Review

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Research review

PBS3: a versatile player in and beyond salicylic acid biosynthesis in *Arabidopsis*

Authors for correspondence:
Ming Chang
Email: changming@njau.edu.cn

Zheng Qing Fu
Email: zfu@mailbox.sc.edu

Fengquan Liu
Email: fqliu20011@sina.com

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Wei Li[1]*, Jinyu He[1]*, Xiuzhuo Wang[1], Matthew Ashline[2], Zirui Wu[1], Fengquan Liu[3,4], Zheng Qing Fu[2] and Ming Chang[1]

[1]Key Laboratory of Soybean Disease and Pest Control (Ministry of Agriculture and Rural Affairs), Key Laboratory of Plant Immunity, College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China; [2]Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA; [3]Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Institute of Plant Protection, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu 210014, China; [4]Key Laboratory of Green Prevention and Control of Tropical Plant Diseases and Pests (Ministry of Education), School of Plant Protection, Hainan University, Haikou, Hainan 570228, China

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Summary

AVRPPHB SUSCEPTIBLE 3 (PBS3) belongs to the GH3 family of acyl acid amido synthetases, which conjugates amino acids to diverse acyl acid substrates. Recent studies demonstrate that PBS3 in *Arabidopsis* plays a key role in the biosynthesis of plant defense hormone salicylic acid (SA) by catalyzing the conjugation of glutamate to isochorismate to form isochorismate-9-glutamate, which is then used to produce SA through spontaneous decay or ENHANCED PSEUDOMONAS SUSCEPTIBILITY (EPS1) catalysis. Consistent with its function as an essential enzyme for SA biosynthesis, PBS3 is well known to be a positive regulator of plant immunity in *Arabidopsis*. Additionally, PBS3 is also involved in the trade-off between abiotic and biotic stress responses in *Arabidopsis* by suppressing the inhibitory effect of abscisic acid on SA-mediated plant immunity. Besides stress responses, PBS3 also plays a role in plant development. Under long-day conditions, PBS3 influences *Arabidopsis* flowering time by regulating the expression of flowering regulators *FLOWERING LOCUS C* and *FLOWERING LOCUS T*. Taken together, PBS3 functions in the signaling network of plant development and responses to biotic and/or abiotic stresses, but the molecular mechanisms underlying its diverse roles remain obscure.

Introduction

Plants have developed a sophisticated immune system to resist the invasion of pathogens. This system not only shares similarities with the innate immune system of animals, but also has some unique defense mechanisms. Plants can produce a powerful defense hormone, salicylic acid (SA), which is required for broad-spectrum disease resistance and induces systemic acquired resistance (SAR) against biotrophic and semibiotrophic pathogens (Klessig *et al.*, 2018; Peng *et al.*, 2021). The SA biosynthetic pathway is well elucidated in the model plant *Arabidopsis thaliana*. A previous study identified *Arabidopsis* ISOCHORISMATE SYNTHASE 1 (ICS1) as an enzyme in pathogen-induced SA biosynthesis (Wildermuth *et al.*, 2001). In the chloroplast, ICS1 catalyzes the formation of isochorismic acid (IC) from chorismic acid. The *Arabidopsis* ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) protein acts as a transporter to transport IC from the chloroplast into the cytoplasm (Rekhter *et al.*, 2019). However, how IC is further catalyzed into functional SA in the cytoplasm remained elusive for a long time. In 2019, two research groups reported that *Arabidopsis* acyl acid amido synthetase AVRPPHB SUSCEPTIBLE 3 (PBS3), also known as GRETCHEN HAGEN 3.12 (GH3.12), conjugates glutamate to IC to produce isochorismate-9-glutamate (IC-9-Glu), which is then used to produce SA (Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019). This groundbreaking discovery fills a long-standing gap in plant SA

*These authors contributed equally to this work.

biosynthesis. However, many studies have shown that the function of PBS3 is not limited to SA biosynthesis.

Plant immune system

The plant immune system consists of two layers of plant defense responses. Pattern recognition receptors (PRRs) on the plant cell surface recognize conserved molecules of plant pathogens called pathogen-associated molecular patterns (PAMPs) to activate PAMP-triggered immunity (PTI), which is the first layer of plant immune responses. To enhance pathogenicity, pathogens secrete effectors into plant cells to suppress PTI. Facing this challenge, plant intracellular resistance (R) proteins, which are also known as nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), can directly or indirectly recognize pathogen-secreted effectors, thereby inducing a stronger immune response than PTI, termed effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones & Dangl, 2006).

Two major classes of plant PRRs that confer PTI are leucine-rich repeat receptor-like kinases (LRR-RLKs) and leucine-rich repeat receptor-like proteins (LRR-RLPs) (W. L. Wan *et al.*, 2019). For example, the LRR-RLK protein FLAGELLIN-SENSITIVE 2 (FLS2) recognizes flg22, a conserved 22-amino-acid peptide from bacterial flagellin (Zipfel *et al.*, 2004); the LRR-RLP protein RECEPTOR LIKE PROTEIN 23 (RLP23) recognizes nlp20, a conserved 20-amino-acid peptide of NECROSIS AND ETHYLENE-INDUCING PEPTIDE 1-LIKE PROTEINS (NLPs) secreted by a wide range of plant-associated microbes (Albert *et al.*, 2015). Unlike LRR-RLKs, LRR-RLPs do not have a cytoplasmic kinase domain. Instead, they form a constitutive heteromeric complex with the LRR-RLK protein SUPPRESSOR OF BIR1 1 (SOBIR1) (Liebrand *et al.*, 2014). Upon ligand binding, LRR-RLKs and LRR-RLP/SOBIR1 complexes recruit the co-receptor protein BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) to transduce downstream immune signaling (W. L. Wan *et al.*, 2019).

Based on the difference in the N-terminus, the majority of NLRs are classified into three types: coiled-coil (CC) NLRs (CNLs), toll/interleukin-1 receptor (TIR) NLRs (TNLs), and RPW8-like coiled-coil (CCR) NLRs (RNLs) (Shao *et al.*, 2019). The CNL protein HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) has been shown to form a narrow pore on the plasma membrane as the Ca^{2+}-permeable channel to trigger cell death and plant immunity (Wang *et al.*, 2019; Bi *et al.*, 2021). Unlike CNLs, some TNLs have been shown to possess the nicotinamide adenine dinucleotide (NAD^+) hydrolase (NADase) activity and the 2′,3′-cAMP/cGMP synthetase activity, which are required for the activation of downstream immune responses (Horsefield *et al.*, 2019; L. Wan *et al.*, 2019; Yu *et al.*, 2022). Notably, the cell death induction activity of TNLs requires the help of other proteins, such as EDS1 and RNLs (RNLs are also known as 'helper' NLRs) (L. Wan *et al.*, 2019). In *Arabidopsis*, ACTIVATED DISEASE RESISTANCE 1 (ADR1) and N REQUIREMENT GENE 1 (NRG1) are two subfamilies of RNLs, and both of them have been proven to form Ca^{2+} channels (Jacob *et al.*, 2021).

Even though PTI and ETI are triggered by different elicitors, they eventually lead to similar downstream defense responses. Both PTI and ETI can induce reactive oxygen species (ROS) burst, mitogen-activated protein kinase pathway, *PATHOGENESIS-RELATED* (*PR*) gene expression, and SA accumulation (Lu & Tsuda, 2021). Increasing evidence suggests that PTI and ETI share common signaling components (Ngou *et al.*, 2021; Pruitt *et al.*, 2021; Tian *et al.*, 2021; Yuan *et al.*, 2021; Chang *et al.*, 2022). The PRR/co-receptor triple mutants *fls2 efr cerk1* (*fec*) and *bak1 bkk1 cerk1* (*bbc*) are markedly impaired in ETI responses, indicating that PTI is necessary for a robust ETI response (Yuan *et al.*, 2021). The ETI responses mediated by NLR proteins can be strongly enhanced by the activation of PRR proteins (Ngou *et al.*, 2021). The NLR proteins potentiate the activation of key PTI signaling components by increasing their mRNA levels or protein accumulation (Ngou *et al.*, 2021). The intracellular signaling protein EDS1 forms distinct protein complexes with PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101) to mediate ETI responses (Lapin *et al.*, 2020; Dongus & Parker, 2021). Recent studies found that the EDS1–PAD4–ADR1 and the EDS1–SAG101–NRG1 nodes are convergent points for PTI and ETI (Pruitt *et al.*, 2021; Tian *et al.*, 2021). Altogether, these data indicate that PTI and ETI responses can be mutually enhanced, and their crosstalk is necessary for conferring stronger plant defense responses.

Diverse roles of SA in plant immunity

Salicylic acid is a phenolic plant defense hormone. Pathogen infection induces the accumulation of SA in plants, and highly accumulated SA modulates the expression of immune-related genes, such as *PR* genes, indicating that SA plays an important role in plant immune signaling (Vlot *et al.*, 2009). Salicylic acid is also closely related to ETI. Usually ETI is accompanied by a rapid localized programmed cell death (PCD) called hypersensitive response (HR). Salicylic acid plays dual roles in regulating PCD of plants (Radojicic *et al.*, 2018). Effector-triggered immunity induced by the *Pseudomonas syringae* type III effector AvrRpm1 or AvrRpt2 results in a dramatic increase in SA level in an ICS1-, EDS5-, and PBS3-dependent manner (Nawrath & Metraux, 1999; Jagadeeswaran *et al.*, 2007; Nobuta *et al.*, 2007; Chen *et al.*, 2022). Salicylic acid strongly accumulates in some lesion mimic mutants, such as *lesion-simulating disease 6* (*lsd6*), *lsd7*, *accelerated cell death 6* (*acd6*), and *acd11* mutants, which exhibit a spontaneous cell death phenotype. The cell death phenotypes can be suppressed by expressing the salicylate hydroxylase gene *NahG* in these mutants to prevent SA accumulation (Weymann *et al.*, 1995; Rate *et al.*, 1999; Brodersen *et al.*, 2005). However, other lesion mimic mutants, such as *lsd2* and *lsd4*, do not require SA accumulation to activate spontaneous cell death (Dietrich *et al.*, 1994; Hunt *et al.*, 1997). In the meanwhile, elevated SA levels have been observed in some autoimmune mutants without the spontaneous cell death phenotype (Yu *et al.*, 1998; Li *et al.*, 2001). These results indicate that PCD in ETI is not a necessary condition for activating SA biosynthesis.

Besides, emerging evidence shows that the activation of SA biosynthesis and signaling can negatively regulate PCD in ETI. Both SA pretreatment and overexpression of SA receptor NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) can inhibit HR activated by the *P. syringae* type III effector AvrRpm1, while *npr1* mutants or *NahG* transgenic plants show a stronger PCD (Rate & Greenberg, 2001; Devadas & Raina, 2002). In the SA biosynthesis defective *eds5* mutants, the *P. syringae* type III effector AvrRpt2 can induce stronger PCD compared with wild-type plants (Radojicic *et al.*, 2018). Preinfection by *P. syringae* carrying *AvrRpt2* can significantly inhibit PCD induced by secondary infection in adjacent leaf tissues, and this inhibition is dependent on SA biosynthesis and signaling (Zavaliev *et al.*, 2020). Mechanistically, NPR1 can accumulate in the cytoplasm by forming protein condensates together with many stress response proteins, and the SA-induced NPR1 condensate (SINC) has been proven to be essential for the inhibition of cell death induced by AvrRpt2 (Zavaliev *et al.*, 2020). Interestingly, the SINC can recruit the Cullin3 E3 ubiquitin ligase in the cytoplasm for protecting cell survival in ETI responses by degrading the SINC-localized substrates, such as EDS1 (Zavaliev *et al.*, 2020).

Salicylic acid is also a core regulator of SAR in plant immunity (Klessig *et al.*, 2018). The biosynthesis and signaling of SA are necessary for plants to establish immune responses in infected and noninfected tissues. The SAR-positive regulator NPR1 and the SAR-negative regulator NPR3/NPR4 have been identified as SA receptors, with NPR1 as a transcriptional coactivator and NPR3/NPR4 as transcriptional co-repressors (Fu *et al.*, 2012; Wu *et al.*, 2012; Ding *et al.*, 2018). Notably, SA itself is not a mobile signaling molecule of SAR (Vernooij *et al.*, 1994). A variety of SAR signaling molecules have been identified, such as SA derivative methyl salicylate (Park *et al.*, 2007), azelaic acid (Jung *et al.*, 2009), glycerol-3-phosphate (Chanda *et al.*, 2011), dehydroabietinal (Chaturvedi *et al.*, 2012), and pipecolic acid (Navarova *et al.*, 2012). Pipecolic acid can be converted to another mobile signaling molecule *N*-hydroxypipecolic acid (NHP) by FLAVIN-DEPENDENT-MONOOXYGENASE 1 (FMO1), which functions as a pipecolate *N*-hydroxylase (Chen *et al.*, 2018; Hartmann *et al.*, 2018). As a mobile signal, NHP plays a crucial role in initiating SAR signaling in *Arabidopsis*, and NHP can be further catalyzed to a nonmobile molecule NHP-*O*-glycoside (NHP-*O*Glc) by the glycosyltransferase UGT76B1 to balance growth and defense responses (Bauer *et al.*, 2021; Holmes *et al.*, 2021; Mohnike *et al.*, 2021).

SA functions in plant responses to abiotic stresses

In addition to regulating biotic stresses, SA also plays a significant role in plant responses to abiotic stresses, such as salinity, drought, heat, osmotic, and metal stresses (Khan *et al.*, 2015). In general, the dramatically reduced or elevated level of SA can lead to enhanced sensitivity of plants to abiotic stresses, indicating that maintaining SA concentration in an appropriate range plays an important role in plants to resist abiotic stresses (Yuan & Lin, 2008). Mechanistically, the regulatory effect of SA on abiotic stresses is related to the accumulation of ROS, which acts as a second messenger to regulate further physiological activities of plants (Qi *et al.*, 2017; Van Butselaar & Van den Ackerveken, 2020). The protective role of SA in abiotic stresses is mainly due to the induction of antioxidant system components, which has been confirmed in *Arabidopsis*, rice, and other plants (Khan *et al.*, 2015; Nadarajah *et al.*, 2021).

Salicylic acid also interacts with other phytohormones to regulate plant responses to abiotic stresses, and the crosstalk between these phytohormones plays an essential role in balancing biotic and abiotic stress responses of plants (Khan *et al.*, 2015). Abiotic stresses can induce the synthesis of plant stress hormone abscisic acid (ABA) and improve plant tolerance to abiotic stresses (Sah *et al.*, 2016). In *Arabidopsis*, studies have found that ABA can inhibit the expression of *ICS1* and promote the degradation of SA receptor NPR1 through the 26S proteasome pathway (Yasuda *et al.*, 2008; Ding *et al.*, 2016), indicating that ABA can suppress the SA-mediated immune response during plant responses to abiotic stresses.

Discovery of *PBS3*

Warren *et al.* (1999) identified three *Arabidopsis* mutants *pbs1*, *pbs2*, and *pbs3*, which show increased susceptibility to the *P. syringae* avirulent effector *AvrPphB* by genetic screening. The *PBS3* gene was also identified by other screening methods and named *GH3-LIKE DEFENSE GENE 1* (*GDG1*) (Jagadeeswaran *et al.*, 2007) and *HOPW1-1-INTERACTING 3* (*WIN3*) (Lee *et al.*, 2007). Loss-of-function of *PBS3* results in increased susceptibility to both virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 strains and *Pst* DC3000 strains carrying the avirulent genes *AvrB*, *AvrRpt2*, *AvrRps4*, or *AvrPphB*, indicating that *PBS3* functions in both basal resistance and R protein (including RPM1, RPS2, RPS4, and RPS5)-mediated resistance (Warren *et al.*, 1999). In the past two decades, many new features of *PBS3* have been found, but the whole picture about the role of *PBS3* in plant cells is still in the mist.

PBS3 is involved in plant immunity

PBS3 is a key SA biosynthesis enzyme

AVRPPHB SUSCEPTIBLE 3, also known as GH3.12, belongs to the GH3 family which can conjugate amino acids to diverse acyl acid substrates through a two-step mechanism. Notably, the subfamily of GH3 proteins that PBS3/GH3.12 is a member of is only found in *Arabidopsis* to date (Jez, 2022). The first step is the adenylation reaction, forming an acyl-adenylate intermediate by transferring AMP from ATP to the carboxylic acid group of an acyl substrate. The second step is the transferase reaction, in which a specific amino acid is conjugated to the acyl substrate. AVRPPHB SUSCEPTIBLE 3 has been proven to be specific for glutamate (Glu) in the transferase reaction (Okrent *et al.*, 2009; Westfall *et al.*, 2012).

The PBS3 protein has a large N-terminal domain and a small C-terminal domain (PDB: 4EPM) (Fig. 1; Westfall *et al.*, 2012). The N-terminal domain (1–419 aa) contains one β barrel, two β sheets, and several α helices. The C-terminal domain (420–575 aa) is composed of four β sheets in the center flanked with two α helices

on each side. One flexible hinge loop (Val^{420}-Glu^{432}) connects the N- and C-terminal domains at the interface between β15 and α15, and this loop structure is essential for the rotation of C-terminal domain during the catalytic process. The active sites of PBS3 are located at the junction of the N- and C-terminal domains (Fig. 1). Ser^{328}, Asp^{398}, and Lys^{550} are the key amino acids for the association of PBS3 with ATP/AMP, and Glu^{329} is crucial for the interaction of PBS3 with Mg^{2+}. Successful bindings of PBS3 with ATP/AMP and Mg^{2+} are the basis of the adenylation reaction. For the transferase reaction, two lysine residues Lys^{428} and Lys^{146} have been proven to be essential for conjugating Glu to the acyl substrate catalyzed by PBS3.

In *pbs3* mutants, SA accumulation induced by pathogen infection is significantly compromised compared with the wild-type *Arabidopsis* Col-0 (Jagadeeswaran *et al.*, 2007; Nobuta *et al.*, 2007). Besides, SA pretreatment can restore the basal resistance deficiency of *pbs3* mutants to *Pst* DC3000 (Jagadeeswaran *et al.*, 2007), suggesting an essential role of SA in PBS3-mediated immunity. Due to the amino acid conjugation activity of GH3 family proteins, PBS3 was originally proposed to be functional in SA biosynthesis or metabolism. However, *in vitro* biochemical and structural studies proved that SA is an extremely poor substrate of PBS3 (Okrent *et al.*, 2009). In contrast, PBS3 shows stronger binding activity to 4-substituted benzoates, such as 4-aminobenzoate and 4-hydroxybenzoate (4-HBA), and the latter is a para isomer of SA (SA also can be seen as 2-HBA) (Okrent *et al.*, 2009). Surprisingly, SA specifically and reversibly inhibits the binding activity of PBS3 to these 4-substituted benzoates (Okrent *et al.*, 2009). These results make the exact enzyme activity of PBS3 seem confusing for a rather long time.

Until 2019, two independent groups reported that PBS3 functions as an acyl acid amido synthetase, which catalyzes the conjugation of Glu to IC to form IC-9-Glu, which is then spontaneously degraded to SA and a by-product (2-hydroxy-acryloyl-*N*-glutamate) (Fig. 2; Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019). Interestingly, another protein called ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) was reported to promote the decomposition of IC-9-Glu to SA by its IC-9-Glu pyruvoyl-glutamate lyase activity (Torrens-Spence *et al.*, 2019). These two studies make the IC pathway of SA biosynthesis in *Arabidopsis* complete. In the chloroplast, chorismate produced from phosphoenolpyruvate and erythrose-4-phosphate is converted to IC by ICS1 (Wildermuth *et al.*, 2001). The chloroplast envelop-localized EDS5 was believed to function as a MATE-transporter protein to transport SA from the chloroplast into the cytoplasm (Serrano *et al.*, 2013). However, there is also evidence, which supports the idea that EDS5 functions to export IC, rather than SA, into the cytoplasm (Rekhter *et al.*, 2019). In the cytoplasm, PBS3 conjugates Glu to IC to generate IC-9-Glu, which can then be converted to SA by the enzyme EPS1 or through spontaneous decay (Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019). Interestingly, another group found that PBS3 also can use chorismate as a substrate with an eightfold higher activity compared with 4-HBA and catalyze the conjugation of Glu to chorismate (Holland *et al.*, 2019), which leads to a new question whether chorismate–Glu can be used as an intermediate in SA biosynthesis or metabolism.

PBS3 is a positive regulator in ETI mediated by both CNLs and TNLs

In the *Arabidopsis* ecotype Col-0 wild-type plants, infection by virulent *Pst* DC3000 or *P. syringae* pv *maculicola* (*Psm*) ES4326 significantly induces the expression of *PBS3*, whereas avirulent *P. syringae* strains carrying the type III effector AvrRpm1, AvrRpt2, or AvrRps4 can activate *PBS3* expression more rapidly (Thilmony *et al.*, 2006; Jagadeeswaran *et al.*, 2007; Lee *et al.*, 2007). These results imply a potential role of *PBS3* in ETI. To analyze the function of PBS3, researchers obtained *pbs3* mutants in different ways. The *pbs3-1* mutant is generated through ethyl methanesulfonate mutagenesis (Warren *et al.*, 1999), while the *pbs3-2* mutant (SALK_018225, also known as *gdg1-1* or *win3-1*) is a T-DNA insertion line (Jagadeeswaran *et al.*, 2007; Lee *et al.*, 2007; Nobuta *et al.*, 2007). Effector-triggered immunity induced by different avirulent effectors were reduced in *pbs3* mutants at different levels

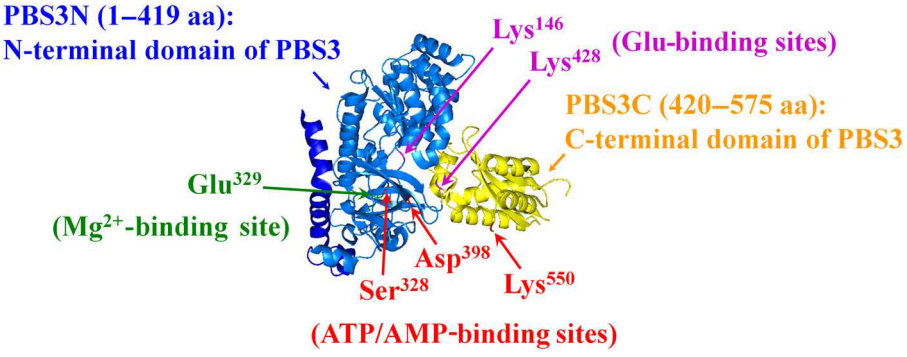

Fig. 1 Domains and key amino acids of AVRPPHB SUSCEPTIBLE 3 (PBS3) (PDB: 4EPM). AVRPPHB SUSCEPTIBLE 3 belongs to the GH3 family of enzymes with adenylation and transferase activities. During salicylic acid biosynthesis, PBS3 catalyzes the conjugation of glutamate (Glu) to isochorismate to generate isochorismate-9-glutamate. The crystal structure of PBS3 shows that it contains a large N-terminal domain (1–419 aa, blue area) and a small C-terminal domain (420–575 aa, yellow area). The active sites are located at the interface of the two domains. Glu^{329} is the key amino acid for Mg^{2+} binding (green arrow). Ser^{328}, Asp^{398}, and Lys^{550} are the key amino acids for ATP/AMP binding (red arrow). Lys^{146} and Lys^{428} are the key amino acids for glutamate binding sites (purple arrow).

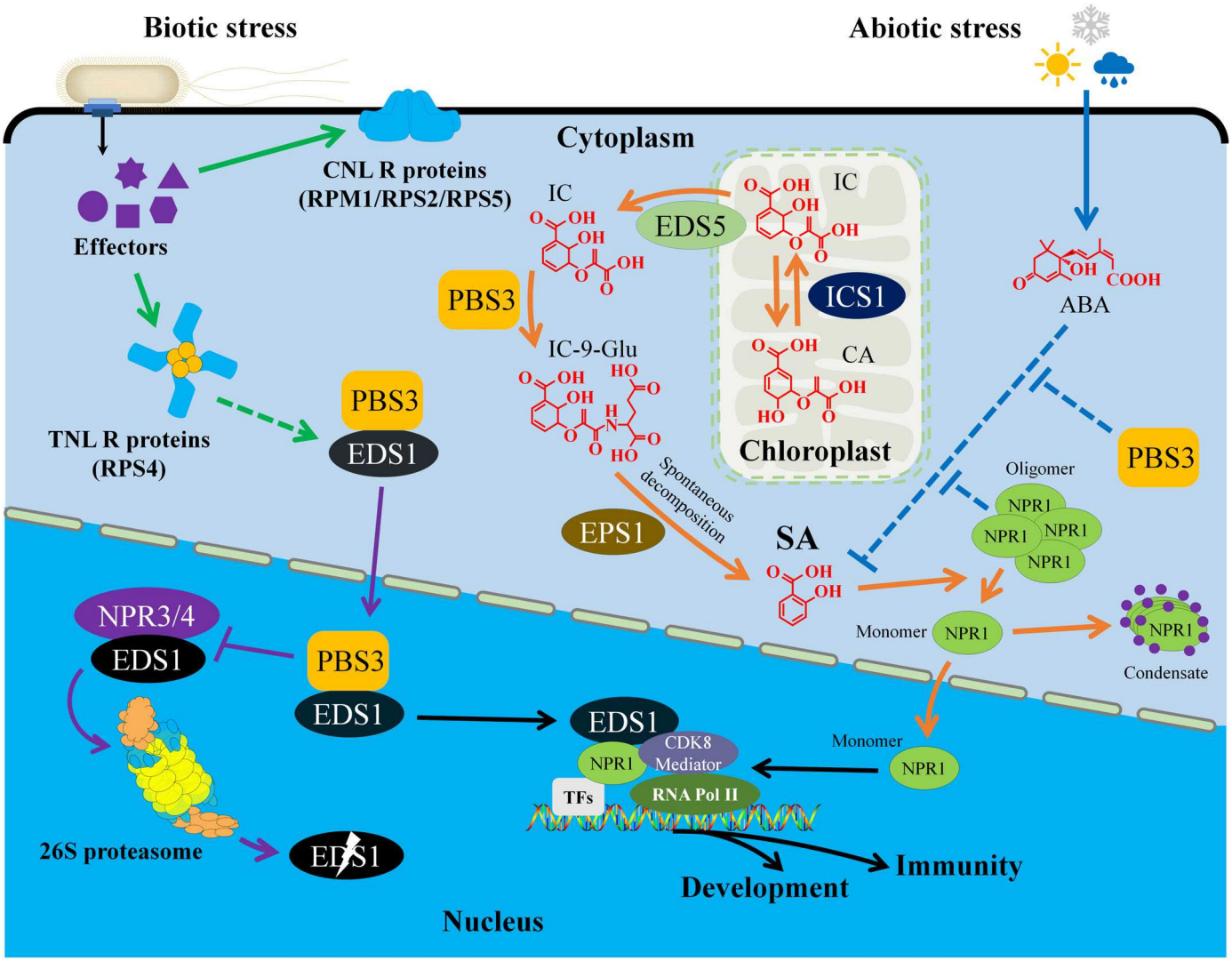

Fig. 2 A proposed model illustrating AVRPPHB SUSCEPTIBLE 3 (PBS3)-mediated signaling network. AVRPPHB SUSCEPTIBLE 3 is a crucial enzyme in the isochorismate (IC) pathway of salicylic acid (SA) biosynthesis in *Arabidopsis*. Biochemically, PBS3 conjugates glutamate (Glu) to IC to form isochorismate-9-glutamate (IC-9-Glu) in the cytoplasm, and IC-9-Glu can be degraded to SA spontaneously or in cooperation with ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1). Salicylic acid is a core phytohormone in plant immune signaling. Upon pathogen infection, SA can induce the formation of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) condensates in the cytoplasm, which are enriched with stress response proteins such as Cullin3 E3 ubiquitin ligases (purple particles surround NPR1 condensates). The SA-induced NPR1 condensate is a hub in suppressing programmed cell death during effector-triggered immunity (ETI). Importantly, PBS3 is also required for ETI mediated by both TIR-type and CC-type resistance (R) proteins. In addition, PBS3 interacts with another immune signaling node protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) in both the cytoplasm and the nucleus, and PBS3 protects EDS1 from the 26S proteasome-mediated degradation. However, whether the PBS3–EDS1 protein complex is also involved in SA biosynthesis remains to be further explored. In the nucleus, EDS1 functions as a transcriptional coactivator by cooperating with NPR1 and Mediator in the transcription machinery. Whether PBS3 is also involved in the transcriptional regulation of downstream genes by associating with EDS1 and/or NPR1 is still an open question. Besides, PBS3 plays a role in balancing the trade-off between abiotic and biotic stress responses by blocking the inhibitory effect of abiotic stress-induced abscisic acid (ABA) on SA-mediated plant immunity. Solid lines indicate direct relationships; dashed lines indicate indirect relationships; blunt-ended arrows indicate inhibition. Orange lines represent the SA biosynthesis and signaling pathway; green lines represent the ETI signaling pathway; blue lines represent the crosstalk between ABA and SA; purple lines represent the protective effect of PBS3 on EDS1 protein stability; black lines represent the transcriptional regulation in the nucleus mediated by transcriptional coactivators EDS1 and NPR1.

compared with the wild-type *Arabidopsis* Col-0 (Warren *et al.*, 1999; Lee *et al.*, 2007). The loss of ETI activated by AvrRpt2 (recognized by the CNL R protein RPS2) and AvrRps4 (recognized by the TNL R protein RPS4) in *pbs3* mutants was relatively more significant (Lee *et al.*, 2007), indicating that PBS3 is involved in ETI signaling mediated by both CNL and TNL R proteins.

EDS1 is a core regulator of both SA accumulation and ETI (Lapin *et al.*, 2020; Dongus & Parker, 2021). One study found that the C-terminus (420–575 aa) of PBS3 can directly interact with EDS1 in both the cytoplasm and the nucleus (Fig. 2; Chang *et al.*, 2019). Importantly, PBS3 promotes the protein stability of EDS1 by inhibiting the 26S proteasome-mediated degradation, and EDS1 and PBS3 contribute additively to both PTI and ETI (Chang *et al.*, 2019).

PBS3 is a target protein of the *Pseudomonas* type III effector HopW1-1

Different bacterial pathogen strains secrete diverse repertoires of type III effectors. In general, effectors can suppress plant immune signaling by interacting with components of the PTI. If this interaction is species- or ecotype-specific, it influences the host range of a pathogen (Greenberg & Vinatzer, 2003). *Psm* ES4326 secretes a rare effector, HopW1-1, while *Pst* DC3000 does not have this effector. In the *Arabidopsis* ecotype Ws-0, HopW1-1 elicits an immune response leading to the accumulation of SA and expression of *HOPW1-1-INDUCED GENE 1* (Guttman *et al.*, 2002; Lee *et al.*, 2008). Three HopW1-1 interacting proteins were identified in *Arabidopsis* plants, among which WIN3 is PBS3 (Lee *et al.*, 2007, 2008). Structurally, PBS3 interacts with the C-terminus (420–774 aa) of HopW1-1, while HopW1-1 is mainly associated with the C-terminus (335–575 aa) of PBS3 (Lee *et al.*, 2008). HopW1-1-induced disease resistance in the Ws-0 ecotype is partially dependent on PBS3 (Lee *et al.*, 2008). Whether and how HopW1-1 induces resistance in the Ws-0 ecotype by regulating the activity of its interacting protein PBS3 still requires more genetic and biochemical studies.

PBS3 has functions beyond SA biosynthesis

PBS3 regulates the balance of abiotic and biotic stress responses in *Arabidopsis* young leaves

It will be intriguing to find out whether PBS3 plays a role in plant responses to abiotic stresses by regulating SA biosynthesis. Berens *et al.* (2019) found that *pbs3* mutants exhibit higher tolerance to salt stress compared with the wild-type *Arabidopsis* Col-0. Interestingly, the regulatory effect of *PBS3* on abiotic stresses is closely related to leaf age. The expression of *PBS3* is decreased in old leaves and increased in young leaves after ABA treatment, and another SA synthase gene, *ICS1*, also has a similar expression pattern (Berens *et al.*, 2019). Importantly, ABA treatment shows similar inhibitory effects on plant immunity in both old and young leaves of *pbs3* mutants, while ABA only shows a strong immunosuppressive effect in old leaves of Col-0 and the SA biosynthesis defective *sid2*/*ics1* mutants, indicating that PBS3 can suppress the inhibitory effect of ABA on plant immunity in young leaves through a mechanism distinct from its role in SA biosynthesis (Fig. 2; Berens *et al.*, 2019).

Interestingly, young leaves of *npr1* mutants also show a similar increased susceptibility to *Pst* DC3000 *hrcC*$^-$ compared with old leaves in the presence of ABA as *pbs3* mutants, indicating that not only PBS3 but also the SA receptor NPR1 is required in young leaves of Col-0 plants to counteract ABA (Fig. 2; Berens *et al.*, 2019). However, data on SA levels in Col-0, *sid2*, *pbs3*, and *npr1* plants challenged with *Pst* DC3000 *hrcC*$^-$ in combination with ABA treatment are still lacking, so it is difficult to make a clear connection between SA levels and ABA-induced susceptibility in young leaves of these plants. Endogenous ABA biosynthesis activated by drought or salt stress also suppresses immune responses in old leaves, while the presence of *PBS3* maintains the resistance of young leaves to biotic stresses (Berens *et al.*, 2019). During the combined stresses, PBS3 plays a role in balancing the trade-off between biotic and abiotic responses, especially in young leaves (Berens *et al.*, 2019), and this function is also a concrete manifestation of the sophisticated environmental adaptability acquired by plants in the long evolutionary process.

PBS3 regulates flowering time under long-day conditions

Another unexpected role of PBS3 is its involvement in regulating flowering time in *Arabidopsis*. The regulation of flowering time by PBS3 is related to photoperiod. According to reported data, PBS3 negatively regulates flowering time mainly under long-day conditions (16 h : 8 h, light : dark) (G. F. Wang *et al.*, 2011; Chang *et al.*, 2019). Notably, the excess SA accumulation mutant *acd6-1* shows little difference in flowering time compared with the wild-type *Arabidopsis* Col-0, while the *acd6-1 pbs3-2* double mutant exhibits a great reduction in SA level and a marked early flowering phenotype compared with *acd6-1* mutants, suggesting that the *PBS3*-mediated early flowering phenotype is largely independent of SA (G. F. Wang *et al.*, 2011). Unlike *pbs3-2* mutants, the SA biosynthesis/accumulation-deficient mutants *sid2-1* and *pad4-1* do not show obvious early flowering phenotypes under long-day conditions (G. F. Wang *et al.*, 2011). These data support the notion that PBS3 regulates flowering time in plants independent of its function in SA biosynthesis.

Under long-day conditions, both *pbs3-2* and *npr1-1* mutants show distinct early flowering phenotypes compared with Col-0 plants (G. F. Wang *et al.*, 2011). There is evidence that the floral repressor gene *FLOWERING LOCUS C* (*FLC*) is suppressed at 16 or 25 d in *pbs3-2* and *npr1-1* mutants compared with Col-0 plants. In contrast, the key flowering gene *FLOWERING LOCUS T* (*FT*) is induced in 25-d-old *pbs3-2* and *npr1-1* mutants compared with Col-0 plants (G. F. Wang *et al.*, 2011). However, future comparison of flowering time among *pbs3-2* and *npr1-1* single mutants and *pbs3-2 npr1-1* double mutants will be required to determine whether PBS3 and NPR1 function together or individually in the regulation of flowering time in *Arabidopsis*.

Concluding remarks and future perspectives

The *PBS3* gene was first reported in 1999 (Warren *et al.*, 1999). Over the past more than 20 yr, PBS3 has been proven to be an important player in many physiological and pathological processes, including SA biosynthesis, balancing of plant responses to biotic and abiotic stresses, and flowering time regulation. Of particular importance, in 2019, two research groups elucidated that PBS3 converts IC to IC-9-Glu through its acyl acid amido synthetase activity, ultimately producing SA with or without the help of EPS1 (Figs 1, 2; Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019). Identifying the enzyme activity of PBS3 is obviously a breakthrough in explaining how PBS3 regulates SA-mediated immunity upon pathogen infection. However, emerging evidence suggests that *pbs3* mutants have distinct phenotypes compared with the SA-deficient mutant *sid2*, indicating that PBS3 has a role beyond SA biosynthesis. For example, PBS3 has a negative effect on ABA-mediated SA pathway inhibition in young *Arabidopsis* leaves

(Berens *et al.*, 2019), and PBS3 can adjust *Arabidopsis* flowering time by regulating the expression of flowering regulators (G. F. Wang *et al.*, 2011).

Salicylic acid is a core plant defense hormone in protecting plants from pathogen infection, but excess levels of SA can be detrimental to plant growth and development (Van Butselaar & Van den Ackerveken, 2020). Therefore, the SA biosynthesis and accumulation processes must be tightly regulated. Since PBS3 is a key enzyme in SA biosynthesis of *Arabidopsis*, it is reasonable to speculate that the expression and/or the activity of PBS3 is finely regulated during pathogen infection. The expression of SA biosynthesis-related genes can be rapidly and highly induced upon pathogen infection. The transcription of *ICS1*, *EDS5*, and *PBS3* can be positively regulated by transcription factors SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 and CAM-BINDING PROTEIN 60G (CBP60g) (Zhang *et al.*, 2010; L. Wang *et al.*, 2011; Ding & Ding, 2020). CBP60a, ABSCISIC ACID-RESPONSIVE NAC 019, and ETHYLENE INSENSITIVE 3 can negatively regulate *ICS1* transcription (Chen *et al.*, 2009; Zheng *et al.*, 2012; Truman *et al.*, 2013), while DP-E2F-LIKE 1 negatively regulates *EDS5* transcription (Chandran *et al.*, 2014). To date, the negative regulator of *PBS3* transcription remains unknown. Besides, considering that the protein stability of PBS3-interacting protein, EDS1, can be regulated by E3 ubiquitin ligase adaptors NPR3 and NPR4 through the 26S proteasome pathway (Fig. 2; Chang *et al.*, 2019), whether the highly induced expression of *PBS3* during pathogen infection can be fine-tuned at the transcriptional level and/or the protein level to reduce the adverse effects of SA on plant growth remains an open question to be answered.

Another fascinating question is whether and how PBS3 plays a role in the transcriptional regulation of gene expression. As mentioned above, PBS3 was originally reported to influence the expression of flowering regulatory genes *FLC* and *FT* together with the transcriptional coactivator NPR1 (G. F. Wang *et al.*, 2011). Large-scale transcriptome studies may be deployed to find out whether PBS3 can regulate gene expression in an SA-dependent or SA-independent manner. Researchers found a set of genes that are similarly affected by *pbs3*, *eds1*, and *pad4* but not by *npr1*, *eds5*, or *sid2* in response to *Psm* ES4326 infection; this supports the notion that *PBS3*/*EDS1*/*PAD4* might regulate processes which are different from those regulated by the classical SA pathway (Wang *et al.*, 2008). Based on the differential gene expression patterns among *pbs3*, *eds1*, *pad4*, *eds5*, *sid2*, and *npr1*, Wang *et al.* (2008) placed *PBS3* upstream of the *EDS5*/*SID2*/*NPR1* node and downstream of the *EDS1*/*PAD4* node in the immune signaling network. What increases the complexity is the fact that the PBS3-interacting protein EDS1 has been shown to be a transcriptional coactivator and cooperates with NPR1 and Mediator in the transcription machinery to enhance the activation of defense genes upon pathogen infection (Fig. 2; Chen *et al.*, 2021). Therefore, it will be intriguing to determine whether PBS3 is also involved in the transcription machinery through its interaction with EDS1.

In conclusion, PBS3, as one of the players in the intracellular signaling network, plays a principal role in plant development and resistance against biotic or abiotic stresses. In addition to the enzyme activity of PBS3 in SA biosynthesis, the molecular mechanism by which PBS3 regulates SA-dependent and SA-independent signaling pathways remains to be explored in the future.

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None declared.

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ORCID

Matthew Ashline https://orcid.org/0000-0001-7869-7387
Ming Chang https://orcid.org/0000-0003-2760-7462
Zheng Qing Fu https://orcid.org/0000-0003-1519-6192
Jinyu He https://orcid.org/0000-0002-5428-5668
Wei Li https://orcid.org/0000-0001-9704-2044
Fengquan Liu https://orcid.org/0000-0001-9325-1500
Xiuzhuo Wang https://orcid.org/0000-0001-7726-6833
Zirui Wu https://orcid.org/0000-0002-2418-0072

Data availability

The data for this review article is publicly available.

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