

PBS3 Protects EDS1 from Proteasome-Mediated Degradation in Plant Immunity

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ABSTRACT

Plant immunity is controlled by both positive regulators such as PBS3 and EDS1 and negative regulators such as NPR3 and NPR4. However, the relationships among these important immune regulators remain elusive. In this study, we found that PBS3 interacts with EDS1 in both the cytoplasm and the nucleus, and is required for EDS1 protein accumulation. NPR3 and NPR4, which function as salicylic acid receptors and adaptors of Cullin3-based E3 ligase, interact with and mediate the degradation of EDS1 via the 26S proteasome. We further discovered that PBS3 inhibits the polyubiquitination and subsequent degradation of EDS1 by reducing the association of EDS1 with the Cullin3 adaptors NPR3 and NPR4. Furthermore, we showed that PBS3 and EDS1 also contribute to PAMP-triggered immunity in addition to effector-triggered immunity. Collectively, our study reveals a novel mechanism by which plants fine-tune defense responses by inhibiting the degradation of a positive player in plant immunity.

Key words: PBS3, EDS1, NPR3, NPR4, the 26S proteasome, PAMP-triggered immunity

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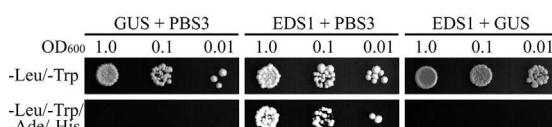
INTRODUCTION

Plants and animals have developed both conserved and unique strategies to fight against infection from a broad range of pathogens (Nurnberger et al., 2004; Ausubel, 2005). During evolution, both plants and animals independently developed the ability to recognize conserved molecules in pathogens called pathogen-associated molecular patterns (PAMPs) (Jones and Dangl, 2006). This layer of defense is called PAMP-triggered immunity (PTI). In plants, PTI is mediated by several pattern recognition receptors, which recognize PAMPs such as flagellin, EF-Tu, and chitin to activate the mitogen-activated protein kinase pathway, the oxidative burst, and callose deposition to restrict pathogen growth (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006; Miya et al., 2007). However, successful plant pathogens can overcome PTI by delivering effectors into plant cells to induce effector-triggered susceptibility (Jones and Dangl, 2006). Facing these challenges, plants have evolved a unique layer of immune response whereby effectors are specifically recognized by different nucleotide binding and leucine-rich repeat (NB-LRR) resistance proteins (Maekawa et al., 2011; Wu et al., 2014). This second layer of defense is termed effector-triggered immunity

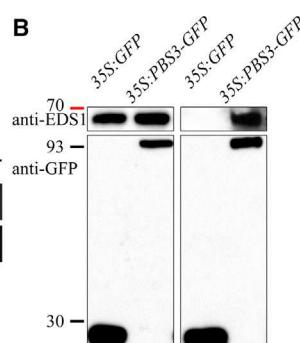
(ETI). Unlike PTI, ETI is a strong immune response and is often associated with the induction of localized programmed cell death called the hypersensitive response (Coll et al., 2011).

Successful plant immunity relies on the coordinated actions of many important regulators (Panstruga et al., 2009; Spoel and Dong, 2012). Among them, *AvrPphB* susceptible3 (PBS3) and enhanced disease susceptibility1 (EDS1) are two central players that positively regulate plant immunity (Parker et al., 1996; Warren et al., 1999). PBS3, also known as *Gretchen Hagen3.12* (GH3.12) (Westfall et al., 2012), *GH3-like defense gene1* (GDG1) (Jagadeeswaran et al., 2007), and *HopW1-1-interacting3* (WIN3) (Lee et al., 2007), was first identified in forward genetic screens for mutants that showed enhanced susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 carrying the avirulent gene *AvrPphB* (Warren et al., 1999). EDS1 was identified in a screen for *Arabidopsis* ecotype Ws-0 mutants showing enhanced susceptibility to the downy mildew pathogen

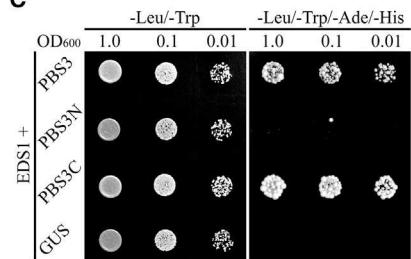
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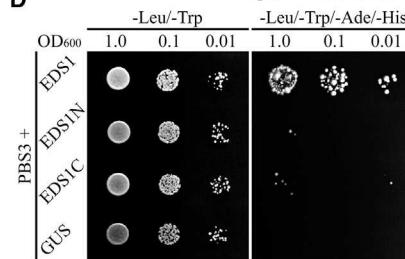
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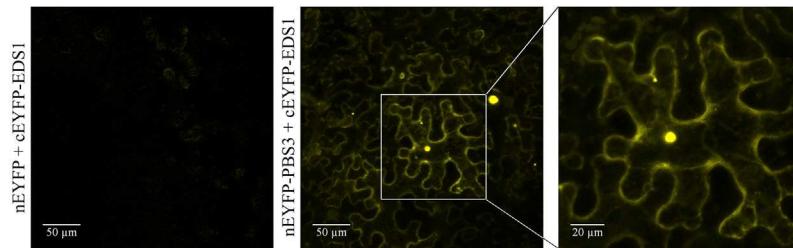
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Hyaloperonospora arabidopsis avirulent isolate Noco2 (Parker et al., 1996). EDS1 functions as a central hub in ETI by activating immune responses mediated by several toll-interleukin-1 receptor (TIR) NB-LRR resistance proteins (Aarts et al., 1998; Falk et al., 1999; Bhattacharjee et al., 2011; Heidrich et al., 2011). Similar to EDS1, PBS3 is also an important positive player in ETI. The *pbs3* mutants exhibit enhanced susceptibility to *Pst* DC3000 pathogens carrying the avirulent gene *AnvRps4*, *AvrB*, *AvrRpt2*, or *AvrPphB* (Warren et al., 1999). In addition, the accumulation of plant defense hormone salicylic acid (SA) was shown to be dependent on both PBS3 and EDS1 (Feys et al., 2001; Jagadeeswaran et al., 2007; Nobuta et al., 2007). These findings imply the common roles of PBS3 and EDS1 in ETI and SA accumulation, but how PBS3 and EDS1 fulfill their overlapping functions in plant immunity remains elusive.

In contrast to PBS3 and EDS1, non-expresser of pathogenesis-related genes1 (NPR1) paralogs NPR3 and NPR4 have been shown to function as negative regulators of plant defense (Zhang et al., 2006; Fu and Dong, 2013). More recently, it was reported that NPR3 and NPR4 bind SA with different affinities and act as SA receptors (Fu et al., 2012; Ding et al., 2018). Both NPR3 and NPR4 function as adaptors of Cullin3 (CUL3)-based E3 ligase to mediate the degradation of the master regulator of plant defense NPR1 in order to maintain an optimal level of NPR1 (Spoel et al., 2009; Fu et al., 2012). In addition, NPR3 and NPR4 also facilitate the degradation of the jasmonic acid transcriptional repressor jasmonate-zim domain (JAZ) proteins

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Figure 1. PBS3 Forms a Protein Complex with EDS1.

(A) Interaction between PBS3 and EDS1 in yeast two-hybrid (Y2H) assays.

(B) Co-immunoprecipitation (Co-IP) assays of PBS3 and EDS1 in transgenic *Arabidopsis* plants.

(C) EDS1 interacts with full-length (1–575 amino acids [aa]) PBS3 and the C-terminal (420–575 aa) domain of PBS3 (PBS3C), but not the N-terminal (1–419 aa) domain of PBS3 (PBS3N) in Y2H assays.

(D) PBS3 interacts with full-length (1–623 aa) EDS1, but not the N-terminal (EDS1N) (1–403 aa) or the C-terminal (EDS1C) (404–623 aa) domains of EDS1 in Y2H assays.

(E) Bimolecular fluorescence complementation (BiFC) assays show co-localization of PBS3 and EDS1 in both the cytoplasm and the nucleus. Confocal microscopy was used to image reconstituted EYFP signals 3 days after infiltration. These experiments were repeated at least three times with similar results.

to promote ETI (Liu et al., 2016). However, we still do not know whether and how NPR3 and NPR4 regulate the other positive central players in plant immunity such as EDS1.

Given the similar functions of PBS3 and EDS1 in plant immunity (Warren et al., 1999; Feys et al., 2001; Jagadeeswaran et al., 2007; Nobuta et al., 2007; Bhattacharjee et al., 2011; Heidrich et al., 2011), we hypothesized that PBS3 and EDS1

contribute to plant immunity by forming a protein complex. In this study, we found that EDS1 indeed interacts with PBS3 in both the cytoplasm and the nucleus. We demonstrated that the EDS1 protein levels are significantly reduced in *pbs3* mutants. Interestingly, we found that NPR3 and NPR4 promote the degradation of EDS1 proteins by the 26S proteasome through CUL3-based E3 ligase and that PBS3 stabilizes EDS1 proteins by protecting EDS1 from NPR3/NPR4-mediated degradation by the 26S proteasome. Our study, therefore, reveals a novel mechanism by which plants fine-tune defense responses by inhibiting the degradation of a positive player in plant immunity.

RESULTS

PBS3 Interacts with EDS1 in Both the Cytoplasm and the Nucleus

To test our hypothesis about the interaction between PBS3 and EDS1, we first performed yeast two-hybrid (Y2H) assays. As shown in Figure 1A, we found a strong interaction between PBS3 and EDS1. To find out whether this interaction is specific, we also tested the interactions between PBS3 and two known EDS1-interacting proteins, phytoalexin deficient4 (PAD4) and senescence-associated gene101 (SAG101) in Y2H assays and found that there was no interaction between PBS3 and PAD4, and between PBS3 and SAG101 (Supplemental Figure 1). Next, we investigated the association between PBS3 and EDS1 by performing co-immunoprecipitation (Co-IP) assays using transgenic *Arabidopsis* plants expressing

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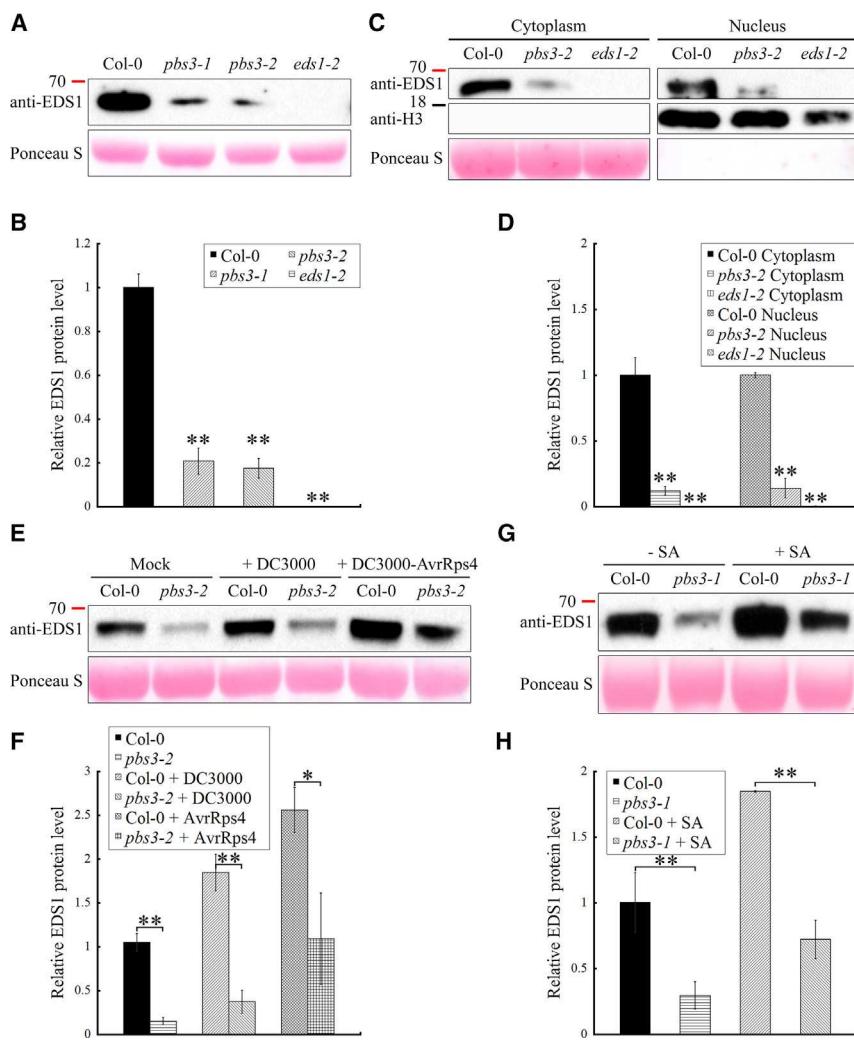


Figure 2. PBS3 Regulates EDS1 Protein Accumulation in Both the Cytoplasm and the Nucleus.

(A) The protein levels of EDS1 are reduced in *pbs3* mutants when compared with Col-0.

(B) Quantification and statistical analysis of EDS1 protein levels based on three biological replicates of the experiment in (A).

(C) The protein levels of EDS1 are reduced in both the cytoplasm and the nucleus of *pbs3-2* mutants when compared with Col-0.

(D) Quantification and statistical analysis of EDS1 protein levels based on three biological replicates of the experiment in (C).

(E) Infection by the pathogen DC3000 or DC3000-AvrRps4 ($OD_{600} = 0.001$ for 24 h) can partially rescue the decrease in EDS1 protein levels in *pbs3-2* mutants.

(F) Quantification and statistical analysis of EDS1 protein levels based on four biological replicates of the experiment in (E).

(G) SA (0.5 mM for 24 h) can partially rescue the decrease in EDS1 protein levels in *pbs3-1* mutants.

(H) Quantification and statistical analysis of EDS1 protein levels based on three biological replicates of the experiment in (G).

Values in (B), (D), (F), and (H) represent the mean \pm SE. Statistical differences were analyzed by Student's *t*-test: * $P < 0.05$, ** $P < 0.01$.

PBS3-GFP (green fluorescent protein) or GFP. EDS1 was co-immunoprecipitated with PBS3-GFP, but not with GFP alone (Figure 1B). Using truncated PBS3 and EDS1 proteins, we found that EDS1 interacted with the C-terminal domain of PBS3 (PBS3C) (Supplemental Figure 2A and Figure 1C), while PBS3 only associated with full-length EDS1 (Supplemental Figure 2B and Figure 1D). To reveal where PBS3 and EDS1 interact inside plant cells, we performed bimolecular fluorescence complementation (BiFC) assays. *Agrobacterium* strains containing constructs expressing fusion proteins of the N-terminal enhanced yellow fluorescent protein with PBS3 (nEYFP-PBS3) and the C-terminal EYFP with EDS1 (cEYFP-EDS1) were co-infiltrated into leaves of *Nicotiana benthamiana* plants. Our data showed that the PBS3-EDS1 protein complex was present in both the cytoplasm and the nucleus (Figure 1E).

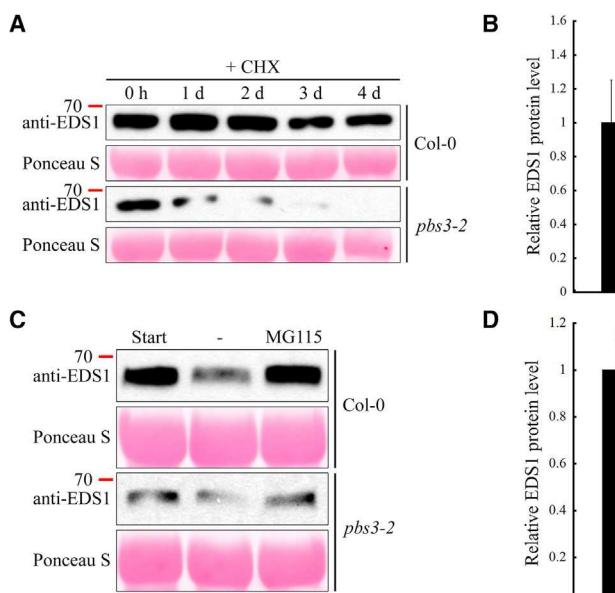
PBS3 Regulates EDS1 Accumulation in Both the Cytoplasm and the Nucleus

To probe the biological significance of the PBS3-EDS1 protein complex, we investigated EDS1 protein levels in *pbs3* mutants and PBS3 protein levels in *eds1-2* mutants. To our surprise, we found that EDS1 protein levels were significantly reduced in *pbs3* mutants when compared with Columbia-0 (Col-0) (Figure 2A).

and 2B), while PBS3 protein levels were similar in *eds1-2* mutants and Col-0 (Supplemental Figure 3). The mRNA levels of *EDS1* were slightly lower in *pbs3* mutants than in Col-0 (Supplemental Figure 4), which can partially explain the reduced EDS1 protein levels in *pbs3* mutants. Because we found that the PBS3-EDS1 protein complex existed in both the cytoplasm and the nucleus (Figure 1E), we decided to determine EDS1 protein levels in the cytoplasm and the nucleus. As shown in Figure 2C and 2D, we found that the accumulation of EDS1 proteins was significantly decreased in both the cytoplasm and the nucleus. Furthermore, the severe reduction in EDS1 protein levels could only be partially restored by infection with *Pst* DC3000 or DC3000 carrying *AvrRps4* (DC3000-AvrRps4) (Figure 2E and 2F) or exogenous treatment with SA (Figure 2G and 2H). These results suggest that PBS3 acts to maintain a steady-state level of EDS1 proteins in both the cytoplasm and the nucleus, and that reduced EDS1 protein levels in *pbs3* mutants are not entirely the result of a defect in SA accumulation.

PBS3 Inhibits the Degradation of EDS1 Mediated by the 26S Proteasome

Because the reduction in EDS1 protein levels in *pbs3* mutants was much larger than the decrease in *EDS1* mRNA levels, we speculated that PBS3 affects the protein stability of EDS1 in addition to its weak effect on *EDS1* mRNA. To test this, we applied cycloheximide (CHX), a protein synthesis inhibitor (Schneider-Poetsch et al., 2010), to Col-0 and *pbs3-2* mutants and collected samples on a daily basis over 4 days. We found that the EDS1 protein levels decreased more rapidly in *pbs3-2* mutants treated with CHX when



compared with Col-0 (Figure 3A and 3B). This indicates that EDS1 proteins are less stable in *pbs3-2* mutants than in Col-0, suggesting that EDS1 is regulated by PBS3 via a post-translational mechanism. Since ubiquitin-mediated degradation through the 26S proteasome is the most important mechanism controlling protein stability in eukaryotes (Collins and Goldberg, 2017), we next performed *in vitro* degradation assays (Osterlund et al., 2000) using protein extracts from Col-0 and *pbs3-2* mutants with and without the proteasome inhibitor MG115. A larger decrease in EDS1 protein levels was observed in the *pbs3-2* mutants than in Col-0, and the addition of MG115 completely prevented EDS1 degradation in Col-0 and the *pbs3-2* mutants (Figure 3C and 3D), indicating that EDS1 degradation is regulated by the 26S proteasome. Together, these data indicate that PBS3 inhibits proteasome-mediated degradation of EDS1.

EDS1 Interacts with NPR3 and NPR4, Two Adaptors of CUL3-Based E3 Ligase

NPR3 and NPR4 contain a broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger (BTB/POZ) domain and act as adaptors of CUL3-based E3 ligase to mediate the degradation of the master regulator of plant defense NPR1 and the jasmonic acid transcriptional repressor JAZ proteins by the 26S proteasome (Fu et al., 2012; Liu et al., 2016; Pintard et al., 2004; Spoel et al., 2009). Since NPR3 and NPR4 function as negative regulators of plant immunity (Zhang et al., 2006), and EDS1 and NPR1 play positive roles in plant immunity (Cao et al., 1994; Parker et al., 1996), we speculated that NPR3 and NPR4 are involved in the regulation of proteasome-mediated degradation of EDS1. To test our hypothesis, we first performed Y2H assays to test whether NPR3 and NPR4 can interact with EDS1. We found that NPR3 and NPR4 strongly interacted with full-length EDS1 and the N-terminal domain of EDS1 (EDS1N) (Figure 4A), but weakly interacted with the C-terminal domain of EDS1 (EDS1C) (Figure 4A). Because NPR3 and NPR4 are receptors of SA and SA affects the interactions between NPR3/NPR4 and their interacting proteins (Fu et al., 2012; Liu et al., 2016), we also tested the effect of SA on the interactions between

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Figure 3. PBS3 Inhibits Proteasome-Mediated Degradation of EDS1 Proteins.

(A) In the presence of 0.1 mM CHX, a protein synthesis inhibitor, EDS1 protein levels decrease more rapidly in *pbs3-2* mutants than in Col-0.

(B) Quantification and statistical analysis of EDS1 protein levels based on three biological replicates of the experiment in (A).

(C) *In vitro* degradation assays showing that EDS1 proteins are regulated by proteasome-mediated degradation. Total proteins were extracted from leaves before (start) and after treatment with (MG115) or without (–) 50 μ M MG115 for 3 h.

(D) Quantification and statistical analysis of EDS1 protein levels based on four biological replicates of the experiment in (C).

Values in (B) and (D) represent the mean \pm SE. Statistical differences were analyzed by Student's *t*-test. **P* < 0.05; ND, no significant difference.

NPR3/NPR4 and EDS1. Surprisingly, as shown in *Supplemental Figure 5*, we found that SA did not significantly affect the interactions between NPR3/NPR4 and EDS1 in Y2H assays, probably because these interactions were already strong in the absence of SA. We also found that PBS3 did not interact with NPR3 or NPR4 in Y2H assays in the presence or absence of SA (*Supplemental Figure 6*). We further validated the observed NPR3/NPR4-EDS1 interactions by performing Co-IP assays using transient expression in *N. benthamiana*. EDS1-3FLAG was co-immunoprecipitated with GFP-NPR3 and with GFP-NPR4, but not with GFP alone (Figure 4B), indicating that NPR3 and NPR4 form a protein complex with EDS1 in plant cells.

EDS1 Protein Stability Is Negatively Regulated by NPR3 and NPR4

If NPR3 and NPR4 function as CUL3 adaptors to mediate EDS1 degradation, EDS1 protein levels should be increased in *npr3* and *npr4* mutants. As shown in Figure 4C and 4D, we found significantly increased EDS1 protein levels in both the *npr3-2* and *npr4-2* single mutants and even higher EDS1 protein levels in the *npr3-2 npr4-2* double mutants when compared with Col-0. In contrast, qRT-PCR data showed that the *EDS1* mRNA levels were lower in *npr3-2* mutants, *npr4-2* mutants, and *npr3-2 npr4-2* double mutants when compared with Col-0 (*Supplemental Figure 7*). To confirm that NPR3 and NPR4 regulate EDS1 protein stability based on their roles as CUL3 adaptors, we measured the EDS1 protein levels in *cul3a/b* mutants. Similar to *npr3-2 npr4-2* double mutants, we observed a significantly higher amount of EDS1 protein in *cul3a/b* mutants than in Col-0 (Figure 4E and 4F). Similarly, qRT-PCR data also showed that the *EDS1* mRNA levels were lower in *cul3a/b* mutants than in Col-0 (*Supplemental Figure 8*). These data suggest that NPR3 and NPR4 negatively regulate EDS1 protein stability by functioning as CUL3 adaptors.

PBS3 Promotes the Stability of EDS1 by Reducing the Association of EDS1 with NPR3 and NPR4

To better understand the function of PBS3 in regulating EDS1 protein stability, we determined whether the polyubiquitination

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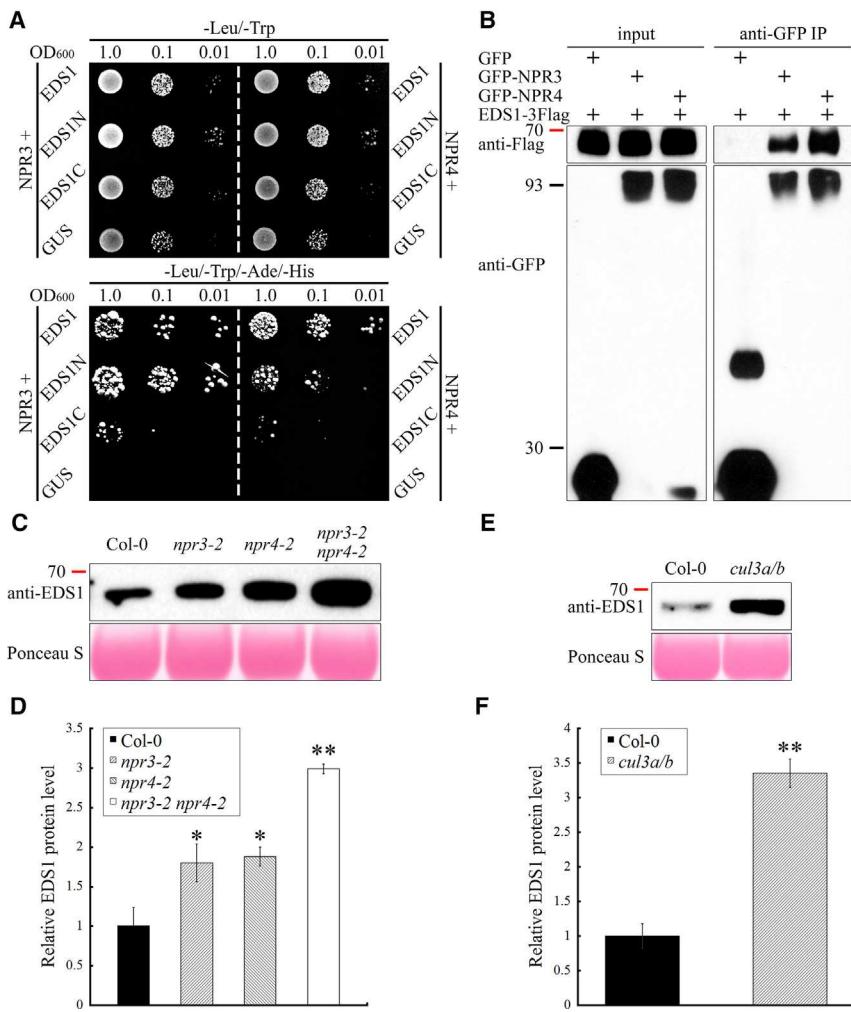


Figure 4. The CUL3 Adaptors NPR3 and NPR4 Interact with EDS1, and EDS1 Protein Levels Are Increased in *npr3-2* Mutants, *npr4-2* Mutants, *npr3-2 npr4-2* Double Mutants, and *cul3a/b* Mutants.

(A) Y2H assays show that NPR3 and NPR4 strongly interact with full-length (1–623 aa) EDS1 and the N-terminal (1–403 aa) domain of EDS1 (EDS1N), but weakly interact with the C-terminal (404–623 aa) domain of EDS1 (EDS1C).

(B) Co-IP assays show that NPR3/NPR4 and EDS1 interact in *N. benthamiana*.

(C) Protein levels of EDS1 are increased in *npr3-2* mutants, *npr4-2* mutants, and *npr3-2 npr4-2* double mutants when compared with Col-0.

(D) Quantification and statistical analysis of EDS1 protein levels based on three biological replicates of the experiment in (C).

(E) Protein levels of EDS1 are increased in *cul3a/b* mutants when compared with Col-0.

(F) Quantification and statistical analysis of EDS1 protein levels based on five biological replicates of the experiment in (E).

Values in (D) and (F) represent the mean \pm SE. Statistical differences were analyzed by Student's *t*-test: ***P* < 0.01.

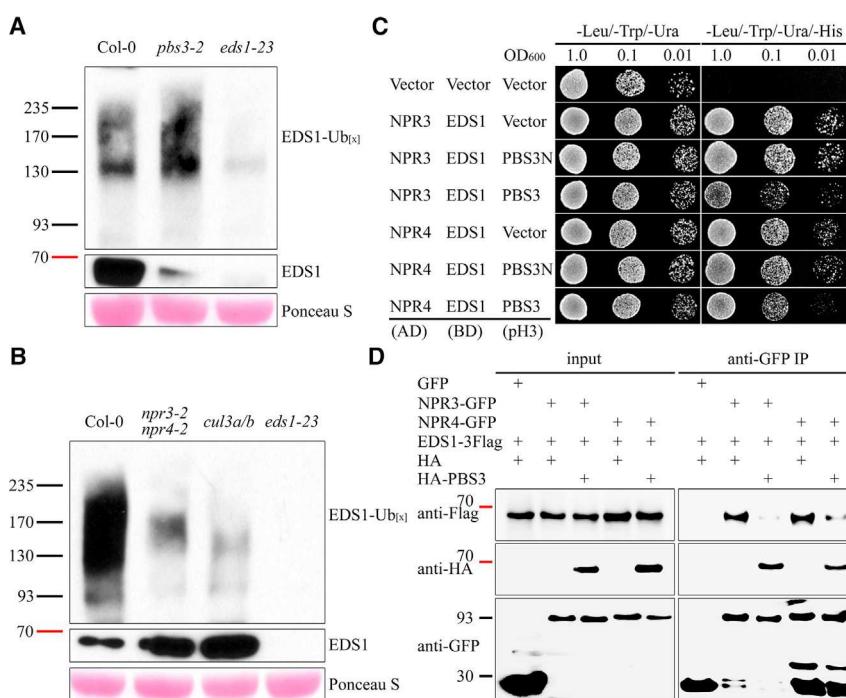
of EDS1 is affected in *pbs3-2* mutants. Total polyubiquitinated proteins were immunoprecipitated from Col-0 and *pbs3-2* mutants using anti-UBQ11 antibodies, then polyubiquitinated EDS1 proteins were detected with anti-EDS1 antibodies. As shown in Figure 5A, although the total amount of EDS1 protein was much lower in *pbs3-2* mutants when compared with Col-0, the levels of polyubiquitinated EDS1 proteins were still higher in the *pbs3-2* mutants than in Col-0, indicating that PBS3 prevents EDS1 polyubiquitination. We further determined the levels of polyubiquitinated EDS1 proteins in *npr3-2 npr4-2* double mutants and *cul3a/b* mutants. As shown in Figure 5B, we found lower levels of polyubiquitinated EDS1 proteins in *npr3-2 npr4-2* double mutants and *cul3a/b* mutants than in Col-0, suggesting that the NPR3/NPR4–CUL3 protein complex promotes EDS1 polyubiquitination. Because we demonstrated that NPR3 and NPR4 negatively regulate the protein stability of EDS1 by functioning as adaptors of CUL3-based E3 ligase, we further hypothesized that PBS3 may inhibit the polyubiquitination of EDS1 by affecting its association with NPR3 and NPR4. To test this hypothesis, we performed yeast three-hybrid (Y3H) assays and found that the co-expression of full-length PBS3 together with EDS1 and NPR3/NPR4 reduced the growth of yeast on quadruple dropout medium when compared with yeast expressing EDS1, NPR3/NPR4, and the pH3 empty vector (Figure 5C); however, when EDS1 and NPR3/NPR4 were co-expressed with

the N-terminal domain of PBS3 (PBS3N), which could not interact with EDS1 in Y2H assays (Figure 1C), this effect was not observed (Figure 5C). Since PBS3 could not interact with NPR3 or NPR4 in Y2H assays (Supplemental Figure 6), the Y3H assay results suggest that PBS3 reduces the normal interactions between EDS1 and NPR3/NPR4 by interacting with EDS1. We

further performed Co-IP assays to confirm the effect of PBS3 in plant cells. As shown in Figure 5D, Co-IP assays using transient expression in *N. benthamiana* showed that less EDS1 was co-immunoprecipitated with NPR3 or NPR4 in the presence of PBS3 when compared with an empty vector control. Together, these data indicate that PBS3 inhibits the polyubiquitination and subsequent degradation of EDS1 by reducing the association of EDS1 with NPR3 and NPR4.

PBS3–EDS1 Protein Complex Functions in Both PTI and ETI

Consistent with previous studies, *pbs3-2* mutants showed an early-flowering phenotype (Supplemental Figure 9) (Wang et al., 2011). Interestingly, we found that this phenotype was enhanced in *pbs3-2 eds1-2* double mutants (Supplemental Figure 9), indicating the existence of a common signaling pathway mediated by PBS3 and EDS1. Next, we investigated the biological function of the PBS3–EDS1 protein complex in plant immunity. We first measured the time-course expression of PBS3 and EDS1 proteins in Col-0 after infection with DC3000 or DC3000-AvrRps4. PBS3 proteins showed an induction pattern similar to that of EDS1 proteins, supporting the role of PBS3 in stabilizing EDS1 proteins by forming a protein complex (Figure 6A). Both the *pbs3-2* and *eds1-2* mutants showed increased susceptibility to DC3000 and DC3000-AvrRps4 infection when



compared with Col-0, and *pbs3-2 eds1-2* double mutants showed even higher susceptibility (Figure 6B), suggesting that PBS3 functions additively with EDS1 in plant basal resistance and AvrRps4-induced ETI. However, *eds1-2* mutants completely lost resistance to DC3000-AvrRps4 (Figure 6B), while *pbs3-2* mutants were only partially compromised in resistance to DC3000-AvrRps4 (Figure 6B), which is consistent with the reduced EDS1 protein levels observed in *pbs3-2* mutants. We further evaluated the accumulation of PR1 proteins induced by DC3000 or DC3000-AvrRps4. We found that both strains strongly induced the accumulation of PR1 proteins in Col-0, whereas PR1 proteins were not detected in *pbs3-2* mutants, *eds1-2* mutants, and *pbs3-2 eds1-2* double mutants (Figure 6C). This result further supports the hypothesis that PBS3 and EDS1 have common functions in the immune signaling pathway. To better understand the function of PBS3 and EDS1 in basal resistance, we further investigated the role of PBS3 and EDS1 in PTI by analyzing flg22-induced callose deposition. As shown in Figure 6D and 6E, we found that callose deposition was obviously lower in both the *pbs3-2* and *eds1-2* single mutants and even lower in the *pbs3-2 eds1-2* double mutants when compared with Col-0, indicating that PBS3 and EDS1 additively contribute to PTI.

DISCUSSION

It is well known that EDS1, which was discovered around two decades ago, plays an essential role in plant immune signaling (Bhattacharjee et al., 2011; Heidrich et al., 2011; Cui et al., 2017). However, how the accumulation of EDS1 proteins is regulated remains elusive. In eukaryotic cells, proteins are linked to the protein ubiquitin and thereafter polyubiquitinated proteins are degraded by the 26S proteasome (Sadanandom et al., 2012; Hu and Sun, 2016; Miricescu et al., 2018). In this study, we found that NPR3 and NPR4, as adaptors of CUL3-based E3 ligase, interact with EDS1 and mediate the degradation of EDS1 by the 26S proteasome (Figure 4). Our data are

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Figure 5. PBS3 Inhibits the Polyubiquitination of EDS1 by Reducing the Association of EDS1 with NPR3 and NPR4.

(A) Levels of polyubiquitinated EDS1 proteins are higher in *pbs3-2* mutants than in Col-0. The *eds1-23* mutant was used as a negative control.

(B) Levels of polyubiquitinated EDS1 proteins are lower in *npr3-2 npr4-2* double mutants and *cul3a/b* mutants than in Col-0. The *eds1-23* mutant was used as a negative control.

(C) PBS3 reduces the interactions between EDS1 and NPR3/NPR4 in yeast three-hybrid (Y3H) assays.

(D) Co-IP assays show that PBS3 reduces the association of EDS1 with NPR3 and NPR4 when transiently expressed in *N. benthamiana*. These experiments were repeated at least three times with similar results.

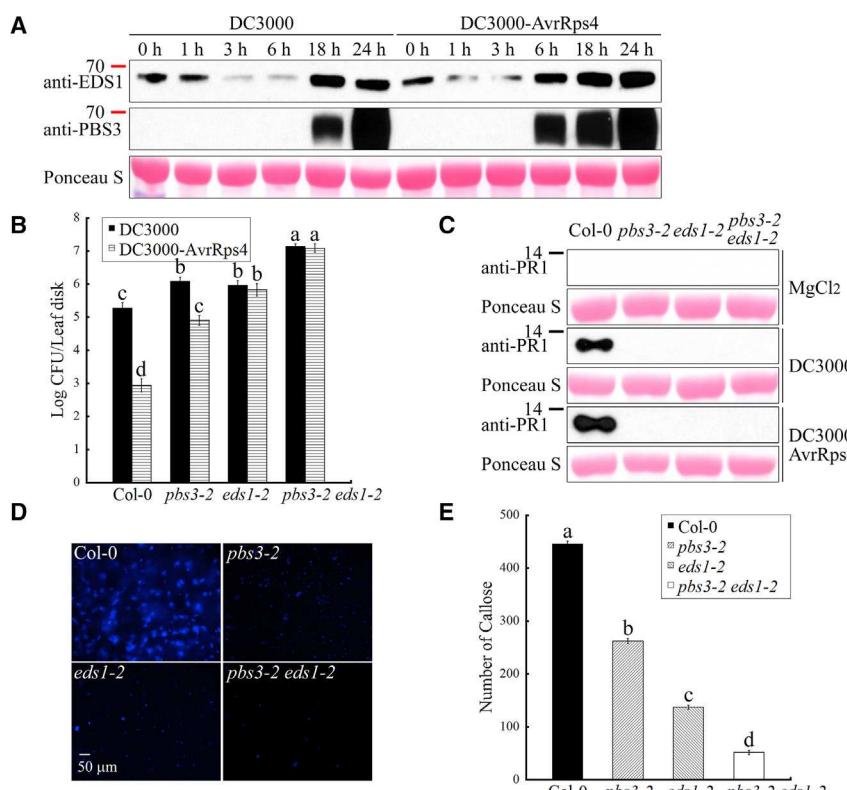
consistent with the notion that EDS1 functions as an essential positive player while NPR3 and NPR4 act as negative regulators in plant immunity (Wiermer et al., 2005; Zhang et al., 2006). It has been demonstrated that NPR3 and NPR4 both

bind SA and function as SA receptors (Fu et al., 2012; Ding et al., 2018). However, our data showed that the interactions between EDS1 and NPR3/NPR4 were not significantly affected by SA in Y2H assays (Supplemental Figure 5) and that SA could only partially rescue the decrease in EDS1 protein levels in *pbs3-2* mutants (Figure 2G and 2H). These results indicate that NPR3 and NPR4 mediate the degradation of EDS1 proteins by functioning as CUL3 adaptors. Therefore, our studies have revealed a novel function of NPR3 and NPR4 as CUL3 adaptors in addition to their known roles in the degradation of NPR1 and JAZ proteins and in the negative regulation of plant defense gene expression (Fu et al., 2012; Liu et al., 2016; Ding et al., 2018).

PBS3 and EDS1 share similar functions in plant immunity, but the relationship between PBS3 and EDS1 is still unclear (Warren et al., 1999; Feys et al., 2001; Jagadeeswaran et al., 2007; Nobuta et al., 2007; Bhattacharjee et al., 2011; Heidrich et al., 2011). PBS3 is thought to act as a benzoate-specific acyl acid amido synthetase (Westfall et al., 2010, 2012), and is required for SA accumulation (Jagadeeswaran et al., 2007; Nobuta et al., 2007). However, PBS3 shows extremely poor SA-binding activity when compared with other benzoates such as 4-hydroxybenzoate (Okrent et al., 2009; Westfall et al., 2012), which points to other functions of PBS3 in plant immunity. In this study, we showed that PBS3 forms a stable protein complex with EDS1 in both the cytoplasm and the nucleus (Figure 1). Furthermore, we demonstrated that EDS1 protein levels are significantly reduced in *pbs3* mutants (Figure 2) and that PBS3 inhibits the polyubiquitination and subsequent degradation of EDS1 by reducing the interactions of EDS1 with NPR3/NPR4 (Figure 5). Three different scenarios may explain our data. First, PBS3 may cover the NPR3/NPR4 interaction sites in the EDS1 protein by forming a protein complex with EDS1. Second, PBS3 may reduce the interactions between EDS1 and NPR3/NPR4 because the PBS3-EDS1 interaction outcompetes the EDS1-NPR3/NPR4 interaction. Third, the

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interaction between PBS3 and EDS1 may cause a conformational change in the EDS1 protein, which reduces the association of EDS1 with NPR3/NPR4.

In this study, we have identified a novel function of PBS3 in protecting EDS1 from proteasome-mediated degradation. Without the protection of PBS3 in *pbs3-2* mutants, EDS1 is degraded by the 26S proteasome, resulting in enhanced susceptibility to pathogen infection (Figures 6 and 7). It was previously reported that two EDS1 sequence-related proteins, PAD4 and SAG101, also interact with EDS1 and promote the protein stability of EDS1 (Feys et al., 2005), but we found that PBS3 interacts with EDS1 but not with PAD4 or SAG101 in Y2H assays (Supplemental Figure 1). Therefore, whether the PBS3-EDS1 and PAD4-EDS1-SAG101 protein complexes exist in common or separate signaling pathways in plant immunity is still an open question.

Previous studies have mainly focused on the functions of PBS3 and EDS1 in ETI and SA accumulation (Warren et al., 1999; Feys et al., 2001; Jagadeeswaran et al., 2007; Nobuta et al., 2007; Bhattacharjee et al., 2011; Heidrich et al., 2011). Our results demonstrate that both PBS3 and EDS1 are required for flg22-induced PTI (Figure 6D and 6E). These data not only greatly increase our understanding of the biological functions of PBS3 and EDS1 in plant immunity, but also suggest that the essential roles of PBS3 and EDS1 in plant basal resistance are partially attributed to their functions in PTI. Notably, our data suggest that PBS3 confers plant immunity in both EDS1-dependent and -independent pathways (Figure 6). This study unravels an EDS1-dependent mechanism underlying PBS3-mediated plant immunity. Further studies are required to elucidate the molecular mechanism through which PBS3 contributes to plant immunity in an EDS1-independent manner.

Figure 6. PBS3-EDS1 Protein Complex Contributes to both PAMP-Triggered Immunity and Effector-Triggered Immunity.

(A) Time-course expression analysis of EDS1 and PBS3 proteins after infection with DC3000 or DC3000-AvrRps4.

(B) PBS3 and EDS1 are required for resistance to DC3000 and DC3000-AvrRps4.

(C) PBS3 and EDS1 are required for accumulation of PR1 proteins induced by DC3000 and DC3000-AvrRps4.

(D) PBS3 and EDS1 are necessary for callose deposition induced by 1 μ M flg22 infiltration. (E) Statistical analysis of callose deposition based on twenty biological replicates of the experiment in (D). In (B) and (E), error bars denote SE. Different letters above bars indicate statistical differences determined by Student's *t*-test ($P < 0.01$). These experiments were repeated at least three times with similar results.

date the molecular mechanism through which PBS3 contributes to plant immunity in an EDS1-independent manner.

METHODS

Plants and Growth Conditions

Arabidopsis thaliana and *Nicotiana benthamiana* seeds were sown on Murashige and Skoog phytagel medium and maintained at 4°C for 2 days, then were transferred to a 20°C–22°C plant growth room with a 12-h light/12-h dark cycle.

The *pbs3-1* mutant (Col-0 genetic background), which is a point mutation line, has been described previously (Warren et al., 1999). The T-DNA insertion line *pbs3-2* (Col-0 genetic background, Salk_018225) was obtained from the Arabidopsis Biological Resource Center (ABRC) (Nobuta et al., 2007). Two gene-specific primers, *pbs3-2*_LP/*pbs3-2*_RP, together with the left border primer Salk_LB1.3 were used for PCR genotyping of the *pbs3-2* mutant using genomic DNA as the template (see Supplemental Table 1). The T-DNA insertion line *eds1-23* (Col-0 genetic background, Salk_057149) (Kim et al., 2012) was obtained from ABRC. Two gene-specific primers, *eds1-23*_LP/*eds1-23*_RP, together with the left border primer Salk_LB1.3 were used for PCR genotyping of the *eds1-23* mutant using genomic DNA as the template (see Supplemental Table 1). The *eds1-2* mutant (Col-0 genetic background) is a fast neutron-bombarded mutant (Aarts et al., 1998) and can be genotyped using two gene-specific primers *eds1-2*_{For}/*eds1-2*_{Rev} (see Supplemental Table 1). The *pbs3-2 eds1-2* double mutant was generated by crossing two single mutant lines, *pbs3-2* and *eds1-2*. The *npr3-2* (Salk_043055), *npr4-2* (Salk_098469), *npr3-2 npr4-2*, and *cul3a/b* mutants have been reported previously (Zhang et al., 2006; Spoel et al., 2009). Ecotype Col-0 was used as the wild-type plant.

Plasmid Construction

Several sets of *attB*-attached gene-specific primers were designed for cloning the full-length coding sequences of PBS3 (PBS3_{For}/PBS3_{Rev}), EDS1 (EDS1_{For}/EDS1_{Rev}), PAD4 (PAD4_{For}/PAD4_{Rev}), SAG101 (SAG101_{For}/SAG101_{Rev}), NPR3 (NPR3_{For}/NPR3_{Rev}), NPR4 (NPR4_{For}/NPR4_{Rev}), and CUL3A (CUL3A_{For}/CUL3A_{Rev}); the genomic DNA sequences (including the predicted promoter sequences) of PBS3 (pgPBS3_{For}/pgPBS3_{Rev}) and EDS1 (pgEDS1_{For}/pgEDS1_{Rev}); the N-terminal domain coding sequence (1–1257 bp) of PBS3 (PBS3_{For}/PBS3N_{Rev}); the C-terminal domain coding sequence (1258–1725 bp) of PBS3 (PBS3C_{For}/PBS3C_{Rev}); the N-terminal

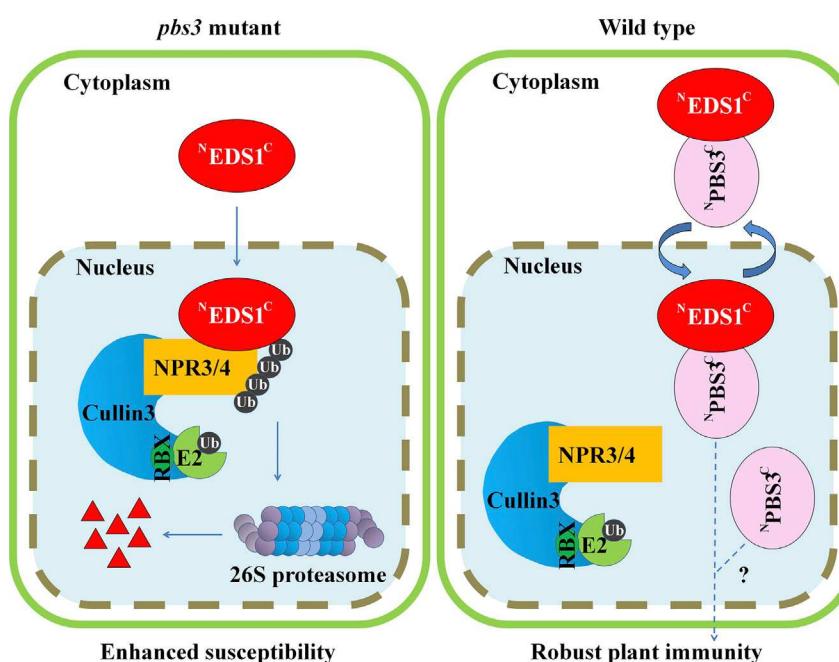


Figure 7. A Proposed Working Model Illustrating the Regulation of EDS1 Protein Stability in Plant Cells.

Left panel: in *pbs3* mutants, NPR3 and NPR4 interact with EDS1 and function as CUL3 adaptors to mediate EDS1 degradation via the 26S proteasome. Right panel: in wild-type plants, PBS3 protects EDS1 from proteasome-mediated degradation by reducing the association of EDS1 with the CUL3 adaptors NPR3 and NPR4. PBS3 confers EDS1-dependent plant immunity by interacting with EDS1, but how PBS3 contributes to EDS1-independent plant immunity remains an open question.

domain coding sequence (1–1209 bp) of *EDS1* (*EDS1*_{For}/*EDS1*_{Rev}); and the C-terminal domain coding sequence (1210–1869 bp) of *EDS1* (*EDS1*_{1C}_{For}/*EDS1*_{Rev}) (see *Supplemental Table 1*). All cloned DNA sequences were recombined into the Entry vector pDONR207 using Gateway BP Clonase II Enzyme Mix (Invitrogen).

For generation of *PBS3* transgenic *Arabidopsis* plants, full-length *PBS3* coding sequence was recombined into the C-terminal GFP-tag fusion Gateway Destination vector pK7FWG2 using Gateway LR Clonase II Enzyme Mix (Invitrogen). This construct was named pK7FWG2 35S:*PBS3*-GFP and was transformed into *pbs3*-1 mutants by floral dipping using the *Agrobacterium tumefaciens* strain GV3101 (Clough and Bent, 1998). For transient expression assays in *N. benthamiana*, several genes (including *PBS3*, *EDS1*, *NPR3*, *NPR4*, and *CUL3A*) were individually recombined into different Gateway Destination vectors using Gateway LR Clonase II Enzyme Mix (Invitrogen); the constructs were named pK7FWG2 35S:*EDS1*-GFP, pK7FWG2 35S:*NPR3*-GFP, pK7FWG2 35S:*NPR4*-GFP, pWGF2K 35S:GFP-*NPR3*, pWGF2K 35S:GFP-*NPR4*, pEarleyGate 201 35S:HA-*PBS3*, pEarleyGate 201 35S:HA-*CUL3A*, pEarleyGate 301 pg*PBS3*-3FLAG, and pEarleyGate 301 pg*EDS1*-3FLAG.

Yeast Two-Hybrid Assays

For investigation of whether *PBS3* interacts with *EDS1* in Y2H assays, the coding sequence of *PBS3* was recombined into the Gateway Destination vector pGBKT7 (containing an N-terminal GAL4 DNA-binding domain); the coding sequence of *EDS1* was recombined into the Gateway Destination vector pGADT7 (containing an N-terminal GAL4 DNA activation domain). The *Saccharomyces cerevisiae* yeast strain Y187 transformed with pGBKT7-*PBS3* was then mated with the yeast strain AH109 transformed with pGADT7-*EDS1* at 30°C overnight. For testing the *NPR3*-*EDS1* or *NPR4*-*EDS1* interaction in Y2H assays, the coding sequence of *EDS1* was recombined into pGBKT7 and pGBKT7-*EDS1* was transformed into the yeast strain Y187; the coding sequence of *NPR3* or *NPR4* was recombined into pGADT7, and pGADT7-*NPR3* or pGADT7-*NPR4* was transformed into the yeast strain AH109. For detecting the possible interactions between *PBS3* and *PAD4*, *SAG101*, *NPR3*, or *NPR4*, the coding sequences of these genes were recombined into the vector pGADT7 or pGBKT7 separately, after which the pGADT7 vector constructs were transformed

into the yeast strain AH109 and the pGBKT7 vector constructs were transformed into the yeast strain Y187. The construct pGADT7-GUS or pGBKT7-GUS was used as the negative control and was separately transformed into the yeast strain AH109 or Y187. The positive mating strains of AH109 and Y187 were selected on double dropout (DDO, -Leu/-Trp) agar medium at 30°C for 2 days and were then placed on the quadruple dropout (QDO, -Leu/-Trp/-Ade/-His, with or without 0.2 mM SA) agar medium at 30°C for 3–5 days to test protein-protein interactions.

Yeast Three-Hybrid Assays

To test the effect of *PBS3* on the interaction between *EDS1* and *NPR3* or *NPR4*, we designed Y3H assays as described previously (Griffiths et al., 2006). The coding sequences of *PBS3* and *PBS3N* were cloned using primers PBS3-pH3_{For}/PBS3-pH3_{Rev} and PBS3N-pH3_{For}/PBS3N-pH3_{Rev} (see *Supplemental Table 1*), respectively, then separately recombined into the vector pH3 using the CloneEZ PCR Cloning Kit (GenScript). The yeast strain PJ69-4A was co-transformed with three types of constructs, namely pGADT7-*NPR3*/*NPR4*, pGBKT7-*EDS1*, and pH3-*PBS3*/*PBS3N*. The empty vectors were used as negative controls. Transformed yeast strains were selected on triple dropout (-Leu/-Trp/-Ura) agar medium at 30°C for 2 days and were then placed on quadruple dropout (-Leu/-Trp/-Ura/-His) agar medium at 30°C for 2–3 days to test protein-protein interactions.

Protein Extraction and Western Blotting

Three rosette leaves of each 4-week-old *Arabidopsis* plant were harvested, frozen in liquid nitrogen, and ground with a 2010 Geno/Grinder (SPEX). Total proteins were isolated from ground leaves by incubating for 1 h on ice with protein extraction buffer (PEB) (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.2% IGEPAL CA-630, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol [DTT], 1× protease inhibitor cocktail, and 50 μM MG132). Crude extracts were subsequently centrifuged at 20 000 g at 4°C for 20 min and supernatants were collected. Centrifugation was repeated three times. Equal amounts of total protein (20–50 μg) extracted from different plants were separated on precast 4%–12% ExpressPlus PAGE Gels (GenScript), and subsequently transferred onto nitrocellulose membranes (GE Healthcare). *EDS1* proteins were detected by the anti-*EDS1* primary antibody (1:1000 dilution) (Agrisera), followed by the secondary antibody goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP) (1:5000 dilution) (Thermo Fisher Scientific). For detection of *PBS3* proteins, the N-terminal *PBS3*-specific peptide (NH₂-MKP1FDINET-FEKQLC-(amidated)) was synthesized by Agrisera and was conjugated to KLH. This peptide was used to immunize rabbits, and the purified antiserum was used as the *PBS3* primary antibody (1:1000 dilution).

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Goat anti-rabbit IgG-HRP (1:5000 dilution) was used as the secondary antibody. PR1 proteins were detected by the anti-PR1 primary antibody (1:5000 dilution) (Agrisera), followed by the secondary antibody goat anti-rabbit IgG-HRP (1:5000 dilution).

Nuclear Protein Extraction

Leaves (2 g) of 4-week-old *Arabidopsis* plants were ground into fine powder in liquid nitrogen using a cold mortar and pestle. Nuclear proteins were extracted using the semi-pure preparation of nuclei method as described in the Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich). Cytoplasmic and nuclear proteins were further analyzed by western blotting. EDS1 proteins were detected by the anti-EDS1 primary antibody (1:1000 dilution), followed by the secondary antibody goat anti-rabbit IgG-HRP (1:5000 dilution). Histone3 (H3) protein was used as the nuclear protein marker and was detected by the anti-H3 primary antibody (1:4000 dilution) (Agrisera), followed by the secondary antibody goat anti-rabbit IgG-HRP (1:5000 dilution).

Co-immunoprecipitation Assays

Leaves (2 g) of 4-week-old *N. benthamiana* or *Arabidopsis* plants were ground into fine powder in liquid nitrogen using a cold mortar and pestle. Total proteins were isolated using PEB as described above. After centrifugation, total proteins were filtered through a 0.2-μm filter and equal amounts of total protein (5–10 mg) extracted from different plants were incubated with 20 μl of GFP-Trap_MA (ChromoTek) beads with gentle rocking at 4°C overnight. The conjugated beads were collected using a magnetic separation stand (Promega) and were washed three times with 500 μl of washing buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.5 mM EDTA [pH 8.0]). Proteins were eluted from the beads by boiling for 10 min with 2x Laemmli Sample Buffer (Bio-Rad) with the additions of 100 mM DTT and 5% β-mercaptoethanol. GFP fusion proteins were detected by the anti-GFP primary antibody (1:3000 dilution) (Clontech), followed by the secondary antibody goat anti-mouse IgG-HRP (1:5000 dilution) (Thermo Fisher Scientific); hemagglutinin (HA) fusion proteins were detected by the antibody anti-HA-peroxidase (3F10) (1:500 dilution) (Roche); and FLAG fusion proteins were detected by the antibody anti-FLAG M2-peroxidase (1:3000 dilution) (Sigma-Aldrich).

In Vivo Ubiquitination Assays

Total proteins were extracted from 2-g of leaves from 4-week-old *Arabidopsis* plants as described for the coIP assays. Total proteins were filtered through a 0.2-μm filter, and equal amounts of total protein (5–10 mg) extracted from different plants were incubated with 5–10 μg of anti-UBQ11 antibody (Agrisera) with gentle rocking at 4°C overnight, after which 20 μl of equilibrated Pierce Protein A/G Magnetic Beads (Thermo Fisher Scientific) were added into the mixture with gentle rocking at room temperature for 1 h. The conjugated beads were collected using a magnetic separation stand and were washed three times with 500 μl of washing buffer (25 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 0.05% Tween 20), then washed once with 500 μl of ultrapure water. Total polyubiquitinated proteins were eluted from the beads by incubating with 2x Laemmli Sample Buffer with the additions of 100 mM DTT and 5% β-mercaptoethanol at room temperature for 10 min. Polyubiquitinated EDS1 was detected by the anti-EDS1 primary antibody (1:1000 dilution), followed by the secondary antibody goat anti-rabbit IgG-HRP (1:5000 dilution).

Bimolecular Fluorescence Complementation Assays

The coding sequence of *PBS3* was recombined into the Gateway Destination vector pSITE-nEYFP (this construct was named nEYFP-PBS3) and the coding sequence of *EDS1* was recombined into the Gateway vector pSITE-cEYFP (this construct was named cEYFP-EDS1). The construct nEYFP-PBS3 or the empty vector pSITE-nEYFP (abbreviated as nEYFP) was transiently co-expressed with the construct cEYFP-EDS1 in *N. benthamiana* leaves using *Agrobacterium*-mediated transformation. *Agrobacterium* strains were inoculated together with another *Agrobacterium* strain carrying silencing suppressor p19 at OD₆₀₀ = 0.5 for each

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strain. Confocal microscopy was used to visualize the EYFP signals 3 days after infiltration.

Quantitative Real-Time PCR

Total RNA was isolated from rosette leaves of 4-week-old *Arabidopsis* plants using TRIzol reagent (Invitrogen), and 2 μg of total RNA was subsequently used for cDNA synthesis with qScript cDNA SuperMix (Quanta) according to the manufacturer's instructions. Each cDNA sample was diluted 5-fold with ultrapure water before being used as a template. For qRT-PCR assays, PCR reaction mixtures were set up in a 96-well microplate (Axygen) with the SYBR Green PCR Master Mix (Applied Biosystems) in volumes of 20 μl, and PCR reactions were performed in a 7300 Real-Time PCR System (Applied Biosystems). For analysis of the *EDS1* mRNA level in Col-0, *pbs3-1*, *pbs3-2*, *npr3-2*, *npr4-2*, *npr3-2 npr4-2*, and *cu3a/b* plants, a C-terminal fragment of the *EDS1* transcript was amplified with the *EDS1*-specific primers *EDS1-qRT_{For}*/*EDS1-qRT_{Rev}* (see *Supplemental Table 1*). *UBQ5* was used as an internal control and was amplified with the *UBQ5*-specific primers *UBQ5-qRT_{For}*/*UBQ5-qRT_{Rev}* (see *Supplemental Table 1*). Each reaction was repeated at least three times.

Chemical Induction

For SA treatment, 0.5 mM SA (dissolved in ultrapure water containing 0.025% Silwet L-77) was evenly sprayed on the rosette leaf surfaces of each 4-week-old *Arabidopsis* plant. After 24 h, three rosette leaves from each plant were harvested as one sample for further analysis.

For CHX treatment, 0.1 mM CHX (dissolved in 100% ethanol) was infiltrated into rosette leaves of 4-week-old *Arabidopsis* plants. Three rosette leaves were collected on a daily basis over 4 days.

In Vitro Degradation Assays

Three rosette leaves from each 4-week-old *Arabidopsis* plant were ground in liquid nitrogen and resuspended in a buffer (25 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 10 mM NaCl, 10 mM ATP, and 5 mM DTT) as described previously (Osterlund et al., 2000). After incubation on ice for 30 min, crude extracts were subsequently centrifuged at 14 000 g at 4°C for 10 min and equal amounts of supernatant were transferred into individual tubes. Inhibitor studies were carried out at room temperature for 3 h in the presence or absence of 50 μM MG115. Reactions were stopped by adding 5× protein loading buffer, and samples were analyzed by western blotting.

Pathogen Infection

For protein expression analysis of EDS1, PBS3, and PR1, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 or DC3000 carrying *AvrRps4* (DC3000-*AvrRps4*) was cultured on selective lysogeny broth (LB) agar plates at 30°C for 2 days. Bacterial colonies were scraped from plates and resuspended in 10 mM MgCl₂ to OD₆₀₀ = 0.001. The bacterial suspension was infiltrated into three rosette leaves of each 4-week-old *Arabidopsis* plant using a 1-ml needless syringe. After 24 h, leaves were harvested for further protein extraction and western blotting.

For *in planta* pathogen growth assays, DC3000 or DC3000-*AvrRps4* was resuspended in 10 mM MgCl₂ to OD₆₀₀ = 0.1 (containing 0.04% Silwet L-77) and the bacterial suspension was evenly sprayed on the rosette leaf surfaces of each 4-week-old *Arabidopsis* plant. Plants were covered with a lid to ensure high humidity, and after 3 days of incubation in a plant growth chamber, three leaf discs from each plant were harvested in 300 μl of 10 mM MgCl₂ as one sample and were ground using a 2010 Geno/Grinder. The mixture was serially diluted in 96-well plates with 10 mM MgCl₂ (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶), and 10 μl of each diluted sample was placed on LB agar plates containing the appropriate antibiotics. Bacterial colony-forming units (cfu) were calculated after incubation at 28°C for 2 days.

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Callose Deposition Assays

Callose deposition assays were performed as described previously (Jin and Mackey, 2017). The flg22 peptide (1 μ M) was infiltrated into three rosette leaves of each 4-week-old *Arabidopsis* plant for about 14–16 h. Aniline blue staining and fluorescent microscopy were used to measure callose deposition in flg22-challenged *Arabidopsis* leaves.

ACCESSION NUMBERS

All data are available from the corresponding authors upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

M.C., F.L., and Z.Q.F. designed experiments and analyzed data. M.C. carried out most of the experiments and prepared the figures. J.Z. demonstrated that PBS3 reduces the association of EDS1 with NPR3 and NPR4 in Co-IP assays. G.L. performed callose deposition assays. H.C., J.C., and M.L. provided assistance in nuclear protein extraction and yeast three-hybrid assays. M.C. and Z.Q.F. wrote the manuscript with the general assistance of I.A.P., J.S., J.R.A., and F.L.

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PBS3 Protects EDS1 from Degradation

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