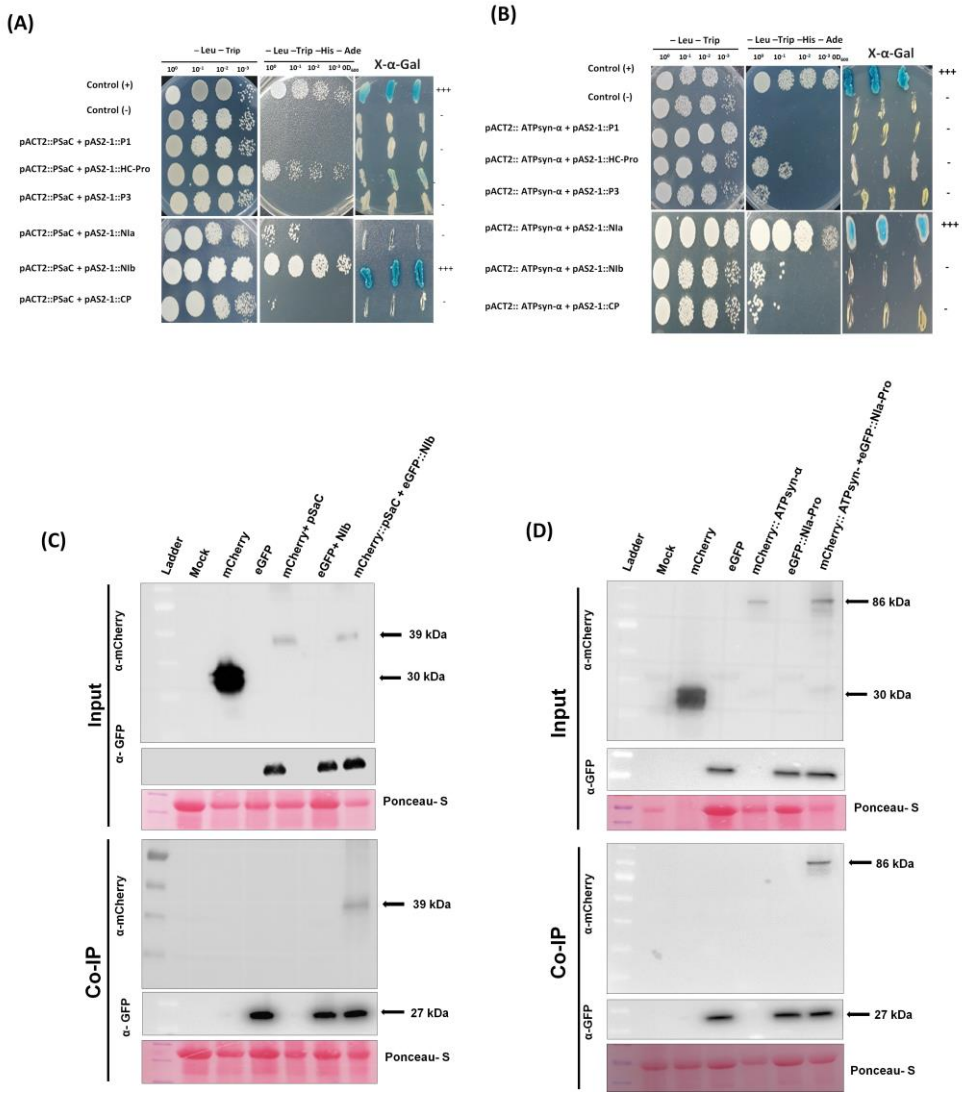

Figure 1. SMV Viral proteins and Soybeans Chloroplast- related proteins interactions.

(a) Analysis of interactions between SMV proteins and GmPSaC in yeast two-hybrid system. (b) Analysis of interactions between SMV proteins and GmATPsyn-α in yeast two-hybrid system. Dilutions of yeast cultures at 10^0, 10^{-1} ,10^{-2} and 10^{-3} OD_{600} were spotted into -Leu –Trip –His –Ade deficient (left) or X-α-Gal -containing (right) plates and grown for 3 d at 28°C. The symbol of "+" indicate intensity of the interaction while "-" means no interaction. The symbol of "+" indicate intensity of the interaction while "-" means no interaction (c) Co-IP analysis showing a direct interaction between SMV NIa- pro and NbATPsyn-α. (d) Co-IP analysis showing a direct interaction between SMV NIb and NbPSaC.

II. Analysis of *in planta* interactions in *Nicotiana benthamiana*

Previously, we confirmed the cellular expression of GmPSaC and GmATPsyn-α in *N. benthamiana* cells by expressing both genes in the binary vector pBin61-HA-mCherry (Alazem et al., 2020; Bwalya et al., 2022). Both PSaC and ATPsyn-α were present in the nucleus, the cytoplasm, and the chloroplast envelope. We used a chloroplast-localized protein from *Arabidopsis*, EMB1303 tagged with GFP as a marker protein for confirmation of chloroplast localization (Bwalya et al., 2022; Huang et al., 2009). This study further investigates possible co-localization between the PSaC and SMV NIb, and between ATPsyn-α and SMV NIa-Pro *in planta* using a Leica confocal microscope. After co-expression of PSaC::mCherry and ATPsyn-α::mCherry with SMV NIb::GFP and SMV NIa-Pro::GFP in *N. benthamiana* through *Agrobacterium*-mediated infiltration, we observed mCherry signal completely overlapped with GFP signals in the cytoplasm and nucleus (Figure 2A and B). Our results prove that GmPSaC and GmATPsyn-α co-localized with SMV NIb and NIa-Pro in *N. benthamiana* cells. The co-localization PSaC::mCherry and ATPsyn-α::mCherry with SMV NIb::GFP and SMV NIa-Pro::GFP resulted in reduced expression of SMV NIb and NIa-Pro. Further studies are needed to elucidate the precise mechanisms underlying these interactions and their impact on viral pathogenesis.

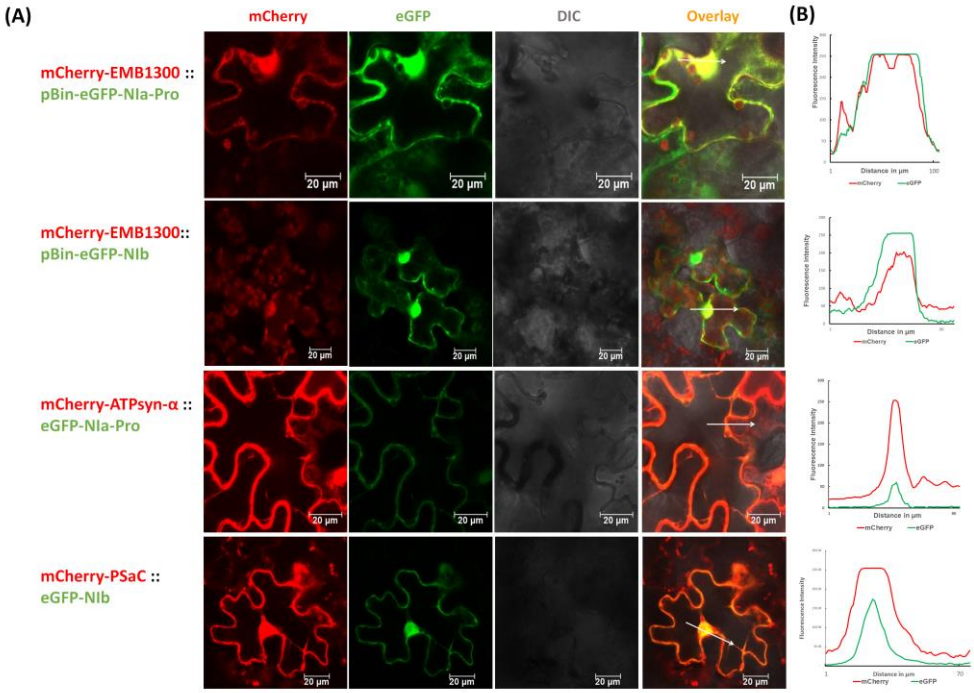

Figure 2. Co-expression of PSaC/ ATPsyn-α with NIb/Nia-pro the *Nicotiana benthamiana* cell.

(a) Co-expression of PSaC/ ATPsyn-α tagged with mCherry with NIb/Nia-pro tagged with green fluorescent protein (GFP) in the Nicotiana benthamiana. The leaves were examined at 3 days post co-infiltration, and fluorescence was assessed by confocal microscopy. (b)Pearson's coefficient of localization represents the degree of fluorescence coincidence. The intensity of eGFP fluorescence (green line) and mCherry fluorescence (red line) are on the right panels. (c)Statistical analysis of eGFP fluorescence intensity of NIa-pro in the control and when Nia-pro intractact with ATPsyn-α

III. The C-terminal region of either ATPsyn-α or PSaC is crucial for protein-protein interaction

To investigate specific regions of GmPSaC and GmATPsyn-α responsible for interaction, we generated five GmPSaC deletion mutants (Figure 3A) and five GmATPsyn-α deletion mutants (Figure 3B). In the Y2H assay, it was determined that two Gm. ATPsyn-α mutants (ATPsyn-$\alpha^{\Delta N\&NBD}$ and ATPsyn-$\alpha^{\Delta N}$) and two GmPSaC mutants (PSaC $^{\Delta N\ \&\Delta Fer}$ and PSaC$^{\Delta N}$) interacted with NIa-Pro and with NIb respectively as they grew very well in media lacking Leu-Trip-His-Ade and turned blue on X-α-Gal -containing media (Figure 3A and B). These interactions, however, were not observed when the mutants without the C-terminal of either ATPsyn-α or PSaC were used (Figure 3A and B), demonstrating that the C-terminal region of either ATPsyn-α or PSaC is responsible for its interaction with NIa-Pro and NIb, respectively.

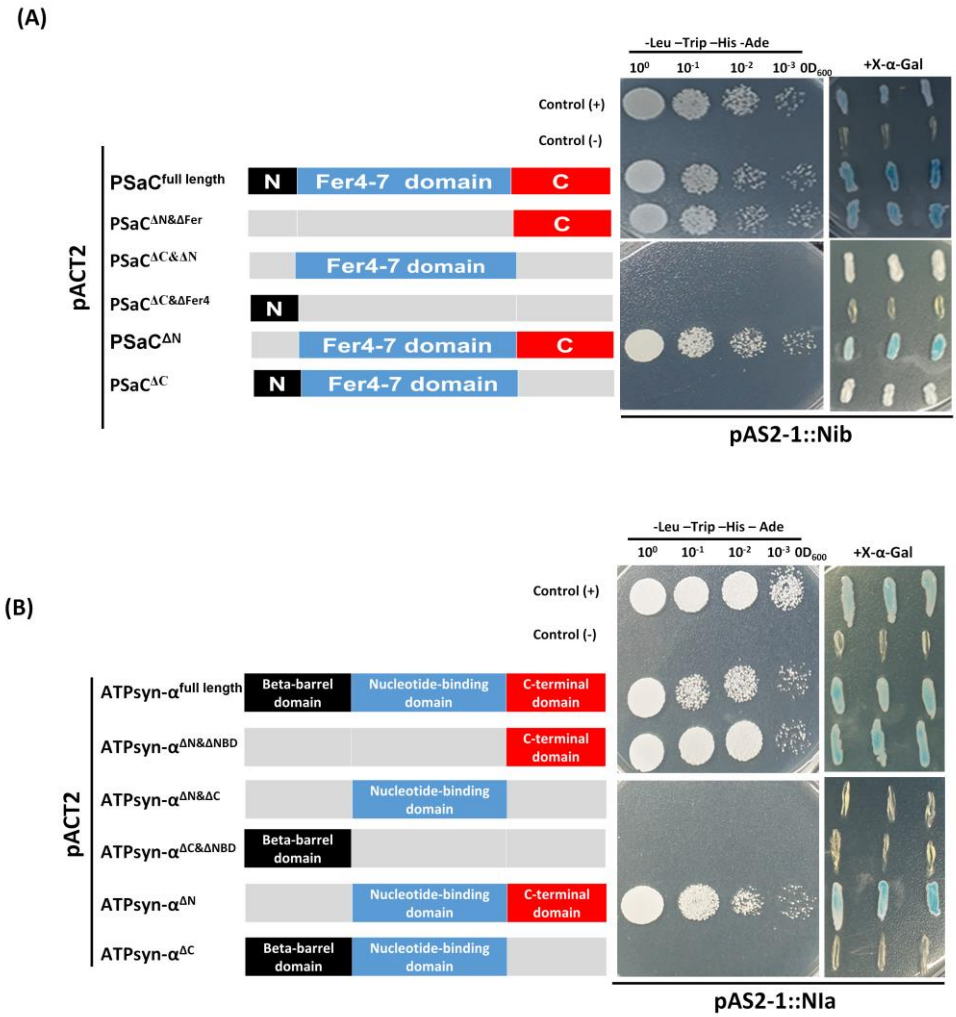

Figure 3. Analysis of SMV NIb/ NIa-pro interaction with GmPSaC/GmATPsyn-α deletions

(a) Schematic representation of GmPSaC and its deletion mutants used in Y2H assays are depicted in the left diagram and their interaction with NIb on right. (b) Schematic representation of GmATPsyn-α and its deletion mutants used in Y2H assays are depicted in the left diagram and their interaction with NIa-pro on right. Dilutions of yeast cultures at 10^0, 10^{-1} ,10^{-2} and 10^{-3} OD_{600} were spotted into -Leu –Trip –His –Ade deficient (left) or X-α-Gal -containing (right) plates and grown for 3 d at 28°C. The symbol of "+" indicate intensity of the interaction while "-" means no interaction.

IV. The C- terminal of either ATPsyn- α or PSaC is required for resistance

To further investigate the region of GmPSaC and GmATPsyn-α responsible for resistance to SMV, we cloned deletion mutants into pSMV-G7H::GFP and overexpressed them on the susceptible cultivar Lee 74 (Figure 4A and B; Figure 5A and B). We discovered that the mutants with N-terminal deletions and middle part deletions of both PSaC and ATPsyn-α did not interfere with the resistance generated by PSaC and ATPsyn-α (Figure 4C and D; Figure 5C and D). Moreover, GFP expression showed that virus replication caused by pSMV-G7H::GFP::PSaC and pSMV-G7H::GFP::ATPsyn-α were similar with or without these deletion mutations in the systemic leaves (Figure 4C and D; Figure 5C and D). These findings were supported by a western protein blot, which showed that both pSMV-G7H::GFP::PSaC and pSMV-G7H::GFP::ATPsyn-α had similar GFP protein accumulation with and/or without N-terminal and middle portion deletions (Figure 4E; Figure 5E). However, when we deleted the C-terminal region of either Gm PSaC or Gm ATPsyn-α, overexpression of these mutants resulted in increased GFP expressions in the systemic leaves, indicating the disruption of resistance to SMV-G7H. Therefore, our results indicate that the C- terminus of either ATPsyn or PSaC is required for resistance (Figure 4C–E; Figure 5C–E).

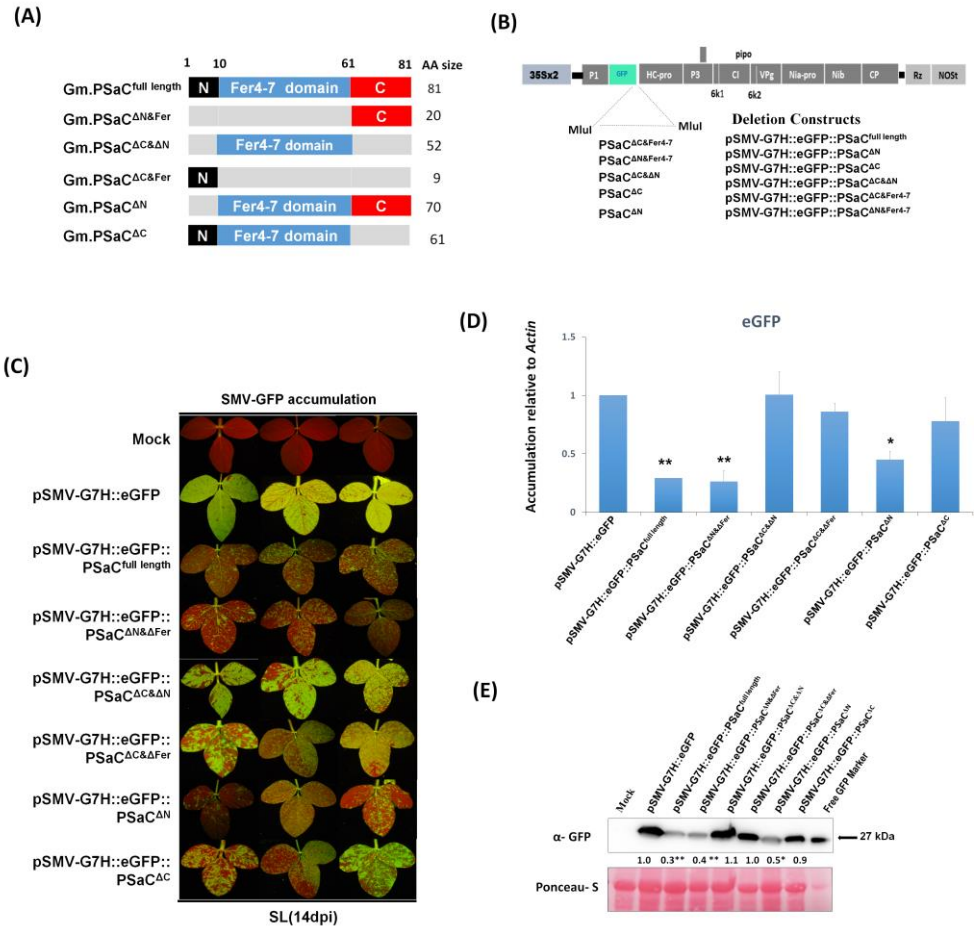

Figure 4. Experimental design, Chimera constructions, and Effect of GmPSaC full length and deletion mutants.

(a) Scheme showing the protocol followed to generate deletion mutants. (b) A schematic diagram showing the genome organization of pSMV-G7H::eGFP on top and deletion constructs below (c) Green fluorescent protein (GFP) visual levels in the systemically infected leaves (SL) of Lee74 plants. The first unifoliate leaves of 12-day-old seedlings were infected with pSMV-G7H::eGFP, pSMV-G7H::eGFP::PSaC, pSMV-G7H::eGFP::PSaC$^{\Delta N\&\Delta Fer}$, pSMV-G7H::eGFP::PSaC$^{\Delta C\&\Delta N}$, pSMV-G7H::eGFP::PSaC$^{\Delta C\&\Delta Fer4}$, pSMV-G7H::eGFP::PSaC$^{\Delta N}$ and pSMV-G7H::eGFP::PSaC$^{\Delta C}$. Fourteen days later, the SL from three plants (1, 2, and 3) were photographed under UV light. (d) Western protein blot for GFP levels (upper panel) in the systemic leaves the SL, and their quantified levels (lower panel). Ponceau S staining of RuBisCO was used on the loading control. (e) Relative expression levels of GFP RNA in SL of Lee74 infected with constructs. Actin11 was used as the internal control. Values are means + SD of three biological replicates. Values were compared to that of the corresponding mock-treated plants (the bar on the left) with one-sided Student's t tests; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively

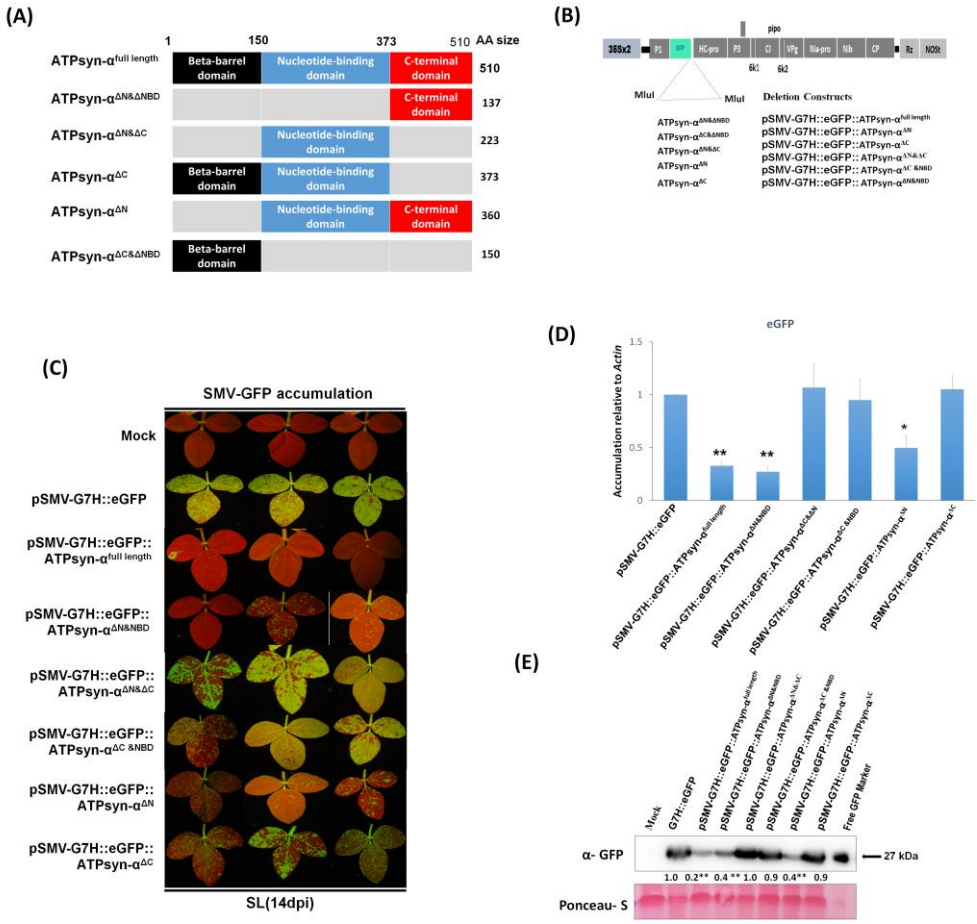

Figure 5. Experimental design, Chimera constructions, and effect of GmATPsyn-α full length and deletion mutants.

(a) Scheme showing the protocol followed to generate deletion mutants. (b)A schematic diagram showing the genome organization of pSMV-G7H::eGFP on top and deletion constructs below(c) Green fluorescent protein (GFP) visual levels in the systemically infected leaves (SL) of Lee74 plants. The first unifoliate leaves of 12-day-old seedlings were infected with pSMV-G7H::eGFP, pSMV-G7H::eGFP::ATPsyn-α, pSMV-G7H::eGFP::ATPsyn-$\alpha^{\Delta N\&NBD}$, pSMV-G7H::eGFP::ATPsyn-$\alpha^{\Delta N\&\Delta C}$, pSMV-G7H::eGFP::ATPsyn-$\alpha^{\Delta C}$, pSMV-G7H::eGFP::ATPsyn-$\alpha^{\Delta N}$ and pSMV-G7H::eGFP::ATPsyn-$\alpha^{\Delta C\&NBD}$. Fourteen days later, the SL from three plants (1, 2, and 3) were photographed under UV light. (d)Western protein blot for GFP levels (upper panel) in the systemic leaves the SL, and their quantified levels (lower panel). Ponceau S staining of RuBisCO was used on the loading control. (e) Relative expression levels of GFP RNA in SL of Lee74 infected with constructs. Actin11 was used as the internal control. Values are means + SD of three biological replicates. Values were compared to that of the corresponding mock-treated plants (the bar on the left) with one-sided Student's t tests; * and ** indicate a significant difference at $p < 0.05$ and $p < 0.01$, respectively

V. Predicted crucial amino residues for resistance to SMV infection

Because the C-terminus of both PSaC and ATPsyn-α is required for resistance to SMV-G7H::GFP, we predicted the essential amino acids in both PSaC and ATPsyn-α using I-TASSER (Chengxin et al., 2017; Yang and Zhang , 2015; Zhang., 2017; Zheng et al., 2021). The analysis predicted arginine at position 45 and aspartic acid at position 76 in C-terminus GmATPsyn-α as crucial residues (Figure 6B). We also identified glycine at position 78 and serine at position 76 in the C-terminal of GmPSaC as the most critical residues in influencing resistance in susceptible cultivars (Figure 6D).

Next, we aligned amino acid sequences of the C-terminal of either GmPSaC or GmATPsyn-α with homologs from five soybean cultivars: William 82 (W82), Lee74, Somyoungkong (SMK), V94, and L29, as well as orthologs from *Arabidopsis thaliana* and *N. benthamiana*. Amino acid sequence analysis indicated that the predicted amino residues in the C-terminal of either ATPsyn or PSaC gene are conserved (Figure 7A, B). It is then reasonable to assume that the predicted conserved amino residues are perhaps necessary for resistance during SMV infection.

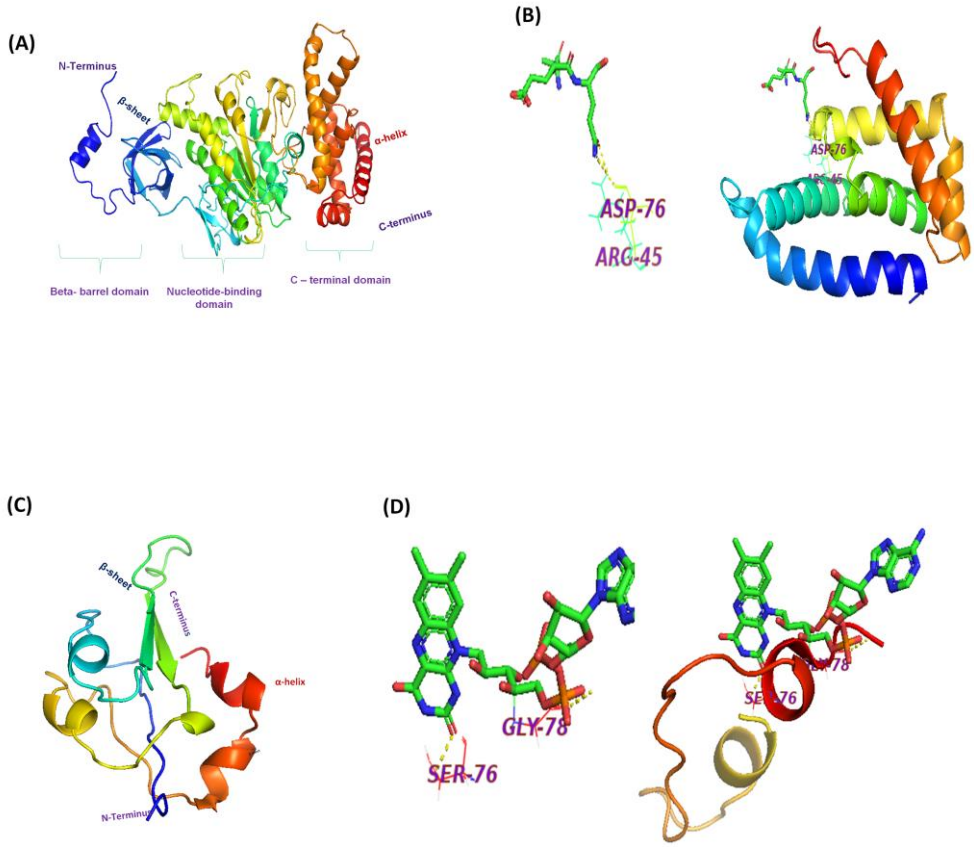

Figure 6. Analysis of predicted crucial amino residues

Analysis of predicted crucial amino residues(a) the amino residues of GmATPsyn-α highlighted with side chains indicate the crucial residue that interact with DNA. (b) the amino residues of GmPSaC highlighted with side chains indicate the crucial residue that interact with DNA

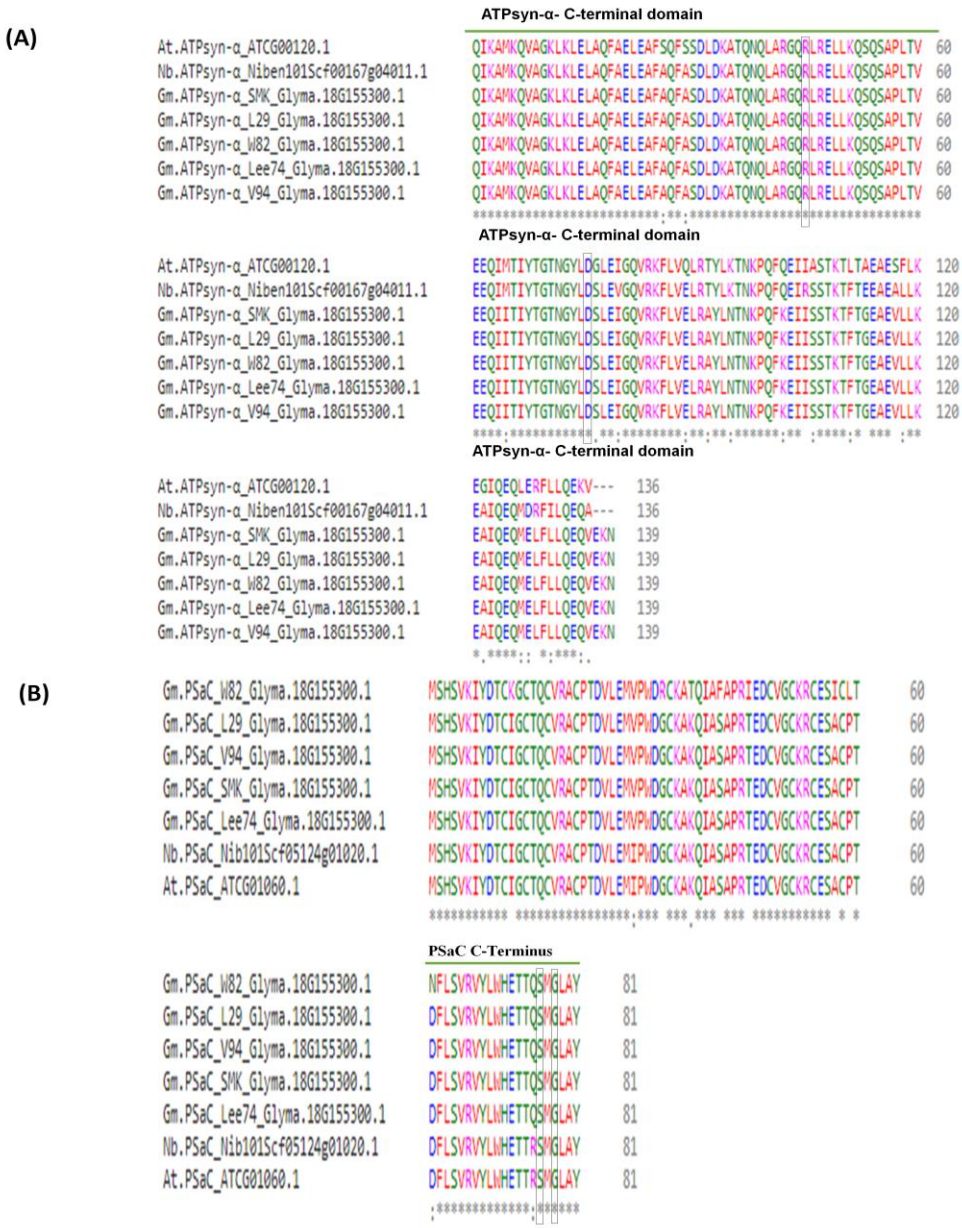

Figure 7. Multiple sequences alignment of PSaC and ATPsyn-α C-terminal with other plant homologous proteins.

(a)Three dimension(3D) structure of PSaC and (b) Amino acid sequences alignment of PSaC sequences with homologs from five soybean cultivars: William 82 (W82), Lee74, Somyoungkong (SMK), V94, and L29, as well as orthologs from *Arabidopsis thaliana* and *Nicotiana benthamiana* (c) Three dimension(3D) structure of GmATPsyn-α and (d) GmATPsyn-α (d) with homologs from five soybean cultivars: William 82 (W82), Lee74, Somyoungkong (SMK), V94, and L29, as well as orthologs from *Arabidopsis thaliana* and *Nicotiana benthamiana*.

DISCUSSION

This study was triggered by the analysis of RNA-Seq data. The transcript levels of GmPSaC and GmATPsyn-α temporarily increased at 8 hours after infection (Bwalya et al., 2022), which may have resulted from shifting dynamics between the virus and host. In addition, the expression of both proteins on susceptible cultivars delayed SMV accumulation in systemic leaves. Here, we have demonstrated that soybean proteins PSaC and ATPsyn-α directly interact with SMV NIb and NIa-Pro, respectively (Figure 1A, B). Through Co-IP assays, we found that both GmPSaC and GmATPsyn-α, attached to mCherry, were present in the pulldown fraction and were pulled down with NIb::GFP and NIa-Pro::GFP, respectively (Figure 1C and D). Furthermore, co-expression of PSaC::mCherry and ATPsyn-α::mCherry with SMV NIb::GFP and SMV NIa-Pro::GFP showed overlapping mCherry and eGFP fluorescence, confirming co-localization in the cytoplasm and nucleus. Interestingly, when two viral proteins, SMV NIb::GFP and SMV NIa-Pro::GFP, were co-expressed with GmATPsyn-α and GmPSaC in *N. benthamiana*, their expression was lower than when they were co-expressed with the chloroplast-localized protein (EMB1303) from *Arabidopsis* (Figure 2). We speculate that the normal movement of NIb and NIa-Pro to the chloroplast membrane was affected by the interaction with PSaC and ATPsyn-α, leading to low expression of these viral proteins. It's

possible that the entry of SMV NIb and NIa-Pro into the replication complex in the chloroplast membrane was delayed because the expression of SMV NIb and NIa-Pro was so low when we co-expressed them with GmATPsyn-α and GmPSaC.

Since GmATPsyn-α and GmPSaC are chloroplast proteins, we expected them to be inside the chloroplast, surprisingly in our study we found that GmATPsyn-α and GmPSaC interact with SMV NIa and NIb in the cytoplasm (Figure 2A and B). Most chloroplast proteins are believed to be synthesized in the cytoplasm, imported, and then targeted to a specific chloroplast compartment (Uniacke et al.,2009). It's conceivable that both SMV NIa and NIb may form a protein complex with GmATPsyn-α and GmPSaC, respectively, and consequently hijack these proteins prior to their entry into the chloroplast to delay virus infection of plants.

The SMV NIb protein was previously found to interact with soybean's poly(A)-binding protein (PABP) (Seo et al., 2007). NIb and PABP interaction has also been reported for another potyvirus, the zucchini yellow mosaic virus (Wang et al., 2000), and its interaction facilitates viral replication. However, unlike the NIb-PABP interaction that promotes viral replication, in our current study, the interaction between SMV NIb and GmPSaC induced a defense response to SMV in the susceptible soybean cultivar Lee 74.

Potyviral NIa-Pro is a multifunctional proteinase that participates in several stages of viral infection. NIa-Pro of papaya ringspot virus has been

reported to interact specifically with the eukaryotic translation initiation factor 3G protein (eIF3G), fructose 1, 6 bisphosphate aldolase class 1 protein (FBPA1), fk 506-binding protein (FK506BP), GTP-binding family protein (GTPBP), methionine sulfoxide reductase B1 protein (MSRB1), and metallothionein-like protein (MTL). Moreover, these proteins which interacted with NIa-Pro play crucial roles in plant protein translation, biotic and abiotic stress responses, energy metabolism, and signal transduction (Broder et al.,1998; Gao et al., 2012). It is also reported that NIa-Pro of potato virus Y functions as an elicitor by cleaving host-encoded proteins to elicit the Ry-mediated disease resistance in potatoes via its structural binding to the proteins (Mestre et al., 2000; 2003). In our study, the host proteins GmATPsyn-α interacted with NIa-Pro and induced resistance to SMV infection.

Other previous reports showed that HC-Pro of potato virus Y interacted with the ATPsyn-β subunit in *Nicotiana tabacum* but did not affect the enzymatic activity of ATP synthase, leading to a reduced ATP synthase content in HC-Pro-transgenic plants (Tu et al., 2015). PSaK, a member of the PSI complex, showed a positive role in resistance against plum pox virus (PPV). The cylindrical inclusion protein of PPV interacted with PSaK and interfered with its function (Jimenez et al., 2006). The influence on resistance can vary depending on the viral group, therefore we cannot generalize about how ATPsyn subunits and PSaC affect host plant resistance to viruses.

Host proteins play important roles in the viral infection cycle and can interact with potyviral proteins to allow or overcome viral infection. Understanding the mechanism of how host factors are involved in virus infection may help in developing a managing strategy for SMV infections. Moreover, we have previously reported that the soybean purple acid phosphatase (GmPAP2.1) from L29 binds with SMV P1 protein and induces robust induction of genes that regulate the SA synthesis pathway (Widyasari et al., 2022). Robust induction of SA-related genes triggers high production and accumulation of active SA that activates SAR in the presence of SMV infection.

In this study, we have highlighted important functions of host proteins (PSaC and ATPsyn-α) that interact with viral proteins (NIb and NIa-Pro) elucidating molecular mechanisms of viral infection and host defense. These two host proteins have functions related to the chloroplast, and we know chloroplast organelles are responsible for photosynthesis and have central roles in response to various biotic and abiotic stresses. However, the function of these two genes (*PSaC* and *ATPsyn-α*) related to photosynthesis in light harvesting and energy production remains unclear and requires further research.

Overall, this study provides a better understanding of the defensive role of GmPSaC and GmATPsyn-α in SMV infection by affecting the functions of NIb and NIa-Pro in viral replication and movement. GmPSaC and GmATPsyn-α are antiviral host factors that are produced vigorously

upon SMV infection and interfere with the functions of SMV NIb and NIa-Pro in the viral infection cycle, ultimately delaying the development of infection in susceptible cultivars. Furthermore, the findings suggest that GmPSaC and GmATPsyn-α can be utilized as resistance genes to delay SMV replication and can be applied in SMV control strategies and genetically engineered plants with better photosynthetic efficiency and resistance to SMV.

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콩 모자이크 바이러스 감염 및 복제에 대한 저항성 관련 엽록체 단백질의 특성 규명

존브왈야

국문초록

엽록체 유전자가 식물-바이러스 상호작용에 관여한다는 증거는 점점 증가하는 반면 광합성 유전자와 바이러스 저항성의 관련은 거의 연구되지 않았다. Rsv3 저항성 유전자를 보유한 콩 재배종 L29 의 RNA-Seq 데이터를 분석한 결과, 콩 모자이크 바이러스(SMV) 중 비병원성 변이 G5H 의 감염시 여러 엽록체 관련 유전자가 강하게 유도되었으나, 병원성 변이 G7H 에는 약하게 유도된 것으로 나타났다. 이중 광계 I 구성 유전자 PSaC 와 ATP-합성효소 복합체의 일부인 ATP-합성효소 α-subunit (ATPsyn-α)로 추가 분석을 진행하였다. rsv3-null 감수성 품종인 Lee74 에서 위의 유전자를 함께 발현하는 G7H 는 야생형에 비해 훨씬 낮은 감염성을 띠었다. 같은 결과가 두 유전자를 발현한 담배 식물에서 G7H 감염시켰을 때도 확인되었다. 두 단백질 모두 엽록체 외피와 핵, 세포질에서 발견되었다. 엽록체는 방어 관련 호르몬의 초기 생합성 부위이기 때문에 ATP syn-α- 및 PSaC 매개 방어에 호르몬 관련 유전자가 관여하는지 여부를 판단했다. 흥미롭게도, ATP

합성-α 를 발현하는 G7H 변이에 감염된 식물에서는 여러 호르몬의 생합성에 관여하는 유전자 발현이 증가하였으나, PSaC 를 발현하는 SMV-G7H 에 감염된 후 자스몬산 및 살리실산 생합성 유전자 발현만 향상되었다. 두 키메라 모두 여러 항바이러스 RNA 침묵기작 유전자의 발현을 유도하였으며, 이는 이러한 저항성이 부분적으로 RNA 침묵 경로를 통해 이뤄질 수 있음을 의미한다. PSaC 와 ATP 합성효소-α 는 각각 NIb 와 NIa-Pro 와 상호작용한다는 것이 공동 면역 침전(Co-IP)에 의해 확인되었다. G7H 유전체로 PSaC 또는 ATP 합성효소-α 의 C 말단 부위를 과발현시키면 감수성 품종 Lee74 에서 바이러스 축적 및 전신 감염이 줄어든다. 본 연구 결과는 PSaC 와 ATP syn-α 유전자가 SMV 복제 및/또는 감염된 식물에서 이동하는 동안 NIb 와 NIa-Pro 의 기능에 영향을 줌으로써 SMV 감염에 대한 저항성을 조절한다는 것을 시사한다.

주요어: 콩모자이크바이러스, 콩, 기주인자, 저항성

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