EPSIN1 modulates the plasma membrane abundance of FLAGELLIN SENSING2 for effective immune responses

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Loss of a trans Golgi network clathrin adaptor impairs immunity to *Pseudomonas* bacteria and defense signaling, correlating with lower receptor kinase and co-receptor levels in the plasma membrane.

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The plasma membrane (PM) provides a critical interface between plant cells and their environment to control cellular responses. To perceive the bacterial flagellin peptide flg22 for effective defense signaling, the immune receptor FLAGELLIN SENSING2 (FLS2) needs to be at its site of function, the PM, in the correct abundance. However, the intracellular machinery that controls PM accumulation of FLS2 remains largely undefined. Arabidopsis (Arabidopsis thaliana) clathrin adaptor EPSIN1 (EPS1) is implicated in clathrin-coated vesicle (CCV) formation at the trans-Golgi Network (TGN), likely aiding transport of cargo proteins from the TGN for proper location; but EPS1’s impact on physiological responses remains elusive. Here, we identify EPS1 as a positive regulator of flg22-signaling and pattern-triggered immunity against Pseudomonas syringae pv. tomato (Pto) DC3000. We provide evidence that EPS1 contributes to modulating the PM abundance of defense proteins for effective immune signaling because in eps1, impaired flg22-signaling correlated with reduced PM accumulation of FLS2 and its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1). The eps1 mutant also exhibited reduced responses to the pathogen/damage-associated molecular patterns elf26 and AtPep1, which are perceived by the co-receptor BAK1 and cognate PM receptors. Furthermore, quantitative proteomics of enriched PM fractions revealed that EPS1 was required for proper PM abundance of a discreet subset of proteins with different cellular functions. In conclusion, our study expands the limited understanding of the physiological roles of EPSIN family members in plants and provides novel insight into the TGN-associated CCV trafficking machinery that impacts plant PM-derived defense processes.
INTRODUCTION

The plasma membrane (PM) serves as a central contact point between a cell and its environment. Proteins at this location perform critical functions to facilitate signal perception as well as initiation, amplification, and attenuation of responses. Overall, eukaryotic cells share similar strategies for perceiving extracellular stimuli; however, plants have independently evolved plant-specific PM proteins (Cock et al., 2002; Nurnberger et al., 2004) to cope with biotic and abiotic stimuli that are distinct from those pertinent to animals. For many biotic stresses, including immunity against the pathogenic flagellated bacteria *Pseudomonas syringae* pathovar *tomato* (*Pto*) DC3000, the cellular mechanisms and molecular machinery regulating the accumulation of plant PM proteins required for effective responses remain largely elusive. One strategy that plant cells utilize to control the protein composition of the host PM is protein cargo trafficking to and from the PM by vesicular trafficking [for review, see (Ben Khaled et al., 2015; Paez Valencia et al., 2016; Ekanayake et al., 2019)].

Vesicular trafficking involves the movement of cargo proteins in small vesicles from a donor to a target membrane. The *trans*-Golgi Network/Early Endosome (TGN/EE) has emerged as a central station for protein sorting to and from the plant cell surface (Gendre et al., 2015; Uemura, 2016). In contrast to animals, the plant TGN and EE functionally overlap and serve as a point of convergence for protein secretion, vacuolar trafficking, and endocytosis (Viotti et al., 2010; Gendre et al., 2015; Uemura, 2016). Protein sorting at the TGN/EE does not appear to occur via simple bulk-flow processes because different cargo proteins seem to utilize distinct trafficking routes with unique vesicle components (Nomura et al., 2011; Gu and Innes, 2012; Sauer et al., 2013; Gendre et al., 2015). The importance of the TGN/EE in plant immunity is underscored by the fact that mutations in genes encoding TGN/EE-resident proteins result in altered susceptibility to plant pathogens [for review, see (Uemura, 2016; Underwood, 2016; LaMontagne and Heese, 2017)]. Furthermore, pathogen effectors target TGN/EE-associated trafficking components to interfere with the plant’s ability to mount effective immunity (Mukhtar et al., 2011; Nomura et al., 2011; Gu and Innes, 2012; Wessling et al., 2014; LaMontagne and Heese, 2017). The plant TGN/EE also contributes to sorting of immune cargo proteins, such as pathogen-related (PR) proteins and proteins for callose deposition, to their correct subcellular
locations for effective immunity (Gu and Innes, 2011; Nomura et al., 2011; Gu and Innes, 2012; Wu et al., 2015).

Another cargo protein that has roles in plant defense and utilizes the TGN/EE for trafficking to and from the PM is the receptor kinase FLAGELLIN SENSING2 (FLS2) (Beck et al., 2012; Choi et al., 2013). FLS2 belongs to a class of pattern recognition receptors (PRRs) that serve as a first line of defense, in that PRRs act as sentinels to detect pathogen- or host-derived molecular patterns, termed microbial/pathogen-associated molecular patterns (M/PAMPs) or damage-associated molecular patterns (DAMPs), respectively (Xin and He, 2013; Couto and Zipfel, 2016; Yu et al., 2017). FLS2 forms a ligand-induced complex with BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) (Chinchilla et al., 2007; Heese et al., 2007), which serves as co-receptor for multiple, diverse PRRs and their cognate ligands, thereby linking perception of structurally diverse P/DAMPs to initiation of a conserved immune signaling network (Chinchilla et al., 2007; Roux et al., 2011; Tang et al., 2014; Yasuda et al., 2017).

FLS2 needs to be at the PM, so that it can utilize its extracellular leucine-rich repeat domain (Sun et al., 2013) to detect the bacterial PAMP flagellin or the active peptide-derivative flg22 (Gómez-Gómez and Boller, 2000; Robatzek and Wirthmueller, 2013; Yu et al., 2017). Perception of flg22 initiates a plethora of immune responses including the production of reactive oxygen species (ROS) as well as the activation of calcium-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), which either directly or indirectly alter the accumulation of thousands of transcripts as part of early, intermediate, and late signaling (Boutrot et al., 2010; Couto and Zipfel, 2016; Yu et al., 2017). These responses are not linear but rather fall into at least three different branches of the flg22-signaling network (Boudsocq et al., 2010; Korasick et al., 2010; Smith et al., 2014; Xu et al., 2014). Notably, absent or reduced levels of functional FLS2 compromises flg22-signaling (Zipfel et al., 2004; Boutrot et al., 2010; Mersmann et al., 2010). Increasing evidence shows that FLS2 requires vesicular trafficking to localize a) correctly to its site of function (the PM); and b) in the correct abundance to induce effective defense signaling [for review, see (Ben Khaled et al., 2015; Ekanayake et al., 2019)]. However, the identity and role(s) of TGN/EE-associated vesicle components that function in FLS2 trafficking to modulate flg22-signaling remain ill-defined.
In this study, we utilized cross-disciplinary approaches including immune assays, live-cell imaging, biochemical fractionation, and quantitative proteomics to identify Arabidopsis \textit{(Arabidopsis thaliana)} EPSIN1 (EPS1) as a modulator of plant immunity. More specifically, we provide evidence that EPS1 contributes to a) pattern-triggered immunity (PTI) against bacterial \textit{Pto} DC3000 strains and b) modulating the PM abundance of a finite set of proteins, including FLS2 and BAK1, for effective P/DAMP-responses. Arabidopsis EPS1, also called EpsinR1 (Lee et al., 2007), is a monomeric clathrin adaptor previously shown to localize to the TGN/EE and Golgi (Song et al., 2006). In animals and yeast, EPSINs and EPSIN-related proteins act as key components of vesicular trafficking by helping to recruit distinct clathrin coat, adaptor, and accessory proteins to either the PM or the TGN, ultimately to initiate budding of cargo-containing vesicles to send them to their target membranes (Duncan and Payne, 2003; Legendre-Guillemin et al., 2004; De Craene et al., 2012). Thus, our results offer novel insights into the clathrin-associated trafficking machinery at the TGN/EE that affects plant PM-derived immune responses.
RESULTS

EPS1 is required for robust Pattern-Triggered Immunity against *Pseudomonas syringae* strains

Previously, we have performed phosphoproteomic screens (Peck et al., 2001; Nuhse et al., 2003; Nuhse et al., 2007) to uncover vesicle components with novel roles in flg22-signaling and plant immunity (Kalde et al., 2007; Smith et al., 2014). Expanding on this work, we focus here on another phosphocandidate, namely the Arabidopsis clathrin adaptor EPS1 (*At5g11710*). To test whether EPS1 has roles in plant immunity against flagellated bacterial strains and/or flg22-signaling, we utilized a previously published Arabidopsis knock-down allele with the T-DNA insertion in its promotor region (Song et al., 2006) that we termed *eps1-1* (Supplemental Fig. S1A). In addition, we isolated a second, independent allele, *eps1-2*, with a T-DNA insertion between its 4th and 5th exons (Supplemental Fig. S1A). With two different affinity purified polyclonal peptide antibodies made against different epitopes in EPS1 (Supplemental Fig. S1B; α130 and α131), we detected reduced or no detectable levels of EPS1 protein in total protein extract from *eps1-1* (knockdown) or *eps1-2* (knockout), respectively, compared to Col-0 (wild type, WT) (Fig. 1A, Supplemental Fig. S1, C and D). Reduced levels of EPS1 protein in *eps1-1* had also been reported previously (Song et al., 2006). In accordance with Kalthoff et al (2002) for animal epsin 1 and Song et al. (2006) for Arabidopsis EPS1, EPS1 migrated more slowly with an apparent molecular weight of about 80 kDa when probing total protein extracts from seedlings with either α130 or α131 antibodies (Fig. 1A, Supplemental Fig. S1, C and D), thus more slowly than its expected molecular weight of about 60 kDa. In agreement with the antibodies made against EPS1 specific peptides, no additional cross-reacting protein bands were detected in WT or the two independent *eps1* mutant alleles on larger immunoblots (Supplemental Fig. S1, C and D). Except for slightly smaller leaves, *eps1* plants did not exhibit any gross developmental defects (Supplemental Fig. S1E).

With two independent *eps1* alleles in hand, we first monitored the growth of virulent, flagellated bacteria *Pto* DC3000 after syringe-infiltration into mature five-to-six week old leaves. Bacterial growth was assessed at 0 and 3 days post-infection (dpi) by bacterial dilution plating as described in (Korasick et al., 2010). At 0 dpi, no differences in bacterial levels were observed between *eps1* mutants and Col-0 plants (Fig. 1B), confirming that equal amounts of bacteria were initially delivered into leaves of the different plant lines. After 3 dpi, both *eps1* alleles...
exhibited increased growth of *Pto* DC3000 compared to Col-0 (Fig. 1B). Loss of *EPS1* also resulted in increased bacterial growth after infiltration with *Pto* DC3000 *hrcC* (Fig. 1C), a bacterial strain that elicits PAMP-signaling but is hypovirulent because its defective type III secretion system fails to suppress PTI (Xin and He, 2013). In conclusion, *eps1* mutant alleles showed increased susceptibility to both pathogenic (*Pto* DC3000) and non-pathogenic (*Pto* DC3000 *hrcC*) flagellated bacteria, with the latter indicating that EPS1 functions as a positive regulator in PTI. Importantly, similar results were obtained with two independent *eps1* mutant alleles confirming that the increased susceptibility to these *Pto* strains was specific to mutations in *EPS1*.

**EPS1 function contributes to P/DAMP-induced immune responses**

To investigate the basis of defective PTI in *eps1* mutants (Fig. 1C), we examined whether loss of *EPS1* resulted in altered molecular PTI responses. First, we assessed mRNA accumulation of early, intermediate, and late defense marker genes (Yu et al., 2017) in response to flg22 using reverse transcription quantitative polymerase chain reaction (RT-qPCR) in *eps1* mutant alleles compared to Col-0 (Fig. 2A-E). Because mutations in genes encoding vesicular trafficking proteins can affect at least three distinct branches of the flg22-signaling network differently (Korasick et al., 2010; Smith et al., 2014), we tested changes in expression of marker genes that represent those different branches. First, we focused on *PATHOGENESIS RELATED 1* (*PRI*), a late defense marker downstream of the plant hormone salicylic acid (SA), and the MAPK-dependent marker gene *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*). Both independent *eps1-1* and *eps1-2* mutant alleles showed reduced *PRI* (Fig. 2A) and *FRK1* (Fig. 2B) mRNA levels after flg22-elicitation providing evidence that it was indeed mutations in the *EPS1* gene that were responsible for impaired immune responses in these mutant plants (see also Fig. 1, B and C). Based on these results, we utilized the *eps1-2* null mutant allele in all subsequent assays. Loss of *EPS1* also resulted in decreased flg22-induced mRNA accumulation of the early MAPK-dependent marker genes *WRKY29* (Fig. 2C) and *WRKY33* (Fig. 2D), as well as the early CDPK-dependent marker gene *PHII* (Fig. 2E). Thus, *eps1* mutant plants showed attenuated flg22-induced accumulation of all transcripts tested. Furthermore, loss of EPS1 function led to defects in another early Ca$^{2+}$-dependent response, namely impaired reactive oxygen species (ROS) production, after flg22-elicitation (Fig. 2F).
Because loss or reduced levels of FLS2 function alone does not have a substantial effect on Pto DC3000 growth when these pathogenic bacteria are syringe-infiltrated for apoplastic infection (Supplemental Fig. S2A) (Zipfel et al., 2004; Boutrot et al., 2010; Mersmann et al., 2010), we tested next whether EPS1 functions in immune responses to unrelated P/DAMPs that are recognized by Arabidopsis PRRs other than FLS2. For these experiments, we focused on responses that represented early/Ca\(^{2+}\)-, intermediate/MAPK-, and late SA-dependent immune signaling branches using the eps1-2 null mutant allele. As was observed for responses to flg22, loss of EPS1 resulted in reduced ROS production (Fig. 3A) as well as PRI and FRK1 mRNA accumulation (Fig. 3B) after elicitation with elf26, the bacterial PAMP recognized by the host EF-TU RECEPTOR (EFR) (Zipfel et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). Similar results were obtained in response to the plant DAMP AtPep1 (Fig. 4, A and B), which is perceived by the PLANT ELICITOR PEPTIDE RECEPTOR1 (PEPR1) and PEPR2 (Zipfel et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010; Ortiz-Morea et al., 2016). Taking these results together, EPS1 is required for PTI signaling in response to multiple P/DAMPs, consistent with EPS1’s positive role in PTI.

**EPS1 is required for correct FLS2 abundance at the PM for effective flg22-response**

Signaling defects in early, intermediate, and late responses that represented the three branches of the immune signaling network (Figs. 2, 3 and 4) indicated a potential impairment at a very early point of defense signaling, such as at the level of the cognate PRRs. Because reduced steady-state mRNA levels of FLS2 can cause reduced flg22-responses due to reduced steady-state protein accumulation of FLS2 (Boutrot et al., 2010; Mersmann et al., 2010), we compared basal FLS2 mRNA and FLS2 protein levels in the eps1-2 null mutant to WT seedlings; but eps1-2 did not show any significant differences in mRNA accumulation of FLS2 (Supplemental Fig. S2B) or total cellular FLS2 protein (Fig. 5A; see also Fig. 6B). Similarly, no defects in the mRNA levels of EFR, PEPR1, and their co-receptor BAK1 were detected (Supplemental Fig. S2B).

Because CCVs are implicated in protein trafficking from the TGN/EE to the PM (Gendre et al., 2015), we examined whether loss of EPS1 affected FLS2 protein abundance at the PM, the site of flg22-perception. First, we utilized live-cell imaging via spinning disc confocal
microscopy (SDCM) (Smith et al., 2014; Leslie and Heese, 2017) to compare PM levels of
FLS2-GFP between eps1-2 null mutant and WT sibling seedlings expressing pFLS2::FLS2-myc-
egFP (FLS2-GFP) (Beck et al., 2012; Smith et al., 2014). First, we confirmed that FLS2-GFP
levels in total protein extracts were similar in eps1-2/FLS2-GFP and WT/FLS2-GFP (Fig. 5A).
However, when comparing PM intensity measurements using SDCM (Smith et al., 2014),
steady-state levels of FLS2-GFP at the PM in eps1-2/FLS2-GFP were reduced compared to
WT/FLS2-GFP (Fig. 5, B and C). We did not detect any obvious accumulation of FLS2-GFP in
defined intracellular puncta in eps1-2 compared to WT (Fig. 5B); but we observed an apparent
diffuse intracellular FLS2-GFP labeling (Fig. 5B), in agreement with increased intracellular pixel
intensity in eps1 over the wild type (Fig. 5D). Furthermore, the PM to intracellular ratio of
FLS2-GFP signals in eps1-2/FLS2-GFP was lower compared to that in WT/FLS2-GFP (Fig. 5E),
indicating that loss of EPS1 function resulted in greater intracellular accumulation of FLS2-
GFP. Note that seedlings used for the immunoblot shown in Fig. 5A were the same homozygous
eps1-2/FLS2-GFP and WT/FLS2-GFP seedlings imaged by SDCM and analyzed for FLS2-GFP
pixel intensities in the PM and intracellular areas (Fig. 5, B to E). EPS1 did not have any
apparent role in ligand-induced endocytosis of FLS2 because we did not observe any statistical
difference in the number of FLS2-GFP endosomal puncta between eps1-2/FLS2-GFP and
WT/FLS2-GFP seedlings (Supplemental Fig. S3) when using SDCM to quantify ligand-induced
endocytosis of FLS2-GFP (Smith et al., 2014; Leslie and Heese, 2017).

As an independent approach to assess the subcellular localization of FLS2, we used a
simple and rapid biochemical method to analyze the PM-associated accumulation of FLS2
protein in eps1-2 null mutant and WT seedlings. This method uses differential centrifugation and
the detergent Brij58 to enrich for PM proteins by depleting contaminating organelles (Zhang and
Peck, 2011; Zhang et al., 2013; Collins et al., 2017). Fractionation efficacy in whole seedling
samples was verified by immunoblot analyses of soluble (S), microsomal (M), and enriched PM
(ePM) subcellular fractions using compartment-specific antibodies (Fig. 6). ePM fractions from
whole seedlings were enriched for PM marker protein AHA H⁺-ATPases while showing reduced
levels of cytosolic (MPK6) and organellar marker proteins including the membrane ER
STEROL-METHYLTRANSFERASE (SMT1), plastid 75 KDA CHLOROPLAST
MEMBRANE TRANSLOCON (TOC75), plastid RIBULOSE BISPHOSPHATE
CARBOXYLASE (RuBisCo), and tonoplast VACUOLAR-ATPase (V-ATPase) (Fig. 6A).
Importantly, while no significant difference in FLS2 accumulation was observed between microsomal fractions, FLS2 levels were lower in ePM fractions of eps1-2 compared to WT (Fig. 6B). Thus, loss of EPS1 did not affect total FLS2 protein accumulation but resulted specifically in reduced levels of FLS2 at the PM.

Taking these results together, using two independent approaches, live-cell imaging (Fig. 5) and biochemical fractionation of ePM (Fig. 6B), we provided evidence that EPS1 contributes to correct FLS2 accumulation in the PM (its site of function), which in turn is necessary for effective flg22-signaling (Fig. 2).

**EPS1 modulates PM abundance of a distinct subset of proteins including BAK1 and BRI1**

To determine if other PM proteins are similarly affected by the absence of EPS1, we probed microsomal and ePM fractions for the FLS2 co-receptor, BAK1, using an αBAK1-specific affinity purified peptide antibody (Supplemental Fig. S4). We observed that BAK1 protein also accumulated to reduced levels in the enriched PM for eps1-2 mutant seedlings (Fig. 6C; ePM) while accumulating to similar levels in the microsomal fraction (Fig. 6C; M/P100). Because BAK1 is the co-receptor for a number of plant PRRs (e.g., FLS2, EFR, and PEPR1), we concluded that in eps1, reduced BAK1 protein accumulation in ePM may contribute to impaired flg22-, elf26-, and AtPepl-responses in eps1 (Figs. 2, 3, and 4, respectively). Similar to FLS2 and BAK1, BRASSINOSTEROID INSENSITIVE 1 (BRI1), the receptor kinase for brassinosteroid signaling and also a protein implicated in plant immunity (Albrecht et al., 2012; Belkhadir et al., 2012; Xiong et al., 2019), showed reduced protein accumulation in ePM but not in microsomal fractions of eps1 (Fig. 6, C and D). Notably, loss of EPS1 did not affect accumulation of all PM proteins with immune function because PM levels of AHA H+-ATPase (Elmore and Coaker, 2011) and SYNTAXIN OF PLANTS132 (SYP132) (Kalde et al., 2007) were not altered in eps1-2 (Fig. 6, B and D).

To obtain a more comprehensive overview of the extent of PM proteins affected by loss of EPS1, we combined ePM fractionation with quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) using previously established methodology (Zhang and Peck, 2011). The mass spectrometry data did not detect/or provide a clear quantification for the PRRs or for BAK1 (Supplemental Dataset S1). However, LC-MS/MS of ePM fractions from eps1-2 and WT
confirmed our immunoblot analyses of ePM (Fig. 6, C and D) that the PM abundance of BRI1 was reduced whereas SYP132 was not affected in *eps1*-2 (Table 1; Supplemental Dataset S1). Overall, the proteomic analysis revealed that only 1.7% of the detected ePM proteome was decreased in *eps1*-2 while the large majority of proteins remained unaffected (Supplemental Dataset S1; see also Table 1). Gene Ontology (GO) analysis of the ePM proteins reduced in *eps1* indicated that they did not segregate into any obvious, distinct structural, molecular function or protein class categories (Supplemental Fig. S5; Supplemental Dataset S2). More specifically, Fisher’s exact test with FDR correction indicated that no terms were over-represented relative to their representation in the whole Arabidopsis genome to a statistically significant degree.

In conclusion, our quantitative proteomics results implicate EPS1 in trafficking of a specific subset of proteins rather than globally regulating transport of all PM proteins, consistent with *eps1* plants exhibiting no gross developmental growth defects (Supplemental Fig. S1E).
DISCUSSION

The correct subcellular localization of proteins rather than their mere presence within a cell determines biological outcomes. Thus, delineating molecular components and cellular mechanisms that regulate the PM proteome is critical to our understanding of how eukaryotic cells interact with and respond to their environment. This work defines EPS1, the TGN/Golgi-associated clathrin adaptor previously implicated in vacuolar trafficking (Song et al., 2006), as a modulator in PM accumulation of FLS2 for effective flg22-responses. EPS1 is not the only known vesicle component with immune function reported to affect more than one trafficking pathway that originates from the TGN/EE. Notably, KEEP ON GOING (KEG), a TGN/EE-localized RING E3 ligase required for plant immunity against fungal Powdery Mildew (Gu and Innes, 2011; Gu and Innes, 2012), participates in regulating multiple endomembrane trafficking routes including cargo transport to both the vacuole and PM (Gu and Innes, 2012; Wu et al., 2015), with TGN/EE to PM trafficking potentially being clathrin-dependent (Wu et al., 2015).

Based on two independent techniques, namely live cell imaging using SDCM (Fig. 5) and biochemical enrichment of PM (Fig. 6), loss of EPS1 resulted in reduced accumulation of FLS2 in the PM. Consistent with having reduced but not abolished PM abundance of FLS2, eps1 null mutant plants displayed impairment rather than lack of flg22-signaling (Figs. 2 and 7). In eps1, the reduced PM accumulation of BAK1 (Fig. 6C) likely contributed to diminished flg22-signaling (Fig. 7). The fact that BAK1 also serves as the co-receptor for EFR (Chinchilla et al., 2007; Roux et al., 2011; Yasuda et al., 2017) and PEPR1/2 (Tang et al., 2014; Yasuda et al., 2017) may explain why elf26 and AtPep1-responses were compromised in eps1 mutants (Figs. 3, 4 and 7). Another possible explanation for the decreased elf26- and AtPEP1-signaling in eps1 is that in addition to FLS2, the PM accumulation of their cognate PRRs, namely EFR and PEPR1/2, may also be at least in part controlled by EPS1 (Fig. 7). In Arabidopsis, lack or reduced levels of FLS2 alone (Supplemental Fig. S2A), of individual other PRRs, or of their co-receptor BAK1 alone do not result in enhanced susceptibility to Pto DC3000 strains during apoplastic defenses (Zipfel et al., 2004; Zipfel et al., 2006; Nekrasov et al., 2009; Roux et al., 2011). Thus, the scenario, in which EPS1 governs PM abundance of multiple PRRs and/or other PM proteins with roles in plant immunity would be consistent with our findings that eps1 mutant plants supported increased growth of Pto DC3000 strains after syringe infiltration. Furthermore,
we cannot exclude the possibility that EPS1 has roles in trafficking of yet unknown protein(s) to
the PM and potentially the vacuole that may contribute to the plant’s ability to mount resistance
against \textit{Pto} DC3000 strains.

The fact that FLS2 (Fig. 5A and 6B) accumulated to similar levels in total protein extract
or microsomal fractions of \textit{eps1} null mutant and WT indicated that in \textit{eps1}, the reduced PM
accumulation of FLS2 was unlikely due to a general defect in protein expression or
accumulation. Furthermore, we did not detect any obvious difference in steady state \textit{FLS2}
mRNA expression between \textit{eps1} mutant and WT, suggesting that gene expression did not appear
to be the cause for the differences in PM protein accumulation between these genotypes. For
\textit{eps1}, the decrease in the PM accumulation of FLS2-GFP was accompanied by an increased
intracellular accumulation (Fig 5, D and E), which is consistent with the notion that EPS1
modulates FLS2 trafficking to the PM. Because EPS1 has been postulated to serve as a clathrin
adaptor for trafficking cargo proteins out of the TGN/EE (Song et al., 2006), an expected
consequence of loss of \textit{EPS1} may have been accumulation of a cargo protein, such as FLS2-
GFP, in TGN/EE-associated puncta. However, despite careful inspection of the \textit{eps1} SDCM
images, we did not detect any difference to WT in defined intracellular puncta accumulating
FLS2-GFP. We observed, however, an apparent increase in diffuse intracellular labeling of
FLS2-GFP. Because EPS1 has been localized to the TGN/EE as well as the Golgi apparatus
(Song et al., 2006), cargo proteins that require EPS1 function for effective accumulation in the
PM may be backed-up and distribute diffusely throughout the endomembrane and/or secretory
system in \textit{eps1} mutants. In the future, it will be interesting to determine where within the plant
cell FLS2 may accumulate in \textit{eps1} mutants.

\textit{Arabidopsis} EPS1 belongs to the ENTH/ANTH/VHS superfamily of proteins that span
across kingdoms, and its members contain an ENTH (EPSIN N-terminal homology), an ANTH
(AP180 N-terminal homology), or a VHS (Vps27, Hrs, and STAM) domain at their N-terminus
(Duncan and Payne, 2003; Legendre-Guillemin et al., 2004; De Craene et al., 2012). The
\textit{Arabidopsis} genome encodes 35 ENTH/ANTH/VHS domain family members, including six with
an ENTH-domain referred to as EPSIN1 to 6 (EPS1 to EPS6) (Holstein and Oliviusson, 2005;
Zouhar and Sauer, 2014). These six EPSs form a subfamily that is distinct from the divergent
ENTH-domain containing MODIFIED TRANSPORT TO THE VACUOLE (MTV1) with roles
in TGN/EE-to-vacuolar cargo trafficking (Sauer et al., 2013). The Arabidopsis ENTH-subfamily also clusters away from the large ANTH-protein family that includes EPSIN-LIKE CLATHRIN ADAPTOR4 (ECA4), PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN5A (PICALM5; also called ECA2), PICALM5b, and AP180, for which physiological roles have been recently described using loss-of-function mutants (Song et al., 2006; Lee et al., 2007; Sauer et al., 2013; Zouhar and Sauer, 2014; Muro et al., 2018; Nguyen et al., 2018; Kaneda et al., 2019). Thus, our identification of EPS1 modulating immune responses expands the short list of Arabidopsis ENTH/ANTH/VHS domain family members with known physiological functions.

In mammalian and yeast EPSINs, the ENTH domains participate in lipid-binding and protein-protein interaction for recruiting EPSINs to the TGN or PM and inducing membrane curvature. Furthermore, sequence divergence in the ENTH domain and differences in peptide/domain architecture are indicative of diverse cellular functions, including the recruitment of different cargo, coat, adaptor, and accessory components to distinct subcellular compartments [for review, see (Ford et al., 2002; Duncan and Payne, 2003; Mills et al., 2003; Legendre-Guillemin et al., 2004; Itoh and De Camilli, 2006; Lee et al., 2007)]. When expanding on previous studies (Holstein and Oliviusson, 2005; De Craene et al., 2012; Zouhar and Sauer, 2014), we found that Arabidopsis EPS1 shares relatively high amino acid sequence identity as well as similar domain and motif architecture with EPS1 orthologs from flowering plants (angiosperms) (Supplemental Fig. S6; 50-88% identity), implicating similar cellular functions. In addition to a highly conserved ENTH domain, EPS1 and its orthologs shared the same peptide motif architecture that include a confirmed (LIDL) (Song et al., 2006) and a putative (LADV) clathrin-interacting motif as well as three putative α-adaptin-binding motifs (DPF), of which two appear relatively well-conserved (Supplemental Fig. S6). Such similarities point toward potentially similar cellular roles of EPS1 orthologs across plant species. We also observed a C-terminal poly-glutamine (polyQ) stretch that is only present in EPS1 orthologs of the Brassicaceae family but not in other plant EPS1 orthologs and paralogs (Supplemental Figs. S6, S7, and S8). Although the function of this domain is unknown for Brassicacea EPS1s, polyQ’s have been implicated in protein aggregation and cell toxicity contributing to neurodegenerative disease in animals (Adegbuyiro et al., 2017; Silva et al., 2017). In contrast, EPS1 shares a less
conserved ENTH domain and differs in peptide motifs and domain architecture with the five
Arabidopsis paralogs (Supplemental Fig. S7; 20-38%), as well as with the animal or yeast
EPSINS (Supplemental Fig. S8; 26-30%) with trafficking roles from the TGN (Legendre-
Guillemin et al., 2004). Arabidopsis EPS2 (also referred to as EpsinR2) (Lee et al., 2007) and
EPS3 share a degenerate LADV motif with EPS1 but lack DPF or LIDL peptide motifs; and the
other three Arabidopsis ENTH proteins are comprised of little more than a divergent ENTH
domain (Supplemental Fig. S7). We propose that the low sequence similarity and diverse peptide
motif structure may indicate distinct cellular and/or physiological functions of the Arabidopsis
EPSINs.

So far, it remains unknown how Arabidopsis EPS1 or related family members may
recognize cargo proteins and whether such recognition occurs through direct or indirect
interaction(s). In support of the latter, EPS1 interacts with γ-adaptin-related protein (γ-ADR) and
VACUOLAR SORTING RECEPTOR1 (VSR1) (Song et al., 2006), both of which belong to
protein families with cargo binding properties (Ahmed et al., 2000; daSilva et al., 2005); but so
far, the physiological role(s) of these protein-protein interactions have not been elucidated. In
mammals and yeast, two different types of EPSINs localize to two distinct locations, the PM and
the TGN. EPSINs that localize to the PM generally contain a C-terminal ubiquitin interacting
motif (UIM) that enables interaction with ubiquitylated cargoes to target these cargoes for
endocytic degradation (Hawryluk et al., 2006; Dores et al., 2009; Chen et al., 2011). In contrast,
TGN-localized mammalian EpsinR or yeast EPSIN Ent3p lack a C-terminal UIM (Duncan and
Payne, 2003). By these distinctions and consistent with EPS1’s localization to the TGN/EE
(Song et al., 2006), Arabidopsis EPS1 more closely resembles the TGN-type in that it also lacks
a UIM (Holstein and Oliviusson, 2005; Zouhar and Sauer, 2014) (Fig. 1 and 2B). Thus, it is
unlikely that EPS1 recognizes ubiquitylated cargo proteins directly. This scenario, however, does
not eliminate the possibility that EPS1 may recruit yet unknown UIM-containing protein(s) for
cargo recognition (Isono et al., 2010).

In conclusion, by identifying novel functions for Arabidopsis EPS1 in plant immunity
against bacteria and modulating FLS2 abundance in the PM for effective flg22-responses, we
advanced the limited information available for plant EPSINs and ENTH/ANTH/VHS
superfamily members and their roles in physiological responses. Beyond FLS2 and the
multitasking co-receptor BAK1, quantitative comparison between the ePM proteome of eps1
mutant and WT identified BRI1 and a small subset of structurally diverse PM proteins that
require EPS1 for their correct PM accumulation, implicating EPS1 in contributing to potential
cellular functions in addition to plant immunity. Because EPS1 serves as a monomeric clathrin
adaptor involved in trafficking from the TGN/EE, analyses of EPS1 may aid in uncovering novel
function(s) for clathrin at the TGN/EE that are distinct from those at the PM.

MATERIALS AND METHODS
Plant material and growth conditions
T-DNA insertion lines Arabidopsis (Arabidopsis thaliana) eps1-1 (SALK_049204) (Song et al.,
2006) and eps1-2 (SAIL_394_G02) (this study) were obtained from the Nottingham Arabidopsis
Stock Centre (NASC) or the Arabidopsis Biological Resource Center (ABRC) in The Ohio State
University, respectively. bak1-4 (SALK_034523) and fls2Δ (SALK_093905) were previously
published (Heese et al., 2007). All mutants were in Arabidopsis ecotype Columbia (Col-0)
background. Plants were genotyped for homozygosity using allele-specific primers
(Supplemental Table S1). eps1-2/FLS2-GFP plants were generated by crossing eps1-2 with
FLS2pro:FLS2-3xMyc-EGFP (Beck et al., 2012; Smith et al., 2014) and genotyped using
primers (Supplemental Table S1). Surface-sterilized seeds were grown on 0.5x MS medium +
1% (w/v) sucrose solidified with 0.6% (w/v) agar at 22°C under continuous light as described
(Korasick et al., 2010). After transplanting of seedlings, plants were grown in individual pots at
22°C with an 8-hour light/16-hour dark photoperiod at 82 μmol m⁻² s⁻¹. Except when noted,
seven to eight-day old seedlings or fully expanded rosette leaves from five-to-six week-old
plants were used for assays as described (Beck et al., 2012; Smith et al., 2014).

P/DAMP Peptides
The peptides flg22 (QRLSTGSRINSAKDDAAGLQIA), elf26 (SKEKERTKPHNVGTVGHVHGKTT), and AtPep1
(ATKVAKQRGKEKVSSGRPGQHN) were synthesized by Genscript and used for elicitation
at indicated concentrations and for indicated times as described.
Gene structure and protein domain structure

Schematics for gene and protein domain structures were created using GSDS 2.0 (Hu et al., 2015) and IBS 1.01 (Liu et al., 2015), respectively.

Bacterial pathogen infections

Syringe-infiltration with *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 *lux* (OD₆₀₀=0.0005) or *Pto* DC3000 *hrcC* (OD₆₀₀=0.02), collection of leaf discs, and quantification of bacterial growth using serial dilution plating was done as described previously (Korasick et al., 2010), except that leaf discs were ground in 100 µl distilled water/leaf disc before serial dilution plating.

RNA isolation and reverse transcription quantitative PCR

RNA isolation, cDNA synthesis, and reverse transcription quantitative PCR (RT-qPCR) were performed as previously described (Libault et al., 2007; Korasick et al., 2010; Anderson et al., 2011). Unless stated otherwise for each sample, three to five seedlings were elicited with the indicated concentration of P/DAMP and placed at 22°C. For *PR1*, leaves of five- to six-week-old plants were syringe infiltrated with the indicated concentration of P/DAMP, allowed to dry, and placed at 22°C. Tissue was flash-frozen in liquid nitrogen at indicated time points. Total RNA was isolated from tissue using Trizol Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. RT-qPCR was performed on cDNA using a 7500 Realtime PCR system (Applied Biosystems) using gene-specific primers (Supplemental Table S1) and normalized to the reference SAND gene (*At2g28390*) (Libault et al., 2007).

Apoplastic ROS production

Luminol-based ROS production was performed as previously described (Leslie and Heese, 2014) with the following modifications. Seedling cotyledons or leaf discs (1.5cm²) from 5-6 week old plants were cut in halves and placed into a 96-well plate for subsequent elicitation with P/DAMP peptides at the indicated concentrations. All ROS experiments shown in the same panel were performed at the same time in a single 96-well plate to allow for direct comparison.
Spinning disc confocal microscopy (SDCM) for quantification of plasma membrane and cytoplasmic intensity, and ligand-induced endocytosis of FLS2-GFP

SDCM was performed as described (Smith et al., 2014; Leslie and Heese, 2017). Briefly, Col-0 FLS2-GFP and eps1-2 FLS2-GFP seedlings were directly mounted in distilled water for imaging. FLS2-GFP was imaged in the epidermal pavement cell layer of the adaxial (top) cotyledon surface. For each experiment, at least 12 seedlings were imaged per genotype, with two fields of view/cotyledon/genotype using a custom Olympus IX-71 inverted microscope (Center Valley, PA) equipped with a Yokogawa CSU-XI 5000 rpm spinning disc unit (Tokyo, Japan), Andor iXon Ultra 897 High Speed EMCCD camera (Belfast, United Kingdom), PZ-2000 XYZ series automated stage with Piezo Z-axis top plate (Applied Scientific Instrumentation; Eugene OR), and a 60x-silicon oil objective (Olympus UPlanSApo 60x/1.30 Sil). GFP was excited with a Spectra Physics 488-nm diode laser (Santa Clara, CA), and fluorescence was collected through a series of Semrock Brightline 488-nm single-edge dichroic beam-splitter and 500-550 nm bandpass filters (Rochester, NY). Camera exposure time was set to 150 msec.

PM intensity measurements were performed as previously described (Smith et al., 2014). In brief, single z-plane images of epidermal pavement cells were captured independently using consistent experimental and imaging parameters across all samples. Brightness and contrast was adjusted consistently between all images using Fiji software. Bright sections of the PM were selected using the Oval Selection tool and analyzed for mean pixel intensity using ImageJ (https://imagej.net/Fiji/Downloads). FLS2-GFP PM intensity for each image was calculated as the average value of the pixel intensity measurements from four selected regions. For each genotype, FLS2-GFP PM intensities are reported relative to the FLS2-GFP PM intensity of Col-0 FLS2-GFP.

For quantifying intracellular compared to PM pixel intensity, single-plane confocal images from seven day old seedling cotyledons were analyzed for pixel intensity using ImageJ (https://imagej.net/Fiji/Downloads) as performed previously (Kitakura et al., 2011; Nguyen et al., 2018). Briefly, the Oval Selection tool was used to select regions of the cell cytoplasm adjacent to the PM and measure the mean pixel intensity. A quotient was calculated using these cytoplasmic values and the PM measurements before normalizing to the average WT control for each dataset.
FLS2-GFP endocytosis experiments were performed as described previously (Smith et al., 2014; Leslie and Heese, 2017). Z-stacks were collected from the adaxial surface of cotyledons in response to 1 μM flg22 in water for either 0 or 50-60 minutes as described above. Images were displayed as maximum intensity projections (MIPs) using ImageJ before brightness and contrast were adjusted uniformly. Stomata were removed from each image using the Freehand Selection Tool before detecting FLS2-GFP puncta using the Advanced Weka Segmentation plug-in from Fiji. Briefly, a single MIP with visible puncta was used to create a “Vesicle Classifier” that was applied to all other MIPs in an experiment. Puncta were analyzed in the resulting binary images using the Analyze Particles selection in Fiji with the following parameters: particle size = 0.25-2.5 μm² and circularity = 0.25-1.00. Each MIP image was manually inspected for accuracy and if necessary, adjustments were made to particle detection.

**Protein sample preparation, immunoblot analysis, and antibodies**

Sample preparation and immunoblot analysis of total, soluble, and microsomal proteins were performed as previously described (Heese et al., 2007; Smith et al., 2014; LaMontagne et al., 2016) with 30 μg loaded per well for seedling extracts. Affinity purified, peptide-specific antibodies were produced in rabbit against EPS1 (#130, CSLSNQRYQSGGFQ; #131, CFADSKPQLQKGDIP; Eurogentec) or BAK1 (CRQDFNYPTHHPAVS; Sigma Genosys) according to manufacturers’ specifications. The following antibody dilutions were used: 1:2500 αEPS1#130 (this study), 1:2500 αEPS1#131 (this study), 1:500 αBAK1 (this study), 1:500 αBR1 (kind gift of Marisa Otegui (Wu et al., 2011)), 1:3000 αMPK6 (Merkouropoulos et al., 2008), 1:500 αFLS2 (Heese et al., 2007), 1:3000 αGFP (JL-8; Clontech Laboratories), 1:3000 αSYP132 (Kalde et al., 2007), 1:10,000 αAHA (AS07 260; Agrisera), 1:1000 αSMT1 (AS07 266; Agrisera), 1:3000 αCNX1/2 (AS12 2365; Agrisera), 1:1000 αV-ATPase (AS07 213; Agrisera), 1:1000 αToc75 (AS06 150; Agrisera).

**Plasma membrane enrichment from seedlings**

Enrichment of plasma membranes (ePM) from whole seedlings were performed as described (Collins et al., 2017). Briefly, approximately 140 seedlings/genotype were homogenized in buffer H (250 mM sucrose, 50 mM HEPES-KOH pH 7.5, 5% v/v glycerol, 50 mM NaPP, 1 mM Na₂MoO₄, 25 mM NaF, 10 mM EDTA, 0.5% w/v polyvinyl pyrrolidone). Homogenate was
filtered through two layers of miracloth and centrifuged for 10 min at 8,000 x g. Supernatant was
then ultracentrifuged for 30 min at 100,000 x g to obtain the microsomal pellet. Pellets were
resuspended in buffer H and microsomal protein was quantified. Microsomal protein was then
incubated for 30 min on ice with 0.02% (w/v) Brij-58 at a protein-to-detergent ratio of 2 μL of
0.02% (w/v) Brij-58 solution per 1 μg microsomal protein followed by centrifugation at 100,000
x g. This Brij-58 incubation and centrifugation step was repeated a second time. Pellets were
then washed with buffer H (no detergent) followed by centrifugation at 100,000 x g. Final pellets
were resuspended for immunoblot analysis.

Quantitative plasma membrane proteomics
Plasma membrane proteomics was performed as described (Zhang and Peck, 2011; Zhang et al.,
2013). Briefly, equal amounts of PM-enriched proteins from each genotype were separated using
8% SDS-PAGE. Each gel lane was cut into 20 slices, proteins in each slice were digested using
trypsin, extracted, and lyophilized. Peptides were resuspended in 1% (v/v) formic acid before
analysis using an LTQ-Orbitrap LC-MS/MS mass spectrometer (Thermoelectron Corp.,
Rockford, IL) controlled by XCalibur v. 2.2.1. Mass spectrometer measurements were obtained
(Zhang and Peck, 2011; Zhang et al., 2013). Mascot distiller 2.0 (Matix Science, London, UK)
was used to de-convolute the mass spectra, and then Mascot (server ver. 2.3 Matrix Science,
London, UK) and X! Tandem (ver. 2007.01.01.1) were used to identify peptides. Scaffold
software (ver. 4.0 Proteome Software, Portland, OR, USA) was used to assign peptide identities
at a greater than 95% probability for a given peptide (Keller et al., 2002). Proteins were
identified when at least two peptides (at greater than 99% probability) were found (Nesvizhskii
et al., 2003). Normalized spectral counts for each genotype were determined using Scaffold
Software (ver. 4.0 Proteome Software, Portland, OR, USA). For each genotype, the mean and
standard deviation were calculated for the spectral counts from each biological replicate. For
each experiment, proteins for which the fold difference between genotypes was greater than 1-
2X the coefficient of variance (CV; CV = standard deviation/mean) were considered changing.
For replicate 1 CV= 1.20, replicate 2 CV= 1.06, and for replicate 3 CV= 1.43.

Bioinformatic analysis
The PANTHER classification system (version 13.1 released 2018-02-03) was used to categorize protein hits into Molecular Function and Protein Class using GO-Slim functional classification tools (Thomas et al., 2003). To determine if any categories were enriched relative to their representation in the whole Arabidopsis thaliana genome, a statistical overrepresentation test was performed (PANTHER, released 20171205), specifically Fisher's Exact test with a False Discovery Rate (FDR) multiple test correction. Categories with an FDR <0.05 were considered statistically significant. Structural classifications were as annotated in UniProt and TAIR.

**Sequence alignment**

Orthologous or paralogous sequences were determined using the amino acids 27-152 of Arabidopsis EPS1 (denoted as AthEPS1 in Supplemental Figs. S6 to S8) for UniProt Basic Local Alignment Search Tool (BLAST). Full-length amino acid sequences were aligned using the Clustal Omega Multiple Sequence Alignment tool. Sequence conservation compared to AthEPS1 was annotated in ExPASy Boxshade using AthEPS1 as the consensus sequence. The fraction of sequences that must agree for shading was set to 0.1.

**Statistical Analysis**

For each experiment, mutant samples were compared to Col-0 wild type (WT) samples, and statistical analyses were performed using n values with each n representing a biological sample as detailed in each figure legend. Statistical significance was based on unpaired two-tailed Student’s t-test determined using GraphPad Prism 4.03 or GraphPad Prism 7.02 Software. Graphpad QuickCalcs outlier calculator (https://www.graphpad.com/quickcalcs/Grubbs1.cfm) with the Alpha = 0.05 (standard) was used to identify outliers. Unless stated otherwise, each experiment represents a biological replicate and was performed at least three independent times with similar results.

**Accession numbers**

*EPSIN1* (At5g11710), *FLS2* (At5g46330), *BAK1* (At4g33430), *EFR* (At5g20480), *PEPR1* (At1g73080), *BRI1* (At4g39400).

**SUPPLEMENTAL DATA**
Supplemental Fig. S1. Isolation and confirmation of eps1 mutant alleles.

Supplemental Fig. S2. Increased susceptibility of eps1-2 null mutant to Pto DC3000 compared to WT and fls2Δ mutant and no difference in mRNA accumulation of PRRs and BAK1.

Supplemental Fig. S3. Ligand-induced endocytosis of FLS2-GFP is not affected in eps1-2 null mutant.

Supplemental Fig. S4. BAK1 antibody specificity.

Supplemental Fig. S5. Proteins reduced in eps1-2 enriched plasma membrane (ePM) fraction do not segregate into any distinct biological or structural categories.

Supplemental Fig. S6. EPS1 is conserved across plant species.

Supplemental Fig. S7. Protein sequence alignment of ENTH-domain containing proteins from Arabidopsis.

Supplemental Fig. S8. Protein sequence alignment of Arabidopsis EPS1 with orthologous TGN localized ENTH-proteins from non-plant species.

Supplemental Table S1. Primer information.

Supplemental Dataset 1. Quantitative Proteomics.

Supplemental Dataset 2. GO term & structure analysis.

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The authors declare no conflict of interest.
Table 1. Representative list of proteins with decreased and similar PM abundance in eps1-2 compared to Col-0 (WT) measured by quantitative mass spectrometry.

| Protein ID                                      | Gene Identifier | Expected Size (kDa) | Average Log2 Fold Change | Predicted Localization |
|------------------------------------------------|-----------------|---------------------|--------------------------|------------------------|
| ABCB14: ABC Transporter Member 14              | At1g28010       | 136                 | -2.581                   | PM                     |
| BRI1: Brassinosteroid Insensitive 1            | At4g39400       | 131                 | -2.497                   | PM                     |
| FLA1: Fasciclin-like Arabinogalactan           | At5g55730       | 45                  | -2.931                   | PM                     |
| PDR9: Pleiotropic Drug Resistance 9            | At3g53480       | 164                 | -4.684                   | PM                     |
| S-lectin Protein Kinase                        | At1g11330       | 94                  | -2.579                   | PM                     |
| ACA11: Autoinhibited Ca^{2+} - ATPase 11       | At3g57330       | 112                 | -0.179                   | PM                     |
| LRR-RLK: Leucine-Rich Repeat Receptor-like Kinase | At2g26730       | 72                  | -0.008                   | PM                     |
| PIP2A: PM Intrinsic Protein 2A                 | At3g53420       | 30                  | 0.319                    | PM                     |
| SYP121: Syntaxin of Plants 121/PEN1            | At3g11820       | 38                  | -0.294                   | PM                     |
| SYP132: Syntaxin of Plants 132                 | At5g08080       | 34                  | -0.029                   | PM                     |

Figure 1. eps1 mutants are hypersusceptible to Pseudomonas syringae pv tomato (Pto) DC3000 and Pto DC3000 hrcC infection.

A, Using two different affinity purified peptide antibodies (α1 30 and α131) made against different EPS1 epitopes (see Supplemental Fig. S1B), immunoblot analyses of total protein seedling extract indicate eps1-1 (e1-1) is a knock-down and eps1-2 (e1-2) is a null mutant compared to Col-0 (wild type, WT). Molecular size markers are indicated in kDa. B and C, Leaves of 5-week-old eps1 mutant or Col-0 (wild type, WT) plants were syringe-infiltrated with Pto DC3000 (OD_{600}= 0.0005) (B) or Pto DC3000 hrcC (OD_{600}= 0.02) (C). Bacterial growth was assessed by serial dilution plating as colony-forming units (cfu) at 0 and 3 days post-infection (dpi). n = 8 samples/genotype,—with each n representing a biological sample taken from an individual leaf from 4 different plants for each genotype and timepoint. Values are means ± SE. Asterisks indicate statistically significant differences compared to WT of same dpi by two-tailed.
Student’s t-test: $P^{**} < 0.01$; $P^{***} < 0.001$; ns = no statistically significant difference. Experiments were performed at least three times with similar results. OD, optical density.

Figure 2. EPS1 is required for robust flg22 signaling.

A, Leaves of 5-to-6-week-old eps1 mutant or Col-0 (wild type, WT) plants were syringe infiltrated with 1 µM flg22 for 0 or 24 hours (h). Using RT-qPCR, relative mRNA levels of PR1 were measured and normalized to the reference gene At2g28390. n = 6 samples/genotype/treatment, with each biological sample (n) consisting of 3 leaf discs taken from 4-6 different plants for each genotype and treatment. B to E, 8-day-old seedlings were treated with 10 nM flg22 for indicated time in hours (h). Using RT-qPCR, relative mRNA levels of FRK1 (B), WRKY29 (C), WRKY33 (D), and PHI1 (E) were measured and normalized to the reference gene At2g28390. For each genotype and treatment, n = 3 biological samples, with each sample consisting of 3 to 4 seedlings. F, Time-course (left) and peak (right) of flg22-triggered (10 nM) ROS production over 30 minutes (min) from leaves of 5-to-6-week-old plants. n = 10 samples/genotype/treatment, which each sample (n) consisting of a leaf disc half taken from 3-4 individual plants. Peak flg22-triggered reactive oxygen species (ROS) production (right) is shown as example for statistical analysis and represents data from same time-course experiment (left). RLU, relative light unit. For all experiments, values are means ± SE. Asterisks indicate statistically significant differences compared to wild type by two-tailed Student’s t-test: $P^{*} < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$; $P^{****} < 0.0001$; ns = no statistically significant difference. All experiments were performed at least three times with similar results.

Figure 3. EPS1 is required for robust elf26 signaling.

A, Time-course and peak for elf26-triggered (10 nM) apoplastic ROS production from leaves of 5-to-6-week-old plants for eps1-2 (■) and Col-0 (wild type, WT; □). n = 15 samples/genotype/treatment with each sample (n) consisting of one leaf disc half taken from 3-4 individual plants for each genotype and treatment. B, For FRK1 mRNA accumulation, 8-day-old seedlings were treated with 100 nM elf26 for indicated time in hours (h). n = 3 to 4 samples/genotype/treatment, with each biological sample (n) containing 3 to 4 seedlings. For PR1 mRNA accumulation, leaves of 5-to-6-week old plants were infiltrated with 1 µM elf26. n = 6 samples/genotype/treatment. Each sample (n) represents 3 leaf discs taken from 3-4 different
plants for each genotype and treatment. Relative mRNA levels of \textit{FRK1} and \textit{PR1} were measured and normalized to the reference gene \textit{At2g28390}. Values represent means ± SE. For all experiments, \textit{eps1-2} (■) and Col-0 (wild type, WT; □). Asterisks indicate statistically significant differences compared to wild type by two-tailed Student’s t-test: $P^* < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$; $P^{****} < 0.0001$. All experiments were performed at least three times with similar results.

**Figure 4.** EPS1 is required for robust \textit{AtPep1} signaling.

A, Time-course and peak for \textit{AtPep1}-triggered (10 nM) apoplastic ROS production from 8-day-old seedlings. n = 20 samples/ genotype/treatment with each sample (n) consisting of one cotyledon half obtained from 5 or more individual seedlings for each genotype and treatment. Experiment was performed twice in seedlings and twice in leaf discs with similar results. B, For \textit{FRK1} mRNA accumulation, 8-day-old seedlings were treated with 100 nM \textit{AtPep1} for indicated time in hours (h). n = 3 to 4 samples/genotype/treatment, with each biological sample (n) containing 3 to 4 seedlings. For \textit{PR1} mRNA accumulation, leaves of 5-to-6-week old plants were infiltrated with 1 μM \textit{AtPep1} for indicated time. n = 6 samples/genotype/treatment, with each sample (n) containing 3 leaf discs taken from 3-4 different plants for each genotype and treatment. Relative mRNA levels of \textit{FRK1} and \textit{PR1} were measured and normalized to the reference gene \textit{At2g28390}. Values represent means ± SE. For all experiments, \textit{eps1-2} (■) and Col-0 (wild type, WT; □). Asterisks indicate statistically significant differences compared to wild type by two-tailed Student’s t-test: $P^* < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$; $P^{****} < 0.0001$. Unless specified otherwise, all experiments were performed at least three times with similar results.

**Figure 5.** Loss of \textit{EPS1} results in reduced plasma membrane and increased intracellular accumulation of FLS2-GFP.

A, In total protein extracts, \textit{eps1-2} \textit{FLS2-GFP} accumulated FLS2-GFP to similar levels as wild type Col-0 \textit{FLS2-GFP}. Total proteins were extracted from Col-0 (wild type, WT) and \textit{eps1-2} (\textit{e1-2}) 7-day-old seedlings that did (+) or did not (-) express \textit{pFLS2::FLS2-GFP} and subjected to immunoblot analysis with indicated antibodies. Col-0 \textit{FLS2-GFP} and \textit{eps1-2} \textit{FLS2-GFP} were from the same homozygous F4 seedlings used for SDCM and pixel intensity analyses in (B) to (E). Antibody against FLS2 detected both endogenous FLS2 (open arrow) and FLS2-GFP.
(closed arrow); antibody against green fluorescent protein (GFP) identified FLS2-GFP (closed arrow); antibody against EPS1 confirmed *eps1-2* mutant; and antibody against Calnexin (CNX, ER membrane marker) served as loading control. B, Representative single z-plane images and zoomed insets (10 x 10 μm in size) for WT *FLS2-GFP* and *eps1-2* (*e1-2*) FLS2-GFP cotyledons using SDCM. Scale bars = 10 μm. C, D, and E, Quantification of FLS2-GFP pixel intensity from selected regions of the PM (C), in the intracellular region (D) and as a PM/ intracellular ratio (E) for WT *FLS2-GFP* (WT, □) and *eps1-2* FLS2-GFP (*e1-2*; ■). Data for *e1-2* are normalized to WT with *n* = 48 images from 24 seedlings/genotype. Values are means ± SE. Asterisks indicate statistically significant differences compared to WT by two-tailed Student’s t-test: *P*** < 0.0001. All experiments were performed three times with similar results.

**Figure 6.** Loss of EPS1 results in reduced protein levels of FLS2, BAK1, and BRI1 in enriched PM fractions but not in microsomal fractions.

A, Efficacy of plasma membrane (PM) enrichment in Col-0 (WT) seedlings using immunoblot analysis. Soluble (S100), microsomal (M/P100), and enriched PM (ePM) fractions were probed for the following subcellular marker proteins: AHA (PM), MPK6 (cytosol), Toc75 (chloroplast), V-ATPase (tonoplast), SMT1 (ER membrane), and PoncS (Ponceau S, general protein dye). PoncS shows staining for stromal RuBisCo (known to be partly released during cell lysis into soluble fraction S100) (asterisk). B, Immunoblot analysis of MP100 and ePM proteins from *eps1-2* (*e1-2*) and WT using antibodies against FLS2. AHA (PM) and SMT1 (ER) serve as PM enrichment and ER depletion markers, respectively. C, Immunoblot analysis of S100, M/P100, and ePM proteins from *e1-2* and WT probed with antibodies against BAK1 and BRI1. Calnexin 1/2 (CNX1/2) and MPK6 served as ER membrane and soluble marker proteins, respectively. D, Immunoblot analysis of SYP132 and BRI1 (both PM proteins) for *eps1-2* (*e1-2*) and WT. CNX1/2 and PoncS served as loading controls. All experiments were performed in 7-8-day old seedlings/genotype and were repeated at least three times with similar results. For (A-D), PoncS served as loading control and showed depletion of RuBisCo (asterisk) in ePM. Stippled lines indicate immunoblots derived from the same gel.

**Figure 7.** Model depicting EPS1-dependent immune signaling defects due to reduced PM levels of pattern recognition receptors (PRRs) and BAK1.
Loss of the TGN/EE-associated clathrin adaptor EPS1 perturbs PM abundance of FLS2 (the PRR for flg22) and its co-receptor, BAK1. Consistent with reduced pre-existing levels of FLS2 and BAK1 protein, eps1 mutants display decreased immune responses upon elicitation with flg22. Based on impaired elf26 and AtPep1 responses, EPS1 may regulate PM abundance of other PRRs including EFR and PEPR1, respectively. EPS1 also impacts a small subset of additional PM proteins with immune and other cellular functions (not depicted in this model).
Figure 1. *eps1* mutants are hypersusceptible to *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000 and *Pto* DC3000 *hrcC* infection. A, Using two different affinity purified peptide antibodies (α130 and α131) made against different EPS1 epitopes (see Supplemental Fig. S1B), immunoblot analyses of total protein seedling extract indicate *eps1-1* (*e1-1*) is a knock-down and *eps1-2* (*e1-2*) is a null mutant compared to Col-0 (wild-type, WT). Molecular size markers are indicated in kDa. B and C, Leaves of 5-week-old *eps1* mutant or Col-0 (wild-type, WT) plants were syringe-infiltrated with *Pto* DC3000 (OD₆₀₀ = 0.0005) (B) or *Pto* DC3000 *hrcC* (OD₆₀₀ = 0.02) (C). Bacterial growth was assessed by serial dilution plating as colony-forming units (cfu) at 0 and 3 days post-infection (dpi). n = 8 samples/genotype, with each n consisting of 3 discs from 3 independent leaves, with each n representing a biological sample taken from an individual leaf from 4 different plants for each genotype and timepoint. Values are means ± SE. Asterisks indicate statistically significant differences compared to WT of same dpi by two-tailed Student’s t-test: $P^{**} < 0.01; P^{***} < 0.001; ns = no statistically significant difference. Experiments were performed at least three times with similar results. OD, optical density.
Figure 2. EPS1 is required for robust flg22 signaling. A, Leaves of 5-to-6-week-old eps1 mutant or Col-0 (wildtype, WT) plants were syringe infiltrated with 1 µM flg22 for 0 or 24 hours (h). Using qRT-PCR, relative mRNA levels of PR1 were measured and normalized to the reference gene At2g28390. n = 6 samples/genotype/treatment, with each biological sample (n) consisting of 3 leaf discs taken from 4-6 different plants for each genotype and treatment. B, to E, 8-day-old seedlings were treated with 10 nM flg22 for indicated time in hours (h). Using qRT-PCR, relative mRNA levels of FRK1 (B), WRKY29 (C), WRKY33 (D), and PHI1 (E) were measured and normalized to the reference gene At2g28390. For each genotype and treatment, n = 3 biological samples, with each sample consisting of 3 to 4 seedlings. F, Time-course (left) and peak (right) of flg22-triggered (10 nM) ROS production over 30 minutes (min) from leaves of 5-to-6-week-old plants. n = 10 samples/genotype, which each sample (n) consisting of a leaf disc half taken from 3-4 individual plants. Peak flg22-triggered ROS production (right) is shown as example for statistical analysis and represents data from same time-course experiment (left). RLU, relative light unit. For all experiments, values are means ± SE. Asterisks indicate statistically significant differences compared to wild-type by two-tailed Student’s t-test: P* < 0.05; P** < 0.01; P*** < 0.001; P**** < 0.0001; ns = no statistically significant difference. All experiments were performed at least three times with similar results.
Figure 3. EPS1 is required for robust elf26 signaling. A, Time-course and peak for elf26-triggered (10 nM) apoplastic ROS production from leaves of 5-to-6-week-old plants for epsI-2 (■) and Col-0 (wildtype, WT; □). n = 15 samples/genotype/treatment with each sample (n) consisting of one leaf disc half taken from 3-4 individual plants for each genotype and treatment. B, For FRK1 mRNA accumulation, 8-day-old seedlings were treated with 100 nM elf26 for indicated time in hours (h). n = 3 to 4 samples/genotype/treatment, with each biological sample (n) containing 3 to 4 seedlings. For PR1 mRNA accumulation, leaves of 5-to-6-week old plants were infiltrated with 1µM elf26. n = 6 samples/genotype/treatment, with each sample (n) representing containing 3 leaf discs taken from 3-4 different plants for each genotype and treatment. Relative mRNA levels of FRK1 and PR1 were measured and normalized to the reference gene At2g28390. Values represent means ± SE. For all experiments, epsl-2 (■) and Col-0 (wild-type, WT; □). Asterisks indicate statistically significant differences compared to wild-type by two-tailed Student’s t-test: P* < 0.05; P** < 0.01; P*** < 0.001; P**** < 0.0001. All experiments were performed at least three times with similar results.
**Figure 4.** EPS1 is required for robust AtPep1 signaling. A, Time-course and peak for AtPep1-triggered (10 nM) apoplastic ROS production from 8-day-old seedlings. n = 20 samples/genotype/treatment with each sample (n) consisting of one cotyledon half obtained from 5 or more individual seedlings for each genotype and treatment. Experiment was performed twice in seedlings and twice in leaf discs with similar results. D, For FRK1 mRNA accumulation, 8-day-old seedlings were treated with 100 nM AtPep1 for indicated time in hours (h). n = 3 to 4 samples/genotype/treatment, with each biological sample (n) containing 3 to 4 seedlings. FRK1 mRNA accumulation, leaves of 5-to-6-week-old plants were infiltrated with 1 μM AtPep1 for indicated time. n = 6 samples/genotype/treatment, with each sample (n) containing 3 leaf discs taken from 3-4 different plants for each genotype and treatment. Relative mRNA levels of FRK1 and PRI were measured and normalized to the reference gene At2g28390. Values represent means ± SE. For all experiments, eps1-2 (■) and Col-0 (wild-type, WT; □). Asterisks indicate statistically significant differences compared to wild-type by two-tailed Student’s t-test: $P^* < 0.05$; $P^{**} < 0.01$; $P^{***} < 0.001$; $P^{****} < 0.0001$. Unless specified otherwise, all experiments were performed at least three times with similar results.
**Figure 5.** Loss of *EPS1* results in reduced plasma membrane and increased intracellular accumulation of FLS2-GFP. A, In total protein extracts, *eps1-2 FLS2-GFP* accumulated FLS2-GFP to similar levels as wild-type Col-0 *FLS2-GFP*. Total proteins were extracted from Col-0 (wild-type, WT) and *eps1-2* (*e1-2*) 7-day-old seedlings that did (+) or did not (-) express *pFLS2::FLS2-GFP* and subjected to immunoblot analysis with indicated antibodies. Col-0 *FLS2-GFP* and *eps1-2* *FLS2-GFP* were from the same homozygous F4 seedlings used for SDCM and pixel intensity analyses in (B) to (E). Antibody against FLS2 detected both endogenous FLS2 (open arrow) and FLS2-GFP (closed arrow); antibody against green fluorescent protein (GFP) identified FLS2-GFP (closed arrow); antibody against EPS1 confirmed *eps1-2* mutant; and antibody against Calnexin (CNX, ER membrane marker) served as loading control. B, Representative single z-plane images and zoomed insets (10 x 10 μm in size) for WT *FLS2-GFP* and *eps1-2* (*e1-2*) *FLS2-GFP* cotyledons using SDCM. Scale bars = 10 μm. C, D, and E, Quantification of FLS2-GFP pixel intensity from selected regions of the PM (C), in the intracellular region (D) and as a PM/intracellular ratio (E) for WT *FLS2-GFP* (WT, □) and *eps1-2* *FLS2-GFP* (*e1-2; ▲*). Data for *e1-2* are normalized to WT with n = 48 images from 24 seedlings/genotype. Values are means ± SE. Asterisks indicate statistically significant differences compared to WT by two-tailed Student’s t-test: 

\[ P^{***} < 0.0001 \] 

All experiments were performed three times with similar results.
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