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The GH3 Acyl Adenylase Family Member PBS3 Regulates Salicylic Acid–Dependent Defense Responses in Arabidopsis

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ABSTRACT

The *pbs3-1* mutant, identified in a screen for *Arabidopsis thaliana* mutants exhibiting enhanced susceptibility to the avirulent *Pseudomonas syringae* pathogen DC3000(*avrPphB*), also exhibits enhanced susceptibility to virulent *Pseudomonas syringae* strains, suggesting it may impact basal disease resistance. As induced salicylic acid (SA) is a critical mediator of basal resistance responses, free and glucose-conjugated SA levels were measured and expression of the SA-dependent pathogenesis related marker *PR1* was assessed. Surprisingly, while accumulation of the salicylic acid glucoside (SAG) and expression of *PR1* were dramatically reduced in the *pbs3-1* mutant in response to *P. syringae* infection, free SA was elevated. However, in response to exogenous SA, the conversion of free SA to SAG and the induced expression of *PR1* were similar in *pbs3-1* and wild-type plants. Through positional cloning, complementation, and sequencing, we determined that the *pbs3-1* mutant contains two point mutations in the C-terminal region of the protein encoded by At5g13320, resulting in non-conserved amino acid changes in highly conserved residues. Additional analyses with T-DNA insertion (*pbs3-2*) and transposon insertion (*pbs3-3*) mutations in At5g13320 confirmed our findings with *pbs3-1*. PBS3 (also referred to as GH3.12) is a member of the GH3 family of acyl-adenylate/thioester-forming enzymes. Characterized GH3 family members, such as JAR1, act as phytohormone-amino acid synthetases. Thus, our results suggest that amino acid conjugation plays a critical role in SA metabolism and induced defense responses with PBS3 acting upstream of SA, directly on SA, or on a competitive inhibitor of SA.
Disease resistance in plants is often dependent on recognition of infecting pathogens by specific disease resistance (R) proteins (Jones and Dangl, 2006). Once activated, R proteins trigger a complex cascade of defense responses, including production of activated oxygen species, fortification of cell walls, accumulation of antimicrobial proteins known as pathogenesis-related (PR) proteins, production of antimicrobial secondary metabolites, and localized programmed cell death known as the hypersensitive response (HR) (Hammond-Kosack and Jones, 1996). The specific signal transduction steps leading to these various responses are poorly understood, although forward genetic screens have identified many potential regulators of these responses (Innes, 1998; Glazebrook, 2005).

A key second messenger involved in inducing production of PR proteins and amplifying the oxidative burst is salicylic acid (SA) (Ryals et al., 1996; Shirasu et al., 1997). SA levels in uninfected dicot plants are normally very low, but rapidly increase upon infection with avirulent pathogens (i.e. those that activate an R protein) (Klessig and Malamy, 1994). Virulent pathogens also induce accumulation of SA, but not as rapidly as avirulent strains (Zhou et al., 1998; Shapiro and Gutsche, 2003; SK Marr and MC Wildermuth, unpublished data). In *Arabidopsis*, the bulk of pathogen-induced SA accumulates as the salicylic acid-O-β-glucoside (SAG). Mutations that reduce SA production (Nawrath and Metraux, 1999; Dewdney et al., 2000), or impair SA perception (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997) increase susceptibility to both virulent and avirulent pathogens, indicating that SA contributes to both basal and R protein-mediated resistance.

In order to identify additional components of the R protein signal transduction pathway, Warren et al. (1999) screened for *Arabidopsis thaliana* mutants that displayed enhanced susceptibility after inoculation with an avirulent strain of *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000(*avrPphB*)). Resistance to this strain by *Arabidopsis* variety Col-0 is mediated by the *RPS5* gene (Simonich and Innes, 1995), which encodes a member of the nucleotide binding
site-leucine rich repeat (NBS-LRR) family of R proteins (Warren et al., 1998). This mutant screen led to the identification of several susceptible alleles of RPS5, plus mutations in three additional genes, which were named PBS1, PBS2 and PBS3 for avrPphB susceptible (Warren et al., 1999). The isolation and characterization of PBS1 and PBS2 have been reported previously (Swiderski and Innes, 2001; Tornero et al., 2002). Here we describe the isolation and characterization of PBS3.

Warren et al. (1999) reported that the pbs3 mutant displayed enhanced disease susceptibility to both virulent and avirulent Pst DC3000 strains, including DC3000(avrPphB) and DC3000(avrRpt2) (Warren et al., 1999). This phenotype is similar to that reported for SA deficient Arabidopsis mutants such as eds5/sid1 (Rogers and Ausubel, 1997; Nawrath and Metraux, 1999; Nawrath et al., 2002) and sid2/eds16 (Nawrath and Metraux, 1999; Dewdney et al., 2000; Wildermuth et al., 2001). Here, we report that the pbs3-1 contains two point mutations in the C-terminal region of the protein encoded by At5g13320. Additional analyses with T-DNA insertion (pbs3-2) and transposon insertion (pbs3-3) mutants in At5g13320 were used to further confirm the findings with pbs3-1. PBS3 (also referred to as GH3.12) is a member of the GH3 protein family of acyl-adenylate/thioester forming enzymes (Staswick et al., 2002). Characterized GH3 family members, such as JAR1, act as phytohormone-amino acid synthetases (Staswick et al., 2002; Staswick and Tiryaki, 2004; Staswick et al., 2005). We found that the pbs3 mutants are compromised in both pathogen-induced accumulation of the SA glucoside (SAG) and expression of the SA-dependent marker gene PR1 (At2g14610). Surprisingly, the level of free SA was about two-fold higher than wild-type plants. Exogenous application of SA was sufficient to restore SAG accumulation, PR1 expression, and enhanced resistance to virulent DC3000. As mutations in PBS3 impact SAG accumulation, SA-dependent gene expression, and disease resistance, PBS3 plays an important role in SA metabolism. Herein, we present the above findings and discuss possible biochemical functions for PBS3 consistent with its observed functional impact and its putative biochemical activity as a GH3 family member.
RESULTS

Positional Cloning of PBS3

Warren et al. (1999) mapped the pbs3 mutation to chromosome 5 in the vicinity of microsatellite marker nga249 at 28.4 cM on the genetic map. To refine this position, an F2 mapping population was inoculated with Pst DC3000(avrRpt2), plants displaying disease symptoms identified and then scored for PCR-based markers adjacent to nga249. These analyses placed the pbs3 mutation between microsatellite markers nga249 and nga151, a map distance of approximately 5.9 cM (Figure 1). We then performed high-resolution genetic mapping by using PCR to pre-select F2 plants containing recombination events between nga249 and nga151. From approximately 800 plants, we identified 70 informative recombinants. These 70 lines were scored for resistance to Pst DC3000(avrRpt2) and for additional markers located between nga249 and nga151. Scores were confirmed in F3 families derived from each F2 plant. Analysis of these lines placed the pbs3 mutation between markers MBJ5NOT and CHS1 (Figure 1A).

The distance between MBJ5NOT and CHS1 is approximately 300 kb. This interval contains 80 predicted genes. We obtained T-DNA insertion lines that disrupted 40 of these genes from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003), and assayed these lines for resistance to PstDC3000(avrRpt2) using dip inoculations. Among the T-DNA insertion lines tested, SALK line 018225 in predicted gene At5g13320, displayed small water-soaked lesions in the inner rosette leaves (data not shown), similar to those observed on pbs3-1 mutant plants. However, we found these symptoms to be variable among individual plants and from one day to another.

Because of this variation, we tested several alternative inoculation methods and pathogen strains to identify a more reproducible assay. We found that the standard “enhanced disease susceptibility” (eds) assay developed by
Glazebrook et al. (1996) gave us the most robust results. This assay employs a needleless syringe to inoculate individual leaves with a low titer of \textit{P. syringae pv. maculicola} strain ES4326 (\textit{Psm}). Using this assay, wild-type Col-0 plants produce little to no disease symptoms, while \textit{pbs3-1} mutants produce marked yellowing with occasional lesions (data not shown). We developed a semi-quantitative disease phenotype scale to score these symptoms, ranging from a value of 1 (no symptoms) to 5 (complete collapse of the inoculated region) (Supplemental Figure 1A). We validated this scoring system by also titering bacterial populations in infected leaves of both wild-type and \textit{pbs3-1} mutant plants at three days post inoculation. Figure 1B shows that the \textit{pbs3-1} mutant supported significantly more growth of strain ES4326 than wild-type Col-0.

Using this scoring system, we found that SALK-T-DNA insertion line 018225 was significantly more susceptible than wild-type plants and similar to the \textit{pbs3-1} mutant (Figure 1C). We also obtained a \textit{Ds} transposon line from the RIKEN collection with an insertion in \textit{At5g13320} (Kuromori et al., 2004). This line, which is in the Nössen genetic background, was also significantly more susceptible to ES4326 than its wild-type sibling (Figure 1C). We therefore named these insertion alleles \textit{pbs3-2} and \textit{pbs3-3} respectively.

The above data strongly suggested that \textit{At5g13320} corresponded to \textit{PBS3}. To confirm this, we amplified \textit{At5g13320} from the \textit{pbs3-1} mutant and sequenced it. We found two point mutations that substitute a lysine for a glutamate (E502K) and threonine for an isoleucine (I519T) (Figure 1D), indicating that \textit{At5g13320} indeed corresponds to \textit{PBS3}.

Further support for this conclusion was obtained by complementing the \textit{pbs3-1} and insertion mutants by transformation with a wild-type genomic copy of \textit{PBS3}, extending from 1,020 bp upstream of the translation initiation site to 127 bp downstream of the stop codon. Disease symptoms of T1 transformant plants were quantified and compared with that of wild-type and mutant plants. As expected, the transformants showed a significant reduction in disease scores, confirming that \textit{At5g13320} is \textit{PBS3} (Figure 1E).

We then examined \textit{PBS3} transcripts in the \textit{pbs3-1} and \textit{pbs3-2} mutants.
As shown in Supplemental Figure 2, PBS3 transcript was detected in wild-type and pbs3-1 plants in response to Pst DC3000(avrRpt2) but not in the insertion mutant pbs3-2 using primers that flank intron 3, the location of the T-DNA insertion (see Figure 1D).

**PBS3 Belongs to the GH3 family of Acyl Adenylases.**

*At5g13320* is a member of the GH3 multigene family that consists of 19 family members in *Arabidopsis* variety Col-0 (Staswick et al., 2002). The first GH3 gene described was isolated from *Glycine max* as an early auxin responsive gene (Hagen and Guilfoyle, 1985). Since then, homologs have been identified in many plants, including *Arabidopsis thaliana*, *Oryza sativa*, *Gossypium spp.*, *Lycopersicon spp.*, *Populus spp.*, *Pinus spp.*, and the moss *Physcomitrella patens* based on ESTs or sequence data (Terol et al., 2006), and in some bacteria (Okrent, R. and Wildermuth, M; unpublished data). The GH3 proteins are members of a large enzyme superfamily of acyl-adenylate/thioester-forming enzymes that catalyze a variety of reactions with a common first step: the transfer of AMP from ATP to the carboxylic acid group of an acyl substrate forming an activated acyl-adenylate intermediate (Staswick et al., 2002). Characterized family members have been shown to catalyze the ATP-dependent conjugation of amino acids to the phytohormones jasmonic acid (JA) or auxin (IAA) with mutants exhibiting altered phytohormone phenotypes (Staswick et al., 2002; Staswick and Tiryaki, 2004; Staswick et al., 2005). For example, the *jar1* mutant was isolated as a jasmonate insensitive mutant and displays enhanced susceptibility to necrotrophic, but not biotrophic, pathogens (Staswick et al., 1998).

Phylogenetic analysis of the 19 AtGH3 family members identified three sequence homology groups, with the known substrate specificity corresponding to their phylogenetic relationships: Group I members, which include JAR1, a JA-amino acid synthetase, Group II members, which are capable of adenylating IAA, and Group III members which include PBS3 (GH3.12) and act on unknown
substrates (Staswick et al., 2002; Staswick and Tiryaki, 2004; Staswick et al., 2005). Recombinant PBS3 did not show significant activity with any of the tested substrates: JA, JA methylester, IAA, abscisic acid, 1-aminocyclopropane-1-carboxylic acid, gibberellic acid, 2-oxophytodienoic acid, linolenic acid, or SA (Staswick et al., 2002) suggesting that the recombinant protein used in these assays was inactive, or that the correct substrate was not provided.

As shown in Figure 1D and Supplemental Figure 3, PBS3 has all three AMP-binding motifs that are necessary for adenylation. Residues of import for substrate specificity have not yet been determined but are likely to reside near the AMP-binding motifs as has been shown for other members of the acyl-adenylate/thioester-forming superfamily (Gulick et al., 2004; Nakatsu et al., 2006). The \textit{pbs3-1} point mutations result in two amino acid changes (E502K and I519T) that are located in the C-terminal domain of the PBS3 protein, not in or near the AMP-binding motifs. Both residues are highly conserved in \textit{Arabidopsis} GH3 family members with amino acids containing negatively charged R groups (E or D) at residue 502 and the nonpolar aliphatic R group members I or V at residue 519. The C-terminal region of these GH3 proteins contains no known protein domains or motifs. It is possible that this region facilitates the conjugation of the amino acid to the activated AMP intermediate, though this remains to be determined.

The \textit{pbs3-1} Mutation Impairs Accumulation of SAG and \textit{PR1} by Pathogens

The enhanced susceptibility of \textit{pbs3} mutant plants to both virulent and avirulent pathogens is similar to previously described \textit{enhanced disease susceptibility (eds)} mutants, many of which are compromised in the accumulation of SA and the expression of the SA-dependent marker \textit{PR1} (Glazebrook et al., 1996; Rogers and Ausubel, 1997; Dewdney et al., 2000). We therefore assayed levels of \textit{PR1} transcript in the \textit{pbs3} mutant before and after infection with two different \textit{P. syringae} strains. As seen in Figure 2A, the avirulent \textit{Pst} strain DC3000 (\textit{avrRpt2}) strongly induced \textit{PR1} by 12 hours post inoculation (hpi) in
wild-type plants, while *PR1* transcript was not detected in *pbs3-1* or *pbs3-2* plants until 48 hpi. Similarly, the virulent strain ES4326 induced *PR1* highly by 24 hpi in wild-type plants, while transcript levels were at least 50 fold lower in the *pbs3-1* mutant (Figure 2B).

The reduced levels of *PR1* transcript in *pbs3* mutants suggested that production and/or perception of SA might be compromised. We therefore measured both free SA and SA glucoside (SAG) levels in *pbs3-1* and *pbs3-2* mutant plants before and 24 hpi with *Pst* DC3000(*avrRpt2*). At this time point, wild-type plants exhibit significant expression of *PR1*, whereas *pbs3* mutants do not (Figure 2A). We found significant accumulation of SAG in wild-type plants, with an average 5-fold reduction in accumulated SAG in the *pbs3* mutant plants (Figure 3A and Supplemental Table 1). Surprisingly, the *pbs3* mutants accumulated approximately 2-fold more free SA than wild-type at this time point (Figure 3A and Supplemental Table 1). Because the majority of SA is found as SAG, the total SA present in the *pbs3* mutants 24 hpi was significantly lower (2-3 fold) than in wild-type plants. Though mutants with reduced (e.g. *pad4* (Zhou et al., 1998), or abrogated (e.g. *ics1/sid2-1/eds16-1* (Nawrath and Metraux, 1999; Dewdney et al., 2000; Wildermuth et al., 2001)) induction of SA and SAG in response to pathogen have been reported, this is the first report of a mutant exhibiting elevated free SA and dramatically reduced SAG accumulation in response to pathogen. A direct comparison of *PR1* induction and SA and SAG accumulation in the *pbs3* mutants with the SA biosynthetic mutant *eds16-1*, confirmed that whereas pathogen-induced *PR1* and SAG accumulation are abrogated in the *eds16-1* mutant, they are dramatically reduced in the *pbs3* mutants (Figs. 2A and 3B).

These results suggest that the PBS3 protein contributes to, or regulates, SAG biosynthesis and total SA accumulation, as well as expression of the SA-dependent marker *PR1*, and resistance to *Pseudomonas syringae* pathogens. The finding that free SA levels were elevated (not reduced) suggests that PBS3 might act directly on SA and that the product formed by PBS3 impacts accumulation of the SA glucose conjugate and expression of *PR1*. 
Exogenous SA restores SAG accumulation, \textit{PR1} expression and resistance

To determine whether \textit{pbs3} mutants have a defect in processing free SA, and whether this processing is required for wild-type pathogen-induced accumulation of SAG and \textit{PR1}, we treated wild-type and \textit{pbs3-1} mutant plants with exogenous SA. Figure 4A shows that application of 2.5 mM SA to \textit{pbs3-1} mutant leaves restores induced \textit{PR1} expression at levels similar to wild-type 24 hrs post SA treatment (hpt). This \textit{PR1} induction is dramatic with relative increases in \textit{PR1} expression at 24 hpt (compared to untreated) of >150-fold for all samples of \textit{pbs3-1} and wild-type.

SAG formation was also comparable for \textit{pbs3-1} and wild-type leaves (Figure 4B) at 24 hpt with SA. SAG is calculated by subtracting measured free SA (no hydrolysis) from measured total SA (after hydrolysis of SAG to SA) as described in Materials and Methods. Significant SAG (~25 µg/FW) was formed in leaves of wild-type and \textit{pbs3-1} mutants 24 hpt with exogenous SA, resulting in reproducible differences in measured total - free SA values. It should be noted, however, that direct measurement of SAG would need to be performed to ascertain subtle changes in SAG in response to exogenous SA. To determine whether exogenous SA treatment conferred enhanced disease resistance, we subsequently (24 hpt) inoculated leaves with \textit{Psm ES4326} (OD$_{600}$= 0.0002) and assessed bacterial growth immediately after inoculation and at 3 dpi. We also performed parallel experiments using the active SA analog INA (2,6-dichloroisonicotinic acid; 0.65 mM) for the pretreatment instead of SA (4 mM). Bacterial growth at 3 dpi was limited by pre-treatment of \textit{pbs3-1} and wild-type leaves with the SA analog INA (data not shown). Though SA pretreatment also conferred resistance to \textit{Psm ES4326} for both the \textit{pbs3-1} and wild-type plants, the results were less consistent than with INA, perhaps due to secondary effects from the high concentration of SA (4 mM) employed in these experiments.
Given these findings, it appears that the *pbs3* mutants are not defective in the perception or processing of SA and that PBS3 function is not required for the conversion of SA to SAG or for the SA-dependent induction of *PR1* expression.

**PBS3 is Induced by Pathogens and is Highly Correlated with ICS1**

Analysis of publicly available *Arabidopsis* ATH1 Affymetrix GeneChip microarray data (Craigon et al., 2004; Zimmermann et al., 2004) revealed that *PBS3* (*At5g13320*) expression is typically quite low, but is strongly induced by *Pseudomonas* pathogens with some induction by oxidative stresses such as ozone and UV-B. As shown in Figure 5A, *PBS3* is expressed most rapidly and strongly in response to non-host strains of *P. syringae*, and to a somewhat lesser degree by avirulent and virulent strains. Furthermore, the type III secretion system (TTSS) is required for maximal induction of *PBS3* expression as *Pst hrcC* mutants, which cannot deliver type III effectors to host cells, do not induce *PBS3* as strongly as does *Pst* (Figure 5A). Average ratios for *PBS3* gene expression in the *Pst hrcC* mutant compared with *Pst* were 0.35, 0.48, and 0.7 at 2, 6, and 24 hpi, respectively. Analysis of the Thilmony et al (2006) microarray dataset (Thilmony et al., 2006) provides even stronger evidence in support of TTSS involvement, as *PBS3* induction is dramatically reduced in the *Pst hrpS* and *hrpA* mutants, which cannot express or secrete TTSS effector genes and proteins, respectively (Figure 5B).

As shown in Figure 5, expression of the pathogen-induced SA biosynthetic gene *ICS1* (*SID2/EDS16*) parallels that of *PBS3*. As PBS3 impacts pathogen-induced free SA and SAG accumulation as well as expression of the SA-dependent marker *PR1*, we assessed whether *ICS1* and *PBS3* expression are correlated across all experiments using wild-type Col-0 plants in the NASCAArray Database (821 ATH1 GeneChips; Craigon et al., 2004). We found that *PBS3* expression is well correlated with *ICS1* (*At1g74710*), with a Pearson correlation coefficient of 0.63 (see Materials and Methods); whereas the Pearson correlation coefficient for *PR1* (*At2g14610*) with *ICS1* is 0.42. As shown in Figure 5C, when
significant expression of *ICS1* and *PBS3* are observed, their expression is very highly correlated. Indeed, *PBS3* is the second most correlated gene with *ICS1* of the >22K genes on the ATH1 GeneChip, while *PR1* is the 140th most correlated gene. We observed no significant correlation in expression between *PBS3* and any of the other Arabidopsis GH3 family members, suggesting that PBS3 plays a unique role among its family.

**DISCUSSION**

In *Arabidopsis* and many other plants, SA is a key molecule that activates plant defense genes, and its accumulation is known to be necessary for local and systemic acquired resistance (Durrant and Dong, 2004). Although SA is necessary for triggering plant defense pathways, an excess amount of free SA may be phytotoxic (e.g. Lee et al., 1995; Kenton et al., 2000). Plants regulate free SA levels in part by glucosylation forming salicylic acid–2-0-β-glucoside (SAG) as the dominant form of SA detected in plants (Enyedi et al., 1992; Malamy et al., 1992). Infection by pathogens such as TMV (tobacco mosaic virus) and *P. syringae* pathovars rapidly induces the accumulation of free SA and SAG (Chong et al., 2001; Malamy et al., 1992; Shapiro and Gutsche, 2003). The conversion of free SA to SAG has been followed using radiolabelled SA (Dean et al., 2005) and appears to be catalyzed by UDP-glucose:salicylic acid glucosyltransferases (SAGT). In tobacco and *Arabidopsis*, putative SAGTs have been identified based on correlation of expression with accumulation of SAG, induction of SAGT expression by free SA, and the ability of these enzymes to catalyze the formation of SAG *in vitro* (Enyedi and Raskin, 1993; Lee and Raskin, 1999; Lim et al., 2002; Song, 2006). However, *sagt* mutants deficient in SAG formation have not yet been reported.

Although SA appears to be synthesized in the chloroplast (Strawn et al., 2006), SAG is formed in the cytoplasm and then transported into the vacuole (Dean and Mills, 2004; Dean et al., 2005). The vacuolar localization of SAG suggests that it is primarily a storage form of SA. Whether SAG can be exported
back out of the vacuole is not known. Because SAG can be rapidly hydrolyzed to form free SA by endogenous hydrolases, SAG is hypothesized to function as an inactive pool for the rapid sustained induction of systemic acquired resistance. In support of this function, exogenous application of SAG, but not the non-hydrolyzable SAG analog thio-SAG, results in the accumulation of free SA and expression of PR1 (Hennig et al., 1993).

The pbs3 mutants exhibit ~5-fold reduction in pathogen-induced SAG accumulation compared to wild-type Arabidopsis plants (Figure 3 and Supplemental Table S1). In contrast to SAG levels, the level of free SA is significantly higher in the pbs3 mutant relative to wild-type at 24 hpi with Pst(avrRpt2). This unusual phenotype suggests that PBS3 may regulate the conversion of SA to SAG. Total SA levels (SA + SAG) are 2- to 3-fold lower in pathogen-infected pbs3 mutant plants than in wild-type, thus PBS3 is not only affecting accumulation of SAG, but somehow is impacting the overall metabolism of SA such that net SA biosynthesis is reduced, or SA turnover is increased.

Pathogen-induced expression of the SA-dependent marker PR1 is severely compromised in the pbs3 mutants with no significant induction by 24 hpi with virulent Psm ES4326 (Figure 2B) and a ~36 hr delay in induction in response to avirulent Pst(avrRpt2) (Figure 2A). The pathogen-induced expression of PR1 requires the ankyrin-repeat containing master regulator NPR1 (Cao et al., 1994; Delaney et al., 1995). Expression of the SA biosynthetic gene ICS1 and PBS3 are correlated (Figure 5C) and ICS1 and PBS3 expression precede the dramatic induction of PR1 expression (Figure 5A, most obvious in response to Psp and avirulent Pst). In the pbs3 mutants, despite the elevated induced free SA levels at 24 hpt with Pst(avrRpt2), PR1 is not induced (Figure 2). This result suggests that elevated free SA alone is not sufficient to activate PR1 expression.

Perhaps, at these time points, total SA (free SA plus SAG) better represents the plant cells’ exposure to free SA during the first 24 hrs after pathogen inoculation. Loss of PBS3 function would then lead to a reduction in PR1 expression and other defenses because it results in a reduction in total SA.
levels. The dramatic reduction of PR1 expression at 24 hpi with Pst(avnRpt2) compared to a 2- to 3-fold reduction in total SA levels in the pbs3 mutants can be explained if an SA threshold (best assessed as total SA) is required for induction of PR1 expression. This SA threshold is consistent with the previously proposed SA amplification loop (e.g. Shah, 2003; Jirage et al. 1999).

A review of the relevant literature indicates that when total leaf SA levels are less than 3 µg/g FW, as reported here for the pbs3 mutants, PR1 induction is not typically observed (e.g. Zeier et al., 2004). In addition, when transgenic plant lines overexpressing bacterial SA biosynthetic genes exhibit constitutive expression of total SA of ≤ 3 µg/gFW, PR1 is not significantly induced (Mauch et al., 2001; Verberne et al., 2000). High levels of SA provided by exogenous SA application would then surpass the SA threshold and restore induced PR1 expression in the pbs3-1 mutant as observed. Exogenous SA application also restores PR1 expression in mutants thought to participate in the SA amplification loop (Parker et al., 1996; Glazebrook et al., 1997; Zhou et al., 1998; Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001; Shah, 2003).

An alternate explanation of our results is that SAG is the active form of SA and is required for PR1 expression. However, SAG is unlikely to be active itself as its hydrolysis is required for activation of PR1 induction (Hennig et al., 1993).

In either case, these findings suggest that PBS3 functions upstream of SA synthesis, either in a regulatory capacity (which may include an amplification loop) or in SA biosynthesis. However, this function still requires an explanation of how free SA levels could be elevated in the pbs3 mutants while SAG (and total SA) is dramatically reduced. Another viable hypothesis is that SA needs to be modified by PBS3 to form SAG and to activate PR1. However, in this case exogenous SA application should not restore wild-type SAG accumulation and expression of PR1 in pbs3-1 plants. Below, we discuss these confounding findings as well as the uncoupling of free SA levels from induced PR1 in the context of proposed biochemical activities for PBS3.

Proposed Biochemical Function of PBS3
PBS3 is a member of the GH3 family of acyl-adenylate/thioester forming enzymes known to conjugate amino acids (AA) to phytohormones (Staswick and Tiryaki, 2004; Staswick et al., 2005), thus it is tempting to speculate that PBS3 may act directly on SA as an SA-amino acid synthetase to form an SA-AA conjugate. Alternatively, PBS3 could act on a competitive inhibitor of SA, or upstream of SA biosynthesis either in a regulatory capacity or through action on a precursor of SA. As discussed earlier, very little is known about SA metabolism. Though SA synthesis appears to be plastid-localized (Strawn et al. 2007), we found no predicted chloroplast transit sequence for PBS3, similar to other GH3 proteins. Thus our expectation is that PBS3, like SAGT, acts in the cytosol, though this requires experimental confirmation.

Could PBS3 act as an SA–AA synthetase? As shown in Figure 6A (top panel), an SA-AA conjugate may itself be the active form of SA required for PR1 induction. This function of PBS3 would be similar to that of JAR1, which activates JA by forming specific JA-AA conjugates (Staswick and Tiryaki, 2004). Though amino acid conjugates of SA have not been reported in Arabidopsis, they have been detected in grape (Steffan et al., 1988) and bean (Bourne et al., 1991). If the SA-AA conjugate itself is the active form of SA required for PR1 induction, then exogenous SA application should not result in PR1 expression in the pbs3 mutant. However, we found that at 24 hpt with SA, PR1 was expressed similarly in leaves of pbs3 and wild-type plants. This suggests that either SA-AA is not the active form of SA required for PR1 induction or that another GH3 family member, perhaps with lower affinity for SA, may compensate for loss of PBS3 function when supplied with a great excess of the substrate SA. One candidate for this compensatory role is GH3.5 (At4g27260), the only tested GH3 family member to exhibit adenylase activity with SA as a substrate in vitro (Staswick et al., 2002). This function of PBS3 would explain why PR1 is not induced in the pbs3 mutants despite elevated free SA levels.

Alternatively (Figure 6A, bottom panel), PBS3 could act on SA to form an
SA-AA conjugate that is required for the proper spatial localization of the active form of SA, which may be free SA. Amino-acid conjugation of auxin appears to regulate its subcellular and tissue-specific distribution and hydrolysis of phytohormone amino acid conjugates by specific amidohydrolases (e.g. IAA-amidohydrolases) allows for fine regulation of active forms of the phytohormone (Woodward and Bartel, 2005). For example, in response to \textit{Pst(avrRpt2)} free SA would be synthesized in the chloroplast of a cell as an early event associated with the hypersensitive response. It could then be modified to SA-AA by PBS3 in the cytosol of the HR-undergoing cell and then exported to neighboring cells. In the neighboring cells the SA-AA conjugate could be hydrolyzed, releasing free SA that could then induce \textit{PR1} expression. SAG formation catalyzed by a cytosolic SAGT could also occur in these neighboring cells. In this case, in the \textit{pbs3} mutant, free SA would be elevated as it would not be converted to SA-AA, and SAG formation in neighboring cells would be reduced. Exogenous SA application would negate this requirement for SA transport, thus restoring \textit{PR1} expression and SAG formation to wild-type levels in the \textit{pbs3} mutant. Consistent with this model, in tobacco free SA accumulation preceded visible HR symptoms by >24 hrs, as observed using an SA reporter strain, with higher levels of free SA detected in neighboring cells surrounding HR lesions as they became apparent (Huang et al., 2006). In addition, induced \textit{PR1} accumulation was not observed in cells undergoing an HR (and subsequently dying) but specifically around HR lesions in tobacco and \textit{Arabidopsis} (e.g. Stone et al., 2000; Dorey et al., 1997).

\textit{Could PBS3 act on a competitive inhibitor of SA?} In this scenario, PBS3 would inactivate a competitive inhibitor X by converting it to an inactive form X-AA (Figure 6B). Amino acid conjugation can either inactivate phytohormones or target them for degradation pathways (Woodward and Bartel, 2005). In the \textit{pbs3} mutants, the competitive inhibitor would compete with SA in binding to the active site of enzymes that use SA as a substrate, such as SAGT. The competitive inhibitor could also bind to enzymes that are regulated by SA-binding. For example, in \textit{E. coli}, binding of SA to the multiple antibiotic resistance repressor
MarR reduces its DNA-binding affinity thus allowing for expression of genes associated with multiple antibiotic resistance (Alekshun and Levy, 1999). In *Arabidopsis*, SA affects the interaction of the master regulator NPR1 with TGA transcription factors enhancing DNA binding (Despres et al., 2003, Fan and Dong, 2002). If PBS3 acted on a competitive inhibitor of SA, free SA would be elevated in the *pbs3* mutants, and SAG and *PR1* would be reduced. The differential reduction in SAG accumulation (5-fold) vs. *PR1* expression (~50-fold) could be explained by different binding affinities of the inhibitor for distinct enzymes controlling SAG synthesis or *PR1* expression. Exogenous SA (2.5 mM) would likely dominate a reversible competitive inhibitor resulting in restored SAG and *PR1* formation. The competitive inhibitor would likely be a small molecule similar to SA, such as a substituted benzoic acid. In support of this hypothesis, the *Arabidopsis* SAGT1 enzyme, in addition to SA, can also use benzoic acid and a subset of other substituted benzoates as substrates (Lim et al., 2002; Song, 2006). Similarly, the DNA-binding affinity of *E. coli* MarR can be regulated by SA and other small phenolics (Alekshun and Levy, 1999). If PBS3 inactivates an inhibitor, the reduction in total SA could then be explained by a feedback inhibition on SA biosynthesis by elevated free SA.

**Could PBS3 act upstream of SA biosynthesis?** PBS3 may act upstream of SA biosynthesis either in a regulatory capacity or through action on a precursor of SA (Figure 6C). For example, adenylation (and amino acid conjugation) of an SA precursor might either be required for its subsequent biochemical conversion or for its proper spatial localization. If PBS3 acted upstream of SA in either a regulatory or biosynthetic capacity, the application of exogenous SA would restore SAG formation and *PR1* expression in the *pbs3* mutants as we observed. However, we would also expect both induced free SA and SAG levels to be reduced in the *pbs3* mutants; this was not observed. One possible explanation for the observed increase in free SA with decreased SAG at 24 hpi is that lack of PBS3 function delays SA biosynthesis. If free SA is first made and then subsequently converted to SAG, it is possible that by 24 hpi with *Pst*(avrRpt2),
the bulk of free SA has been converted to SAG in the wild-type and thus free SA levels have decreased from their maximum, whereas SAG levels are still increasing. This decrease in free SA levels as SAG continues to increase has been observed (e.g. Zhou et al., 1998). In contrast, if SA synthesis is delayed in the pbs3 mutant, free SA might be at its maximum at 24hpi, with less of it having been converted to SAG thereby resulting in higher free SA and lower SAG levels in the pbs3 mutant. In this scenario, the induction of PR1 expression requires some threshold of total SA (free + SAG) that was not met at 24 hpi with Pst(avrRpt2) in the pbs3 mutants.

In summary, our results suggest that PBS3 plays an important role in pathogen-induced SA metabolism and that this function is critical to SAG accumulation, PR1 expression, and both basal and R-protein mediated resistance. Given the function of GH3 family members of acyl-adenylase thioester-forming enzymes, it is likely that PBS3 acts as a small molecule-amino acid synthetase, either upstream of SA biosynthesis, on SA, or on a competitive inhibitor of SA. Further work is clearly needed to unravel the complexity of SA synthesis, activation, processing, and catabolism. In addition, our work with the pbs3-1 mutant, which contains two point mutations resulting in non-conserved amino acid changes in the C-terminus of the PBS3 protein, highlights the importance of this uncharacterized region of GH3 family members and provides a framework for detailed mechanistic analyses of GH3 function.

MATERIALS AND METHODS

Bacteria, Plants, and Growth Conditions

P. syringae strains Psm ES4326 and Pst DC3000 have been described previously (Dong et al., 1991; Whalen et al., 1991). The avirulence gene avrRpt2 was cloned into broad host-range vector pVSP61 and introduced into these strains by triparental mating as described by Kunkel et al. (1993). Bacteria were
cultured on King’s B medium (protease peptone, 10 mg/ml; glycerol 15 mg/ml; K₂HPO₄, 1.5 mg/ml; MgSO₄, 4 mM pH7.0) supplemented with 100 µg/ml streptomycin (ES4326) or 100 µg/ml rifampicin (DC3000) plus 50 µg/ml kanamycin when *avrRpt2* was present in the strain. *Arabidopsis* varieties Columbia-0 (Col-0) and Nössen-0 (No-0) were grown in Metromix 360 in growth rooms under a 9 hr-light/15 hr-dark cycle at 23°C. Isolation of the *pbs3-1* mutant was described by Warren et al. (Warren et al., 1999). T-DNA insertion lines (Col-0 genetic background) were obtained from the Arabidopsis Biological Resource Center at Ohio State (Columbus, OH) (Alonso et al., 2003). A *Ds* transposon insertion allele of *PBS3* (No-0 genetic background) was obtained from the RIKEN Plant Functional Genomics Research Group (Kanagawa, Japan) (Kuromori et al., 2004).

**Genetic Mapping of *pbs3-1***

F2 progeny of a *pbs3-1* cross to *Arabidopsis* variety Landsberg *erecta* (Ler) were used to genetically map the *PBS3* gene. The F2 plants were inoculated with *Pst* DC3000(*avrRpt2*) and scored 3 days after inoculation. Plants displaying a *pbs3-1* phenotype were used for mapping. Initially, the *pbs3-1* mutation was mapped to chromosome 5 between microsatellite markers nga249 and nga151. To further localize the *PBS3*, we scored approximately 800 F2 plants with these two markers and identified 70 plants with recombination events within this interval. F3 progeny of these plants were assayed for resistance to DC3000(*avrRpt2*). Analysis of these lines placed the *pbs3* mutation between markers MBJ5NOT and CHS1, an interval of ~300 kb. A collection of 40 Arabidopsis T-DNA insertion lines with insertions in genes in this interval was then obtained from the Arabidopsis Biological Resource Center at Ohio State and assayed for resistance. SALK line 018225 displayed a susceptible phenotype. Location of the T-DNA insertion in this line was confirmed by PCR amplification and sequencing of the T-DNA junction fragments. A *Ds* insertion in the same gene was obtained from the RIKEN Plant Functional Genomics Research Group.
and the insertion site also confirmed by sequencing junction fragments. The 
\textit{pbs3}-1 mutant allele was amplified by PCR and the PCR product directly 
sequenced. All sequencing reactions were performed using BigDye Terminator 
Kits (Applied Biosystems, Foster City, CA) and separated on an ABI 3730 
automated DNA sequencer.

\textbf{Complementation of \textit{pbs3} Mutants}

A full-length \textit{PBS3} genomic sequence, including the promoter region and 
3' untranslated region was amplified from Col-0 genomic DNA using the 
Eppendorf TripleMaster PCR System (Eppendorf, Westbury, NY) and the 
following primers: 5'-CTGCAGAAATTTTGCAGAAGTTT-3' and 5'-
CTGCAGTAACGAAGGGTTTGGTTTCA-3', which contain \textit{PstI} restriction sites at 
their 5' ends. The PCR product was ligated into the pGEM-T Easy plasmid vector 
(Promega) and transformed into \textit{E. coli} strain DH10B. The \textit{PBS3} insert was then 
removed from this clone by digestion with \textit{PstI} and ligated with the binary vector 
pGreen0229 digested with \textit{PstI} (Hellens et al., 2000). This DNA sequence 
contains a full-length \textit{At5g13320} gene including 1,020 bp upstream of the 
translation initiation site to 127 bp downstream of the stop codon.

The pGreen0229:At5g13320 construct was transformed into \textit{Agrobacterium tumefaciens} strain GV3101 carrying helper plasmid pSOUP and 
disarmed Ti plasmid pMP90 by electroporation and selected on LB plates 
containing 50 \(\mu\)g/mL kanamycin sulfate (Sigma). Arabidopsis plants were 
transformed using the floral dip method (Clough and Bent, 1998). Transgenic 
plants were selected by spraying seedlings growing in flats of Metromix with 
Finale herbicide (Farnam Companies, Inc., Phoenix, AZ) at a concentration of 0.1 
g/L glufosinate ammonium (0.5 mM). T1 plants surviving herbicide selection were 
transplanted to pots and grown for 5 weeks then assayed for disease phenotypes 
after inoculation with \textit{Psm} strain ES4326.

\textbf{Measurement of Bacterial Growth in \textit{Arabidopsis} Leaves}
Leaves of 5 week old plants were injected with *Psm* ES4326 at a dose of $10^3$ cfu/cm$^2$ leaf area (OD$_{600}$ = 0.0002). At 1 and 72 hours, a 0.7 cm diameter disc from each of 12 leaves was excised using a cork borer. These 12 discs were divided into 4 replicates of 3 leaf discs each and ground in 1 mL 10 mM MgCl$_2$ with a plastic pestle. Appropriate dilutions were plated on King’s medium B containing streptomycin and bacterial colonies were counted. Data are reported as means and standard deviations of the log (cfu/cm$^2$) of 4 replicates. Growth assays were performed twice with similar results.

**Semiquantitative Scoring of Disease Phenotypes**

Leaves of 5 week old plants grown in chambers were injected with *Psm* ES4326 at a dose of $10^3$ cfu/cm$^2$ leaf area (OD$_{600}$ = 0.0002). At 72 hours, 10 leaves from each line were evaluated for disease symptoms and given a qualitative score: 1 = no symptoms, 2 = slight chlorosis, 3 = severe chlorosis, 4 = severe chlorosis and some necrotic lesions, 5 = leaf collapse (see Supplemental Figure 1A). Data are reported as means and standard deviations of the qualitative disease score. The disease assay was performed twice with similar results. A pairwise T-test was used to determine whether differences between lines were significant.

**Measurement of SA and SAG levels in *Arabidopsis* Leaves**

*Arabidopsis* plants for salicylic acid analysis were grown in Scotts Metro-Mix 200 (Scotts, Marysville, OH) with a 12 h photoperiod at a PAR of 100-150 µEm$^{-2}$s$^{-1}$. The EMS mutant *pbs3-1*, the SALK T-DNA insertion line *pbs3-2*, *eds16-1*, and Col-0 were infected at 4 weeks with OD$_{600}$ 0.0001 *Pst* DC3000 containing the avirulence gene *avrRpt2* on the pVSP61 plasmid (Kunkel et al., 1993). Overnight cultures of *Pst* DC3000(*avrRpt2*) grown in King’s B with rifampicin to OD$_{600}$ 0.7 were pelleted, resuspended in sterile 10 mM MgSO$_4$, and
diluted to OD$_{600}$ 0.0001. Three leaves per plant were infiltrated with either OD$_{600}$ 0.0001 Pst in 10 mM MgSO$_4$ or 10 mM MgSO$_4$ as a negative control using a needleless syringe. Mature, fully expanded rosette leaves were collected at 1 dpi, frozen in liquid nitrogen, and stored at -80º C.

The protocol for SA extraction and analysis was adapted from Dewdney et al. (2000). Frozen leaf samples (~0.5 g) were ground to a powder in a prechilled mortar and pestle using liquid nitrogen. The ground leaf material was transferred to a glass tube and suspended in 3 mL of 90% MeOH. 500 ng o-anisic acid (Aldrich) in 100% MeOH was added to each sample as an internal standard. Samples were vortexed, sonicated in a water bath sonicator for 20 min, and centrifuged at 5000 rpm for 15 min at 4º C. The supernatant was transferred to a new tube, and the brown pellet was resuspended in 2 mL 90% MeOH with vortexing. This suspension was sonicated for 20 min and centrifuged for 15 min at 4º C. The two supernatants from each sample were combined, vortexed to mix, and divided into two equal portions in new tubes (for free and total SA measurement). The solvent was evaporated using a dry vacuum at ~5 torr.

For total SA, 500 µL of 80U/mL β-glucosidase (Fluka) in 100mM sodium acetate (pH 5.2) was added. The samples were sonicated 5 min, vortexed, and incubated for 90 min at 37º C. For both total and free SA, 2.5 mL 5% trichloroacetic acid (Sigma) was added, and samples were vortexed, sonicated 5 min, and centrifuged at 5000 rpm for 15 min at 4º C. The supernatant was transferred to a new tube, and extracted two times with 2.5 mL of a 1:1 mixture of ethylacetate and cyclopentane. Organic phases were combined in a new tube, and the solvent was evaporated under vacuum as above. The evaporated samples were stored at -80º C until ready to load on the HPLC. Prior to loading, samples were resuspended in 125 µL 20% MeOH, vortexed, sonicated 5 min, and filtered through 0.45 µm PTFE filter (Millipore).

HPLC separation of leaf extracts was performed on a Shimadzu SCL-10A system with a Shimadzu RF-10A scanning fluorescence detector and Shimadzu SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan). Samples were separated on a 5 µm, 15 cm x 4.6 mm ID Supelcosil LC-ABZ+Plus column.
(Supelco) preceded by a LC-ABZ+Plus guard column maintained at 27º C. Prior to loading 50 µL sample, the column was equilibrated with 15% acetonitrile in 25 mM KH₂PO₄ pH 2.5 at a flow rate of 1.0 mL/min. The concentration of acetonitrile was increased linearly to 20% over 15 min, followed by isocratic flow at 20% for 5 min, followed by a linear increase from 20% to 43% over 23 min, a linear increase from 43% to 66% over 2 min, isocratic flow at 66% for 5 min, a linear decrease from 66% to 15% over 5 min, and isocratic flow at 15% for 3 min.

α-Anisic acid and salicylic acid were quantified using a fluorescence detector set at 305 nm excitation/365 nm emission for α-anisic acid and 305/407 for salicylic acid. The calibration curves used were $y = 4104.6x$ ($r^2 = 0.9997$) for α-anisic acid and $y = 3893.8x$ ($r^2 = 0.9988$) for salicylic acid, with $x$ in ng and $y$ in area units. Under these HPLC conditions, salicylic acid eluted at approximately 22 min and α-anisic acid at 10 min. The percent recovery of salicylic acid was estimated from that of α-anisic acid and ranged from 60-70% in the three separate experiments. The detection limit for α-anisic acid and salicylic acid were approximately 0.5 ng. SAG is calculated for paired samples as total SA – free SA.

**Analysis of PBS3, PR1, and ICS1 mRNA Levels**

Publicly available Affymetrix Gene Chip data were accessed through the Genevestigator web portal (http://www.genevestigator.ethz.ch/at/) (Zimmermann et al., 2004). Using the “Digital Northern” tool on this site, we determined that PBS3 was expressed at low levels (typically called “absent”) under the vast majority of experimental conditions (data from 2507 whole genome ATH1 chips). Exceptions were from experiments examining biotic stress.

Two particularly informative experiments compared expression in plants infected with different bacterial strains. Expression data from PBS3 (At5g13320) and ICS1 (At1g74710) were downloaded from NASCArrays (Craigon et al., 2004). Expression in response to infection with strains of *P. syringae* was examined in the dataset AtGenExpress: Response to virulent, avirulent, type III-
secretion system deficient and nonhost bacteria (NASCArrays-120) performed by Thorsten Nürnberg. Expression in response to *E. coli* and *P. syringae* lacking functional flagellin or type III secretion systems was examined in the dataset: Genome-wide transcriptional analysis of the compatible *Arabidopsis thaliana-Pseudomonas syringae* pv. *tomato* DC3000 interaction (NASCArrays-340) performed by William Underwood (Thilmony et al., 2006).

Correlation analyses were performed by downloading the full ATH1 expression dataset from NASCArrays (Craigon et al., 2004) and then limiting the dataset to those slides (GeneChips) using plant stock code N1092 (Col-0), a total of 821 GeneChips. The Pearson correlation (Freedman, 2005) between \( \log_2 \) expression values of *ICS1* (At1g74710) and the remaining >22K genes on the ATH1 GeneChip and between *PBS3* (At5g13320) and the remaining genes was computed across these 821 GeneChips.

For quantitative RT-PCR (qRT-PCR), RNA was isolated from infected leaf tissue immediately before inoculation, and 6 hrs, 12 hrs and 24 hrs after inoculation. RNA was purified using an RNeasy Plant Mini Kit from Qiagen. cDNA was generated using a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Figure 2) and random primers. qRT-PCR analyses were performed using the SYBR Green PCR Mastermix Kit from Applied Biosystems (Figure 2) or the SYBR Premix *Ex Taq* kit from TaKaRa Bio USA (Figure 4), and reactions were run on a Stratagene Mx3000 QRT-PCR system. Primer sequences for qRT-PCR reactions are listed in supplemental Table II. For all primer pairs, amplification of a single product was confirmed using a melting curve analysis. Efficiency of amplification was calculated by generating a standard curve using known dilutions of a wild-type Col-0 cDNA preparation. Default parameters of the Mx3000 instrument were used for calculating Ct values for each sample (i.e. the cycle number at which the detectable fluorescence signal began to increase exponentially). Relative expression values for each sample were normalized to \( \alpha \)-*TUBULIN3* (*TUA3*) using the formula \( 2^{(\text{Ct}_{\text{tubulin}} - \text{Ct}_{\text{target gene}})} \). Data presented in Figures 2 is the mean of three technical repeats employing the same cDNA template. The entire experiment (inoculations, RNA
extractions, cDNA synthesis and qRT-PCR) was repeated three times with similar results. Data presented in Figure 4 is the mean of three biological repeats.

For RT-PCR, RNA was isolated from infected tissue immediately before inoculation, 12, 24, and 48 hrs post inoculation with Pst DC3000(avsRpt2) OD600=0.0001. RNA was purified using the TRIzol method (Invitrogen), and cDNA was generated using SuperScript III (Invitrogen) with random primers. Primers used to amplify UBQ5 (ubiquitin 5; At3g62250) were UBQ5F (5' GTGGTGCTAAGAAGAGGAAGA 3') and UBQ5R (5' TCAAGCTTCAACTCCTTCTTT 3') yielding a 250 bp product. Primers used to amplify PR1 (At2g14610) were PR1F (5' TAGCCCACAAGATTATCTAAGG 3') and PR1R (5' CTCGTTCACATAATTCCCAC 3') generating a 391 bp product. Primers used to amplify PBS3 (At5g13320) were PBS3F (5' GAAGATGTGAAACTTGGGTGCAC 3') and PBS3R (5' CCTCCATTACAAACAACACG 3') yielding a 391 bp product. The PBS3 primers flank intron 3, the site of the T-DNA insertion in pbs3-2.

**Exogenous SA Treatment**

Five week old plants were sprayed with a 2.5 mM SA solution adjusted to pH 7.0. Leaves were collected immediately before spraying and 24 hours after spraying. qPCR and SA analyses were performed as described above. For bacterial growth assays following SA or INA pretreatment, either 4 mM SA or 0.65 mM of the active SA analog INA (2,6-dichloroisonicotinic acid) were employed. Bacterial counts were performed as detailed above.

**Phylogenetic Analysis of the Arabidopsis GH3 Family**

Protein sequences for the nineteen Arabidopsis GH3 proteins were obtained from TAIR and aligned using Megalign with the ClustalW method (Thompson et al., 1994) from the DNASTAR software package. Identical residues were highlighted in black and conserved residues in gray. The three
groups are those determined by Staswick et al. (2002) based on phylogeny (Staswick et al., 2002). The three motifs that form an AMP-binding domain in acyl-adenylases (Chang et al., 1997) are present in all 19 proteins.

**Accession Numbers**

Sequence data from this article can be found in the GenBank data library under accession numbers NM_121335 (cDNA sequence for \textit{PBS3}/At5g13320) and NP_196836 (protein sequence for \textit{PBS3}/At5g13320).

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Table S1.** Salicylic acid levels in \textit{pbs3} mutants normalized to wild-type Col-0 in pathogen infected leaves.
- **Supplemental Table S2.** Primers used for qRT-PCR.
- **Supplemental Figure S1.** Semi-quantitative disease scoring scale.
- **Supplemental Figure S2.** \textit{PBS3} transcript in the \textit{pbs3-1} and \textit{pbs3-2} mutants.
- **Supplemental Figure S3.** Protein sequence alignment of Arabidopsis GH3 family.

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FIGURE LEGENDS

Figure 1. Identification of the PBS3 gene. A, Genetic and physical map of the region containing PBS3. B, Growth of P. syringae strain ES4326 in wild-type and pbs3-1 mutant leaves. C, Semi-quantitative disease scores of wild-type and pbs3 mutant plants inoculated with ES4326. D, Sequence of PBS3 highlighting locations of pbs3-1 mutations, Ds and T-DNA insertions, along with locations of conserved domains and ATP binding site. E, Complementation of pbs3 mutants with the wild-type PBS3 gene (semi-quantitative disease scores).

Figure 2. Pathogen-induced expression of PR1 is delayed and reduced in the pbs3 mutant. A, RT-PCR analysis of PR1 expression in wild-type, pbs3 mutants, and eds16-1 in response to Pst DC3000(avrRpt2) (OD600 0.0001). B, qRT-PCR analysis of PR1 expression in wild-type and the pbs3-1 mutant in response to Psm ES4326 (OD600 0.002).

Figure 3. SAG accumulation is dramatically reduced in pbs3 mutant plants in response to Pst DC3000(avrRpt2). A, Free SA and SAG levels in wild-type Col-0, pbs3-1, and pbs3-2 inoculated with Pst DC3000(avrRpt2) (OD600 0.0001), or with 10 mM MgSO4 at 24 hrs post inoculation. B, Free SA and SAG levels in wild-type, pbs3-1, and pbs3-2 compared with the SA biosynthetic mutant eds16-1 at 24 hpi with Pst DC3000(avrRpt2) (OD600 0.0001). Each bar represents the average of three replicates +/- standard deviation. *= below detection limit.

Figure 4. Exogenous treatment with 2.5 mM SA of pbs3-1 restores induced PR1 expression and SAG accumulation at 24 hr post treatment. A. qRT-PCR analysis of PR1 expression in pbs3-1 and wild-type in response to exogenous SA treatment. Shown is the average with standard deviation of three biological replicates, from independent experiments. B, SAG levels in pbs3-1 and wild-type plants in response to exogenous SA treatment. Each bar represents the average of three replicates with standard deviation.
Figure 5. Comparative expression of PBS3 and ICS1 in response to bacteria. A, Comparative expression of PBS3, ICS1, and PR1 in response to P. syringae. Expression in response to non-host (Psp), avirulent (Pst DC3000 (avrRpt2)), virulent (Pst DC3000), a type III secretion deficient mutant (Pst DC3000 (hrcC)), and mock MgCl2 treatment is shown. Expression data was acquired from NASCArrays (Craigon et al., 2004) for the experiment performed by Nürnberg (NASCArrays-120). Dosage for each inoculation was 1x10⁸ cfu/mL. B, Ratio of expression induced by type III secretion deficient versus type III secretion wild-type P. syringae strains for the PBS3 and ICS1 genes. The Pst hrpA and hrpS mutants are deficient in type-III secretion, and Pst COR is deficient in the synthesis of the toxin coronatine. Expression data was acquired from NASCArrays for the experiment performed by Underwood (NASCArrays-340), published in Thilmony, et al. (2006). The Pst and Pst hrpA inoculations were performed at a dosage of 1x10⁸ bacteria/mL and samples were collected after 7 hrs. The Pst COR hrpS and COR inoculations used 5x10⁷ cfu/mL with collection 10 hpi and 1x10⁶ cfu/mL with collection 24 hpi respectively. C, NASCArray Gene Correlation Plot for log PBS3 (At5g13320) vs. log ICS1 (At1g74710) expression using the full ATH1 Affymetrix wild-type NASCArray dataset. Perfect positive correlation would be an upward diagonal line. Note the high degree of correlation in slides (GeneChips) with significant induction of both genes.

Figure 6. Proposed biochemical functions of PBS3. These models assume PBS3 activity results in conjugation of an amino acid to a small molecule (such as a phytohormone), similar to characterized GH3 family members. A. PBS3 acts directly on salicylic acid (SA) to form an SA-amino acid conjugate. In this case, SA-AA, itself, may be the active form of SA required for SAG accumulation and induction of PR1 expression (TOP). Alternatively, the formation of SA-AA may be required for a subsequent conversion to an unknown active form of SA. In the bottom panel, the formation of an SA-AA conjugate is
required for the proper spatial localization of SA. The SA-AA conjugate is translocated and then hydrolyzed to release SA, resulting in SAG accumulation and PR1 induction. B. PBS3 acts on a competitive inhibitor (X) of SA to inactivate it by forming X-AA, resulting in full SAG accumulation and activation of SA-dependent transcriptional responses. The differential impact of X on SAG accumulation vs. PR1 expression could be explained by different binding affinities of the inhibitor for distinct enzymes controlling these processes. C. PBS3 acts upstream of SA either in a regulatory capacity or on a precursor of SA. In this case, net SA biosynthesis is reduced, resulting in reduced SAG accumulation and PR1 expression. Activators of SAG accumulation and PR1 expression are shown in green whereas inhibitors are shown in red.
Supplemental Figure Legends

Supplemental Figure 1. Semi-quantitative disease scoring scale. Leaves of wild-type Col-0 and pbs3-1 mutant plants were inoculated with Psm strain ES4326 (OD$_{600}$ 0.0002) and removed for photography 72 hrs post inoculation.

Supplemental Figure 2. PBS3 transcript in the pbs3-1 and pbs3-2 mutants. RT-PCR was performed using RNA isolated from mature Arabidopsis leaves treated with Pst DC3000(avrRpt2) at OD$_{600}$=0.0001 before (0), 12, 24, and 48 hrs post inoculation. M= molecular weight marker. Primers are detailed in Materials and Methods. The PBS3 primers flank intron 3, the site of the T-DNA insertion in pbs3-2.

Supplemental Figure 3. Protein sequence alignment of Arabidopsis GH3 family. The Arabidopsis GH3 protein sequences were aligned using Megalign with the ClustalW method from the DNASTAR software package using default parameters. Identical residues are highlighted in black and conserved residues in gray. The three GH3 family subgroups (Staswick et al., 2002) are indicated and gene names associated with mutant characterization (PBS3, DFL1, YDK1, JAR1, and DFL2) are noted next to the gene locus. The three motifs composing the AMP-binding domain of acyl-adenylases are indicated above the sequence. Asterices above the sequences indicate positions mutated in pbs3-1 (E502K and I519T).
A

Normalized Expression

PR1

Untreated SA 24 h

Col-0 pbs3-1

B

µg SAG/g FW

Untreated SA 24 h

Col-0 pbs3-1

See inset
A. PBS3 acts on SA

\[
\text{SA} \xrightarrow{\text{PBS3}} \text{SA-AA} \xrightarrow{\text{translocation}} \text{SA} \\
\text{• SAG accumulation} \\
\text{• Activation of SA-dependent transcriptional responses (e.g. PR1)}
\]

B. PBS3 acts on a competitive inhibitor (X) of SA

\[
\text{SA} \xrightarrow{\text{PBS3}} \text{SA-AA} \\
\text{X} \xrightarrow{\text{PBS3}} \text{X-AA} \\
\downarrow \text{SAG accumulation} \\
\downarrow \text{Activation of SA-dependent transcriptional responses (e.g. PR1)}
\]

C. PBS3 acts upstream of SA

\[
\text{PBS3} \xrightarrow{\text{}} \text{SA} \\
\text{• SAG accumulation} \\
\text{• Activation of SA-dependent transcriptional responses (e.g. PR1)}
\]