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


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The pbs3-1 mutant, identified in a screen for Arabidopsis (Arabidopsis thaliana) mutants exhibiting enhanced susceptibility to the avirulent Pseudomonas syringae pathogen DC3000 (avrPphB), also exhibits enhanced susceptibility to virulent P. syringae strains, suggesting it may impact basal disease resistance. Because 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 (PR) marker, PR1, was assessed. Surprisingly, whereas accumulation of the SA glucoside and expression of PR1 were dramatically reduced in the pbs3-1 mutant in response to P. syringae (avrRpt2) infection, free SA was elevated. However, in response to exogenous SA, the conversion of free SA to SA glucoside 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 nonconserved amino acid changes in highly conserved residues. Additional analyses with Arabidopsis containing 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; S.K. Marr and M.C. Wildermuth, unpublished data). In Arabidopsis (Arabidopsis thaliana), the bulk of pathogen-induced SA accumulates as the SA-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.
To identify additional components of the R-protein signal transduction pathway, Warren et al. (1999) screened for Arabidopsis 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 var. Columbia-0 (Col-0) is mediated by the *RPS5* gene (Simonich and Innes, 1995), which encodes a member of the nucleotide-binding site Leu-rich repeat 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 *enhanced disease susceptibility5* (eds5)/SA induction deficient1 (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 *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, 2005; Staswick and Tiryaki, 2004). We found that *pbs3* mutants are compromised in both pathogen-induced accumulation of the *SAG* and expression of the SA-dependent marker gene *PR1* (*At2g14610*). Surprisingly, the level of free SA was about 2-fold higher than wild-type plants. Exogenous application of SA was sufficient to restore *SAG* accumulation, *PR1* expression, and enhanced resistance to virulent DC3000. Because 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*), and plants displaying disease symptoms were 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 (Fig. 1). We then performed high-resolution genetic mapping by using PCR to preselect 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 (Fig. 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 Pst DC3000 (*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 1 d 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 *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 *P. syringae pv maculicola* strain ES4326 (*Psm*). Using this assay, wild-type Col-0 plants produce little to no disease symptoms, whereas *pbs3-1* mutants produce marked yellowing with occasional lesions (data not shown). We developed a semiquantitative disease phenotype scale to score these symptoms, ranging from a value of 1 (no symptoms) to 5 (complete collapse of the inoculated region; Supplemental Fig. S1A). We validated this scoring system by also titering bacterial populations in infected leaves of both wild-type and *pbs3-1* mutant plants at 3 d post-inoculation (dpi). Figure 1B shows that the *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 *pbs3-1* mutant (Fig. 1C). We also obtained a Dissociation (*Ds*) transposon line from the RIKEN collection with an insertion in *At5g13320* (Kuromori et al., 2004). This line, which is in the Nössen (No-0) genetic background, was also significantly more susceptible to ES4326 than its wild-type sibling (Fig. 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 Lys for a Glu (E502K) and a Thr for an Ile (I519T; Fig. 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 significant reduction in disease scores, confirming that \textit{At5g13320} is \textit{PBS3} (Fig. 1E).

We then examined \textit{PBS3} transcripts in the \textit{pbs3-1} and \textit{pbs3-2} mutants. As shown in Supplemental Figure S2, \textit{PBS3} transcript was detected in wild-type and \textit{pbs3-1} plants in response to 	extit{Pst DC3000 (avrRpt2)}, but...
not in the insertion mutant \textit{pbs3-2} using primers that flank intron 3, the location of the T-DNA insertion (see Fig. 1D).

**PBS3 Belongs to the GH3 Family of Acyl Adenylasases**

\textit{At5g13320} is a member of the GH3 multigene family that consists of 19 family members in Arabidopsis var. Col-0 (Staswick et al., 2002). The first GH3 gene described was isolated from soybean (\textit{Glycine max}) as an early auxin-responsive gene (Hagen and Guilfoyle, 1985). Since then, homologs have been identified in many plants, including Arabidopsis, rice (\textit{Oryza sativa}), \textit{Gossypium} spp., \textit{Lycopersicon} spp., \textit{Populus} spp., \textit{Pinus} spp., and the moss \textit{Physcomitrella patens} based on ESTs or sequence data (Terol et al., 2006) and in some bacteria (R. Okrent and M. Wildermuth, 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: 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 indole acetic acid (IAA) with mutants exhibiting altered phytohormone phenotypes (Staswick et al., 2002, 2005; Staswick and Tiryaki, 2004). For example, the \textit{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 \textit{JAR1}, a JA-amino acid synthetase; group II members, which are capable of adenylyating IAA; and group III members, which include PBS3 (GH3.12) and act on unknown substrates (Staswick et al., 2002, 2005; Staswick and Tiryaki, 2004). Recombinant PBS3 did not show significant activity with any of the tested substrates—JA, JA methyl ester, IAA, abscisic acid, 1-aminocyclopropane-1-carboxylic acid, gibberellic acid, 2-oxo-phytodienoic 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 S3, 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 E519T) 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 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 conjugation of the amino acid to the activated AMP intermediate, although 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{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 h postinoculation (hpi) in wild-type plants, whereas \textit{PR1} transcript was not detected in \textit{pbs3-1} or \textit{pbs3-2} plants until 48 hpi. Similarly, the virulent strain ES4326 induced \textit{PR1} highly by 24 hpi in wild-type plants, whereas transcript levels were at least 50-fold lower in the \textit{pbs3-1} mutant (Fig. 2B).

The reduced levels of \textit{PR1} transcript in \textit{pbs3} mutants suggested that production and/or perception of SA might be compromised. We therefore measured both free SA and SAG levels in \textit{pbs3-1} and \textit{pbs3-2} mutant plants before and 24 hpi with \textit{Pst} DC3000 (\textit{avrRpt2}). At this time point, wild-type plants exhibit significant expression of \textit{PR1}, whereas \textit{pbs3} mutants do not (Fig. 2C).
We found significant accumulation of SAG in wild-type plants, with an average 5-fold reduction in accumulated SAG in pbs3 mutant plants (Fig. 3A; Supplemental Table S1). Surprisingly, the pbs3 mutants accumulated approximately 2-fold more free SA than wild type at this time point (Fig. 3A; Supplemental Table S1). Because the majority of SA is found as SAG, the total SA present in the pbs3 mutants 24 hpi was significantly lower (2- to 3-fold) than in wild-type plants. 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 pathogens have been described. In contrast, here, we report on a mutant exhibiting elevated free SA with dramatically reduced SAG accumulation in response to pathogens. 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 P. 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 Glc conjugate and expression of PR1.

Exogenous SA Restores SAG Accumulation, PR1 Expression, and Resistance

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

SAG formation was also comparable for pbs3-1 and wild-type leaves (Fig. 4B) at 24 hpt with SA. SAG is calculated by subtracting measured free SA (no hydrolysis) from measured total SA (after hydrolysis of

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; OD<sub>600</sub> 0.0001), or with 10 mM MgSO<sub>4</sub> at 24 hpi. 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; OD<sub>600</sub> 0.0001). Each bar represents the average of three replicates ± sd. *, Below detection limit.

Figure 4. Exogenous treatment with 2.5 mM SA of pbs3-1 restores induced PR1 expression and SAG accumulation at 24 hpt. A, qRT-PCR analysis of PR1 expression in pbs3-1 and wild type in response to exogenous SA treatment. Shown is the average with SD 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 SD.
SAG to SA), as described in “Materials and Methods.” Significant SAG (approximately 25 μg/g fresh weight) was formed in leaves of wild-type and 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 Psm ES4326 (OD<sub>600</sub> = 0.0002) and assessed bacterial growth immediately after inoculation and at 3 dpi. We also performed parallel experiments using the active SA analog 2,6-dichloroisonicotinic acid (INA; 0.65 mM) for the pretreatment instead of SA (4 mM). Bacterial growth at 3 dpi was limited by pretreatment of pbs3-1 and wild-type leaves with the SA analog INA (data not shown). Although SA pretreatment also conferred resistance to Psm ES4326 for both the 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 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 PRI 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 UVB. As shown in Figure 5A, PBS3 is expressed most rapidly and strongly in response to nonhost 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 do *Pst* (Fig. 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 Thimony et al. (2006) microarray dataset provides even stronger evidence in support of TTSS involvement because PBS3 induction is dramatically reduced in the *Pst, hrpS*, and *hrpA* mutants, which cannot express or secrete TTSS effector genes and proteins, respectively (Fig. 5B).

As shown in Figure 5, expression of the pathogen-induced SA biosynthetic gene ICS1 (SID2/EDS16) parallels that of PBS3. Because PBS3 impacts pathogen-induced free SA and SAG accumulation, as well as expression of the SA-dependent marker PRI, we assessed whether ICS1 and PBS3 expression are correlated across all experiments using wild-type Col-0 plants in the NASCArray 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 PRI (At2g14610) with ICS1 is 0.42. As shown in Figure 5C, when significant expression of ICS1 and PBS3 is observed, their expression is very highly correlated. Indeed, PBS3 is the second most correlated gene with ICS1 of the >22 K genes on the ATH1 GeneChip, whereas PRI 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 SAG as the dominant form of SA detected in plants (Enyedi et al., 1992; Malamy et al., 1992). Infection by pathogens such as *Tobacco mosaic virus* and *P. syringae* pathogens rapidly induces the accumulation of free SA and SAG (Malamy et al., 1992; Chong et al., 2001; Shapiro and Gutsche, 2003). The conversion of free SA to SAG has been followed using radiolabeled SA (Dean et al., 2005) and appears to be catalyzed by UDP-Glc:SA glucosyltransferases (SAGT). In tobacco (*Nico-tiana tabacum*) 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., 2007), 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 nonhydrolyzable SAG analog thio-SAG, results in the accumulation of free SA and expression of PRI (Hennig et al., 1993).
The pbs3 mutants exhibit approximately 5-fold reduction in pathogen-induced SAG accumulation compared to wild-type Arabidopsis plants (Fig. 3; 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 not only affects accumulation of SAG, but somehow impacts 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 pbs3 mutants with no significant induction by 24 hpi with virulent Psm ES4326 (Fig. 2B) and an approximately 36-h delay in induction in response to avirulent Pst (avrRpt2; Fig. 2A). 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 is correlated (Fig. 5C).
and ICS1 and PBS3 expression precedes the dramatic induction of PR1 expression (Fig. 5A; most obvious in response to Fsp and avirulent Pst). In pbs3 mutants, despite the elevated induced free SA levels at 24 hpt with Pst (avrRpt2), PR1 is not induced (Fig. 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 + SAG) better represents plant cell exposure to free SA during the first 24 h 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 (avrRpt2) 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., Jirage et al., 1999; Shah, 2003).

Review of the relevant literature indicates that when total leaf SA levels are less than 3 μg/g fresh weight, 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/g fresh weight, PR1 is not significantly induced (Verberne et al., 2000; Mauch et al., 2001). 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 because 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, whereas 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 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-amino acid 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. Although 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, although this requires experimental confirmation.

Could PBS3 Act as an SA-Amino Acid Synthetase?

As shown in Figure 6A (top), an SA-amino acid 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-amino acid conjugates (Staswick and Tiryaki, 2004). Although amino acid conjugates of SA have not been reported in Arabidopsis, they have been detected in grape (Vitis vinifera; Steffan et al., 1988) and bean (Phaseolus vulgaris; Bourne et al., 1991). If the SA-amino acid 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 hpi with SA, PR1 was expressed similarly in leaves of pbs3 and wild-type plants. This suggests that either SA-amino acid 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 greater excess of the substrate SA. One candidate for this compensatory role is GH3.5 (At4g27260), the only tested GH3 family member to exhibit amidohydrolase 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 (Fig. 6A, bottom), PBS3 could act on SA to form an SA-amino acid 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 Pst (avrRpt2), free SA would be synthesized in the chloroplast of a cell as an early event associated with HR. It could then be modified to SA-amino acid by PBS3 in the cytosol of the HR-undergoing cell and then exported to neighboring cells. In the neighboring cells, the SA-amino acid conjugate could be hydrolyzed, releasing free SA that could then induce PR1 expression. SAG formation catalyzed by a cytosolic SAGT could also occur in these
neighboring cells. In this case, in the pbs3 mutant, free SA would be elevated because it would not be converted to SA-amino acid, and SAG formation in neighboring cells would be reduced. Exogenous SA application would negate this requirement for SA transport, thus restoring PR1 expression and SAG formation to wild-type levels in the pbs3 mutant. Consistent with this model, in tobacco, free SA accumulation preceded visible HR symptoms by >24 h, 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 PR1 accumulation was not observed in cells undergoing an HR (and subsequently dying), but specifically around HR lesions in tobacco and Arabidopsis (e.g. Dorey et al., 1997; Stone et al., 2000).

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-amino acid (Fig. 6B). Amino acid conjugation can either inactivate phytohormones or target them for degradation pathways (Woodward and Bartel, 2005). In 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 *Escherichia 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 (Cao et al., 1997) with TGA transcription factors enhancing DNA binding (Fan and Dong, 2002; Despres et al., 2003). 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) versus PR1 expression (approximately 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 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 (Fig. 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,
application of exogenous SA would restore SAG formation and PR1 expression in the \textit{pbs3} mutants as we observed. However, we would also expect both induced free SA and SAG levels to be reduced in the \textit{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 \textit{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 \textit{pbs3} mutant, free SA might be at its maximum at 24 hpi, with less of it having been converted to SAG, thereby resulting in higher free SA and lower SAG levels in the \textit{pbs3} mutant. In this scenario, the induction of \textit{PR1} expression requires some threshold of total SA (free + SAG) that was not met at 24 hpi with \textit{Pst (avrRpt2)} in \textit{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 SA accumulation, \textit{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 \textit{pbs3-1} mutant, which contains two point mutations resulting in nonconserved 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

\textit{Pseudomonas syringae} strains \textit{Psm} ES4326 and \textit{Pst DC3000} have been described previously (Dong et al., 1991; Whalen et al., 1991). The avirulence gene \textit{avrRpt2} was cloned into broad host-range vector pVS61 and introduced into these strains by triparental mating as described by Kunkel et al. (1993). Bacteria were cultured on King’s B medium (10 mg/mL protease peptone; 15 mg/mL glycerol; 1.5 mg/mL K2HPO4; 4 mM MgSO4, pH 7.0) supplemented with 100 \mu g/mL streptomycin (ES4326) or 100 \mu g/mL rifampicin (DC3000) plus 50 \mu g/mL kanamycin when \textit{avrRpt2} was present in the strain. \textit{Arabidopsis (Arabidopsis thaliana)} var. Col-0 and var. No-0 were grown in Metromix 360 in growth rooms under a 9-h light/15-h dark cycle at 23°C. Isolation of the \textit{pbs3-1} mutant was described by Warren et al. (1999). T-DNA insertion lines (Col-0 genetic background) were obtained from the ABRC (Alonso et al., 2003). A Ds transposon insertion allele of \textit{PBS3} (No-0 genetic background) was obtained from the RIKEN Plant Functional Genomics Research Group (Kuramori et al., 2004).

Genetic Mapping of \textit{pbs3-1}

\textit{F2} progeny of a \textit{pbs3-1} cross to \textit{Arabidopsis} var. Landsberg erecta were used to genetically map the \textit{PBS3} gene. \textit{F2} plants were inoculated with \textit{Pst DC3000 (avrRpt2)} and scored 3 dpi. Plants displaying a \textit{pbs3-1} phenotype were used for mapping. Initially, the \textit{pbs3-1} mutation was mapped to chromosome 5 between microsatellite markers nga249 and nga151. To further localize the \textit{PBS3}, we scored approximately 800 \textit{F2} plants with these two markers and identified 70 plants with recombinant events within this interval. \textit{F3} progeny of these plants were assayed for resistance to DC3000 (avrRpt2). Analysis of these lines placed the \textit{pbs3} mutation between markers MBJSN01 and CHS1, an interval of approximately 300 kb. A collection of 40 Arabidopsis T3000A insertion lines with insertions in genes in this interval was then obtained from the ABRC 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 \textit{Dis} 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) and separated on an ABI 3730 automated DNA sequencer.

Complementation of \textit{pbs3} Mutants

A full-length 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) and the following primers: 5′-CTGCAGAAATTTTGCAGAATTCCTT-3′ and 5′-CTGCAGAATACGAA-GGTGTGTCATCA-3′, which contain Pst restriction sites at their 5′ ends. The PCR product was ligated into the pGEM-T Easy plasmid vector (Promega) and transformed into Escherichia coli strain DH10B. The PBS3 insert was then removed from this clone by digestion with PstI and ligated with the binary vector pGreen0229 digested with PstI (Hellens et al., 2000). This DNA sequence contains a full-length At5g13320 gene, including 1,020 bp upstream of the translation initiation site to 127 bp downstream of the stop codon. The pGreen0229:A5g13320 construct was transformed into Agrobacterium tumefaciens strain GV3101 carrying helper plasmid pSoup and disarmed Ti plasmid pMP90 by electroporation and selected on Luria-Bertani 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) at a concentration of 0.1 g/L sulfosulfate 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.

Measurement of Bacterial Growth in \textit{Arabidopsis} Leaves

Leaves of 5-week-old plants were injected with \textit{Psm} ES4326 at a dose of 108 cfu/cm² leaf area (OD600 = 0.0002). At 1 and 72 h, a 0.7-cm-diameter disc from each of 12 leaves was excised using a cork borer. These 12 discs were divided into four replicates of three leaf discs each and grown in 1 mL 10 mM MgCl2 with a plastic pestle. Appropriate dilutions were plated on King’s B medium containing streptomycin and bacterial colonies were counted. Data are reported as means and sds of the log (cfu/cm²) of four 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 \textit{Psm} ES4326 at a dose of 106 cfu/cm² leaf area (OD600 = 0.0002). At 72 h, 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 Fig. S1A). Data are reported as means and sds 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 \textit{Arabidopsis} Leaves

Arabidopsis plants for SA analysis were grown in Scotts Metro-Mix 200 with a 12-h photoperiod at a photosynthetically activated radiation of 100 to 150 \text{μEm}^{-2} \text{s}^{-1}. The ethyl methanesulfonate mutant \textit{pbs3-1}, the SALK T-DNA

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insertion line pbs3-2, eds16-1, and Col-0 were infected at 4 weeks with OD600 0.0001 Pst DC3000 containing the avirulence geneavrRpt2 on the pVSP91 plasmid (Kunkel et al., 1993). Overnight cultures of Pst DC3000 (avrRpt2) grown in King’s B with rifampicin to OD600 0.7 were pelleted, resuspended in sterile 10 mM MgSO4, and diluted to OD600 0.0001. Three mature, fully expanded rosette leaves per plant were infiltrated with either OD600 0.0001 Pst in 10 mM MgSO4 or 10 mM MgSO4 as a negative control using a needleless syringe. Leaves were collected at 1 dpi, frozen in liquid nitrogen, and stored at –80°C. The protocol for SA extraction and analysis was adapted from Dewdney et al. (2000). Frozen leaf samples (approximately 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. Five hundred nanograms of 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 5,000 rpm for 15 min at 4°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 at 15 min at 4°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 approximately 5 Torr. For total SA, 500 μL of 80 units/mL β-glucosidase (Fluka) in 100 mM sodium acetate (pH 5.2) were added. The samples were sonicated for 5 min, vortexed, and incubated for 90 min at 37°C. For both total and free SA, 2.5 mL 5% TCA (Sigma) was added, and samples were vortexed, sonicated for 5 min, and centrifuged at 5,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube and the two supernatants were combined in a new tube and the solvent was evaporated under vacuum as above. The evaporated samples were stored at –80°C until ready to load on the HPLC. Prior to loading, samples were resuspended in 125 μL 20% MeOH, vortexed for 5 min, and filtered through a 0.45-μm polytetrafluoroethylene filter (Millipore). HPLC separation of leaf extracts was performed on a Shimadzu SCL-10A system with a Shimadzu RF-10A scanning fluorescence detector and a C18 column (Cotlove et al., 1991) using acetonitrile-water gradient elution. Calibration curves were created using a fluorescence detector set at 380/405 for o-anisic acid and 360/407 for SA. Calibration curves were y = 1140.6x2 (r2 = 0.9997) for o-anisic acid and y = 3893.8x2 (r2 = 0.9988) for SA, with x in nanograms and y in area units. Under these HPLC conditions, SA eluted at approximately 15 min at 10 μl/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 45% 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. o-Anisic acid and SA were quantified using a fluorescence detector set at 305 nm excitation/365 nm emission for o-anisic acid and 360/407 for SA. The percent recovery of SA was estimated from that of o-anisic acid and ranged from 60% to 70% in three separate experiments. The detection limit for o-anisic acid and SA 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 absence) under the vast majority of experimental conditions (data from 2,507 whole-genome ATH1 chips). Exceptions were from experiments examining biotic stress. For quantitative reverse transcription PCR (qRT-PCR), RNA was isolated from infected leaf tissue immediately before inoculation and 6, 12, and 24 hpi. RNA was purified using an RNaseasy plant mini kit cdna was generated using a high-capacity cdna reverse transcription kit from Applied Biosystems (Fig. 2) and random primers. qRT-PCR analyses were performed using the SYBR Green PCR Mastermix kit from Applied Biosystems (Fig. 2) or the SYBR Premix Ex Taq kit from TaKaRa Bio USA (Fig. 4), and reactions were run on a Stratagene MX3000 qRT-PCR system. Primer sequences for qRT-PCR reactions are listed in Supplemental Table S2. For all primer pairs, amplification of a single product was confirmed using 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 threshold cycle (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 α-TUBULIN3 (TUA3) using the formula 2-Cttarget gene – Ct target gene. Data presented in Figure 2 are 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 are the means of three biological repeats.

RT-PCR, RNA was isolated from infected tissue immediately before inoculation, 12, 24, and 48 hpi with Pst DC3000 (avrRpt2) 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 (At1g74710) and the remaining genes was PB3-primers flanks intron 3, the site of the T-DA 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 h after spraying. qRT-PCR 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 were employed. Bacterial counts were performed as detailed above.

Phylogenetic Analysis of the Arabidopsis GH3 Family

Protein sequences for the 19 Arabidopsis GH3 proteins were obtained from The Arabidopsis Information Resource 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. The three motifs that form an AMP-binding domain in acyl-adenylases (Chang et al., 1997) are present in all 19 proteins.

Sequence data from this article can be found in the GenBank data library under accession numbers NM_121335 (cdna sequence for PBS3/At1g3320) and NP_196836 (protein sequence for PBS3/At1g3320).

Supplemental Data

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

Supplemental Figure S1. Semiquantitative disease-scoring scale.

Supplemental Figure S2. PBS3 transcript in pbs3-1 and pbs3-2 mutants.
Supplemental Figure S3. Protein sequence alignment of the Arabidopsis GH3 family.

Supplemental Table S1. SA levels in pbs3 mutants normalized to wild-type Col-0 in pathogen-infected leaves.

Supplemental Table S2. Primers used for qRT-PCR.

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LITERATURE CITED


PB53 Regulates Salicylic Acid-Dependent Defense Responses
Supplemental Table S1

**Supplemental Table S1.** *Salicylic acid levels in pbs3 mutants normalized to Col-0 in pathogen infected leaves.*

Free SA and SAG in µg/g fresh weight normalized to Col-0 24 hpi with *Pst avrRpt2* at OD$_{600}$ 0.0001 for three independent experiments each done in triplicate. SA and SAG levels were calculated based on the average percent recovery of an internal o-anisic acid standard for each experiment.

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### Supplemental Table S2. Primers used for qRT-PCR

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Supplemental Figure 1
Supplemental Figure 2

Col-0  eds16-1  pbs3-1  pbs3-2
24 48 0 12 24 48 0 12 24 48 0 12 24 48  M

PBS3
UBQ5
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(avnRpt2) 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).