

The *Arabidopsis* *PBS1* resistance gene encodes a member of a novel protein kinase subfamily

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Summary

Specific recognition of *Pseudomonas syringae* strains that express the avirulence gene *avrPphB* requires two genes in *Arabidopsis*, *RPS5* and *PBS1*. Previous work has shown that *RPS5* encodes a member of the nucleotide binding site-leucine rich repeat class of plant disease resistance genes. Here we report that *PBS1* encodes a putative serine-threonine kinase. Southern blot analysis revealed that the *pbs1-1* allele contained a deletion of the 3' end of the *PBS1* open reading frame. DNA sequence analysis of the *pbs1-2* allele showed it to be a missense mutation that caused a glycine to arginine substitution in the activation segment of *PBS1*, a region known to regulate substrate binding and catalytic activity in many protein kinases. The identity of *PBS1* was confirmed using both transient transformation and stable transformation of mutant *pbs1* plants. Comparison of the predicted *PBS1* amino acid sequence with other plant protein kinases revealed that *PBS1* belongs to a distinct subfamily of protein kinases that contains no other members of known function. The Pto kinase of tomato, which is required for specific resistance to *P. syringae* strains expressing *avrPto*, did not fall in the same subfamily as *PBS1* and is only 42% identical in the kinase domain. These data suggest that *PBS1* and Pto may fulfil different functions in the recognition of pathogen avirulence proteins. We discuss several possible models for the roles of *PBS1* and *RPS5* in *AvrPphB* recognition.

Keywords: pathogen recognition, gene-for-gene, *Pseudomonas syringae*, avirulence gene, resistance gene, *RPS5*.

Introduction

The resistance of plants to pathogens is often governed by the presence of dominant or semidominant resistance (*R*) genes in the plant and dominant avirulence (*avr*) genes in pathogen (Flor, 1971). Resistance is usually correlated with the induction of programmed cell death within the infected tissue. This response is known as the hypersensitive response or HR (Greenberg, 1997). An *R*-gene dependent HR can also be initiated by transgenic expression of some pathogen *avr* genes in plants (Gopalan *et al.*, 1996; Leister *et al.*, 1996). These data indicate that such *avr* proteins are being recognized directly or indirectly by cognate *R*-gene products in the host cell cytoplasm. Consistent with this view, most bacterial *avr* proteins are believed to be secreted directly into host cells by a bacterial type III secretion system (Alfano and Collmer, 1996). How *R*-gene products mediate recognition of pathogen *avr* products is poorly understood, however.

Several *R*-genes from various plant species have been cloned in recent years. The largest group of *R*-genes encodes products that are characterized by the presence of a nucleotide-binding site (NBS) and leucine rich repeats (LRR) (Jones and Jones, 1997). LRR domains are found in many proteins that fulfill diverse functions in the cell and are believed to mediate protein–protein interactions (Kobe and Deisenhofer, 1995). Mutations that compromise resistance to specific pathogen strains are often localized in the LRR region of *R*-gene products (Gassmann *et al.*, 1999; Grant *et al.*, 1995; Mindrinis *et al.*, 1994; Warren *et al.*, 1998). In addition, comparison among *R*-gene LRR sequences have revealed a rapid rate of amino acid substitutions, implying that they are under divergent selection (McDowell *et al.*, 1998; Meyers *et al.*, 1998; Parniske *et al.*, 1997). This has led to speculation that *R* proteins might function as receptors for *Avr* factors

(Hammond-Kosack and Jones, 1997). Consistent with this model, the *R*-gene product RPS2 and the bacterial protein AvrRpt2 can be coimmunoprecipitated when proteins are transiently expressed in *Arabidopsis thaliana* mesophyll protoplasts (Leister and Katagiri, 2000). Additionally, the fungal avr protein AVR-Pita from *Magnaporthe grisea* has recently been shown to interact with the leucine rich domain of the Pi-ta protein from rice, using both a yeast two-hybrid assay and an *in vitro* nitrocellulose filter assay (Jia *et al.*, 2000). Cumulatively, these data suggest that at least some NBS-LRR proteins may function directly as receptors for pathogen derived protein ligands.

A second class of *R*-genes is represented by the *Pto* gene of tomato, which encodes a serine/threonine protein kinase. *Pto* confers resistance to *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *avrPto*. A direct interaction between *Pto* and *AvrPto* has been revealed using yeast two-hybrid assays (Scofield *et al.*, 1996; Tang *et al.*, 1996). Furthermore, mutations in either protein that disrupt their interaction in yeast also block elicitation of the hypersensitive response in plants (Tang *et al.*, 1996), indicating that this interaction is biologically relevant. *Pto* also interacts with another serine/threonine protein kinase, *Pti1*, and with the transcription factors *PTI4/5/6* (Zhou *et al.*, 1995; Zhou *et al.*, 1997), but the contribution of these proteins to *Pto*-mediated disease resistance has not yet been established.

Specific recognition of *AvrPto* by tomato requires at least one gene in addition to *Pto*, the *Prf* gene. *Prf* belongs to the NBS-LRR class of *R*-genes (Salmeron *et al.*, 1996). Rathjen *et al.* (1999) recently showed that specific mutations in the activation loop of the *Pto* kinase can cause *Pto* to induce an HR-like response in tomato in the absence of *AvrPto* (Rathjen *et al.*, 1999); however, this response was dependent on *Prf*. These data suggest that the *Prf* protein may not directly interact with *AvrPto*. Thus it is unclear whether NBS-LRR proteins generally function as receptors for pathogen-derived ligands, or as intermediate signal transduction components.

To address this question, we have been studying recognition of the *AvrPphB* protein from *P. syringae* pv. *phaseolicola* by *Arabidopsis thaliana*. Specific recognition of *AvrPphB* requires at least two genes, *RPS5* and *PBS1* (Warren *et al.*, 1998; Warren *et al.*, 1999). *RPS5* encodes an NBS-LRR protein. Here, we report that *PBS1* encodes a protein serine/threonine kinase; thus *RPS5/PBS1*-mediated resistance is structurally analogous to *Prf/Pto*-governed resistance in tomato. However, phylogenetic comparison of plant protein kinases indicates that *Pto*, *Pti1* and *PBS1* belong to different subfamilies. We discuss several alternative models for the function of protein kinases in pathogen recognition.

Results

Genetic mapping and identification of *PBS1*

Previous genetic mapping showed that *PBS1* is located between simple sequence length polymorphic (SSLP) markers nga249 and nga106, which are 10 cM apart on chromosome V (Warren *et al.*, 1999). More detailed mapping was accomplished by searching for recombination events between these SSLP markers in 830 F2 lines. From this analysis, 72 informative recombinant lines were identified, which were then scored in the F3 generation for resistance/susceptibility to *P. syringae* strain DC3000 (*avrPphB*). The informative recombinants were also scored with cleaved amplified polymorphic markers (CAPS) (<http://www.arabidopsis.org/aboutcaps.html>), which allowed us to locate *PBS1* between markers CHS and P104a (Figure 1a). To further delineate the position of *PBS1*, new markers were developed based on single nucleotide polymorphisms between Col-0 and Ws-0 (see Experimental procedures). These new markers enabled us to localize *PBS1* to two overlapping bacterial artificial chromosome (BAC) clones, F5H2 and F7J12 (Figure 1b).

To prepare for complementation of *pbs1* mutants, cosmid libraries were made from BAC clones F5H2 and F7J12 in the binary vector pCLD04541 (Bent *et al.*, 1994). Subclones were organized into contigs by identifying overlapping clones using a PCR-based screen. Primer pairs used during cosmid contig assembly were derived from end sequences and internal sequences of BACs and from end sequences of cosmids (see Experimental procedures).

In parallel with the contig assembly process, we tested all primer pairs for their ability to amplify from *pbs1-1* mutant plants. This analysis was undertaken because the *pbs1-1* allele, which was induced by fast neutrons, is associated with a chromosome translocation event (Warren *et al.*, 1999). We therefore speculated that the *pbs1-1* mutation might be caused by a chromosome breakage and/or deletion event, and that primer pairs that spanned this break would not amplify from the *pbs1-1* mutant. A primer pair corresponding to the SP6 end sequence of BAC T27G13 (accession number B74054; Figure 1b) failed to produce a PCR product from the *pbs1-1* allele, but amplified the expected product from wild-type Col-0 plants (data not shown), suggesting that this region was disrupted in the *pbs1-1* mutant. This end sequence was included in the analysis because BAC T27G13 overlaps BAC clones F5H2 and F7J12 in the *Arabidopsis* BAC fingerprint database (<http://genome.wustl.edu/gsc/arab/arabidopsis.html>). Southern hybridization with this PCR product as a probe revealed that it is deleted from the *pbs1-1* genome (Figure 1d). Only a single hybridizing band was observed in wild-type *Arabidopsis*, suggesting that this sequence is represented by a single

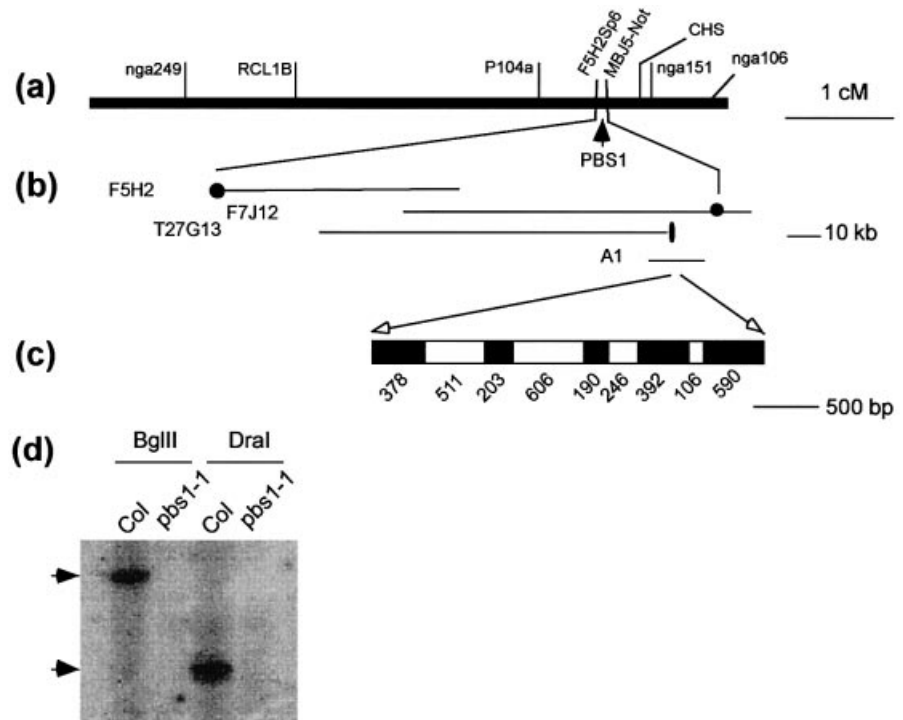
Figure 1. Map-based cloning of *PBS1*.

(a) Genetic map of the *PBS1* region. The molecular markers used for localization of *PBS1* are indicated. Markers F5H2Sp6 and MBJ5-Not were each separated from *PBS1* by a single recombination event (out of 1660 meioses).

(b) The BAC contig spanning the genetic interval containing *PBS1*. Filled circles indicate positions of molecular markers shown in part (a). The filled oval represents the SP6 end of BAC T27G13 used as a probe in part (d). The smaller bar below BAC clone T27G13 represents cosmid A1, which was used to complement the *pbs1-2* mutation.

(c) Structure of the *PBS1* gene. Exons and introns are marked as shaded and open bars, respectively. Numbers below indicate sizes of exons and introns in bases.

(d) Southern blot analysis of the *pbs1-1* mutant. Genomic DNA from *pbs1-1* and wild-type plants was digested with the indicated enzymes, separated by agarose gel electrophoresis, and transferred to a nylon membrane. The blot was probed with a [³²P]-dATP-labeled PCR product representing the SP6 end of BAC T27G13. Arrows point to hybridizing DNA fragments. Only DNA from wild-type Col-0 hybridized.



gene in *Arabidopsis*. Sequencing of the same fragment from the *pbs1-2* mutant, an ethyl methanesulfonate-induced allele, revealed a G to A transition event. Discovery of mutations in the same DNA sequence in two different *pbs1* alleles strongly indicated that this gene represented *PBS1*. The identity of *PBS1* was confirmed by functional complementation of both the *pbs1-1* and *pbs1-2* mutations (see below).

PBS1 is a serine/threonine kinase

The full sequence of the *PBS1* gene was obtained by direct sequencing of cosmid templates using a primer walking strategy. A search of the NCBI dbEST sequence database using the BLASTN algorithm (Altschul *et al.*, 1997) identified one cDNA clone, 130C10T7 (accession number T45087), corresponding to this genomic sequence. Complete sequencing of this EST clone revealed that it lacked the 5' end of the gene. The full-length cDNA sequence (Figure 2) was obtained by sequencing several 5' RACE clones that overlapped with the EST sequence by 450 bp. Comparison of the genomic and cDNA sequences showed that *PBS1* contains 5 exons and 4 introns (Figure 1c).

The predicted *PBS1* protein contains 456 amino acid residues, has a mass of 50.4 kDa and a pI of 6.47. A BLASTP search of the NCBI non-redundant protein database revealed that *PBS1* is highly similar to a large number of protein kinases. As shown in Figure 2, the kinase

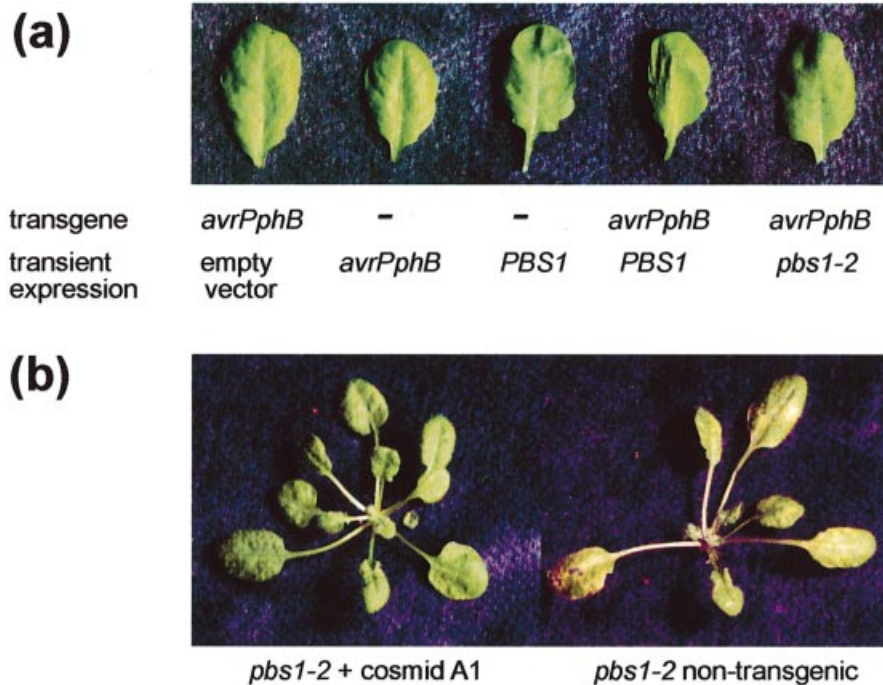
domain occupies the central part of *PBS1* (residues 78–360) and contains all 11 conserved subdomains of protein kinases (Hanks and Quinn, 1991). Subdomain VI contains a lysine (K) residue in the DFKSSN motif and subdomain VIII contains a G-T/S-XX-Y/F-X-APE motif. These features strongly suggest that *PBS1* is a serine/threonine protein kinase (Stone and Walker, 1995). The *pbs1-2* mutation changes a highly conserved glycine in subdomain VIII (position 252) to arginine (Figure 2).

The presence of an EST clone corresponding to *PBS1*, together with the successful amplification of *PBS1* from leaf cDNA, indicates that the *PBS1* gene is expressed in *Arabidopsis*. RNA gel blot analysis revealed that *PBS1* is not significantly induced by inoculation with either virulent or avirulent strains of *P. syringae* (not shown). In addition, analysis of hybridization data from over 100 experiments deposited in the Stanford Microarray Database (<http://genome-www4.Stanford.EDU/MicroArray/SMD/>) revealed no conditions that induced or suppressed *PBS1* mRNA levels greater than four fold. In particular, *PBS1* mRNA levels in plants inoculated with a virulent strain of *P. syringae* (DC3000) did not differ from the levels detected in plants inoculated with a non-pathogenic mutant of DC3000. Similarly, infection with a virulent strain of the powdery mildew fungus *E. cichoracearum* had no significant effect on *PBS1* mRNA levels at 48 h after infection. Expression of *PBS1* was also unaffected by the *cpr5* mutation, which causes elevation in salicylic acid (SA) levels (Bowling *et al.*, 1997), or by expression of the

Figure 3. Complementation of *pbs1-1* and *pbs1-2* mutants.

(a) Transient expression of *PBS1* induces *avrPphB*-dependent cell death. Transgenic *pbs1-1* plants containing a pTA7002/*avrPphB* construct (+) or non-transgenic *pbs1-1* controls (-) were infiltrated with *Agrobacterium* strains carrying the indicated pTA7002 constructs. Dexamethasone was sprayed on leaves 48 h after infiltration, and leaves detached from plants and photographed 24 h after dexamethasone application. Each leaf was infiltrated on the left side only. The experiment was repeated three times, with each construct infiltrated into three leaves each time. For all experiments, only the wild-type *PBS1* construct induced leaf collapse, and only on *avrPphB* transgenic plants.

(b) Complementation of *pbs1-2* by stable transformation with a cosmid containing wild-type *PBS1*. Cosmid A1 (Figure 1b) was used to transform *pbs1-2* mutant plants. Plants from the T2 generation were inoculated with *P. syringae* strain DC3000(*avrPphB*) and disease phenotypes scored four days later. All resistant plants (left photograph) were subsequently shown to contain the A1 cosmid DNA by PCR analysis, while all susceptible plants (right photograph) lacked the cosmid.



ment was 57% for *Apk2a* and 51% for *ARSK1*. Similarity to *Apk2a* extends beyond the kinase domain to the N-terminus; however, the identity drops to 33% in this region. The highest identity to any *Arabidopsis* kinase was 79% in a 310 amino acid overlap with a sequence represented by gene index 7413555 (EST AI994944).

We also compared the *PBS1* protein with the tomato protein kinases *Pto* and *Pti1*, since these proteins have been implicated in *AvrPto* recognition (Zhou *et al.*, 1995). The *PBS1* kinase domain is 50% identical to the kinase domain of *Pti1* and 42% identical to the kinase domain of *Pto*. The higher similarity to *Pti1* is of interest because *Pti1* does not interact with *AvrPto* in the yeast two-hybrid assay, whereas *Pto* does (Scofield *et al.*, 1996; Tang *et al.*, 1996).

PBS1, *Pti1* and *Pto* are located on different branches of the kinase phylogenetic tree

The relationship between the above kinases was analyzed by construction of a phylogenetic tree. Multiple alignment of the 50 full-length proteins most similar to *PBS1* was performed using Clustal X (Thompson *et al.*, 1997). A phylogenetic tree was then constructed using the part of the alignment that embraced the kinase domains. Several receptor-like kinases were included in the alignment, as they have been shown to group closely with NAK-like kinases (Hardie, 1999). In addition, the receptor-like kinase *Xa-21* from rice was included, as this kinase has recently been shown to induce a HR-like

responses in rice cells (He *et al.*, 2000). An unrooted tree was drawn to visualize how the kinases grouped (Figure 4a). This analysis revealed that *PBS1*, *Pti1*, *Pto* and *Apk1a/NAK*-like kinases are on separate branches that have high bootstrap support.

To see if other plants contain *PBS1*-like sequences we searched the NCBI dbEST database using the *PBS1* amino acid sequence and the TblastN algorithm (Altschul *et al.*, 1997). ESTs representing kinase domains with high similarity to *PBS1* were found from *Lotus japonicus* (AI967314), *Glycine max* (AI794805), *Zea mays* (AI770970) and *Lycopersicon esculentum* (AI896006). Since the overlaps between the EST sequences and *PBS1* included only part of the kinase domain, we constructed a phylogenetic tree based only on the overlapping region between conserved residues, H/Y-RD, in subdomain VI and APE in subdomain VIII. This tree was very similar to the tree based on the whole kinase domain, with identical *PBS1* and *Apk2A/NAK* branches (Figure 4b). The sequences from soybean, maize, tomato and *Lotus japonicus* were located on the same branch as *PBS1*, indicating that the *PBS1* subfamily likely arose early in angiosperm evolution.

The *PBS1* protein can autophosphorylate

To confirm that *PBS1* is a functional protein kinase, we performed an autophosphorylation assay on *PBS1* protein produced using an *E. coli* expression system (see

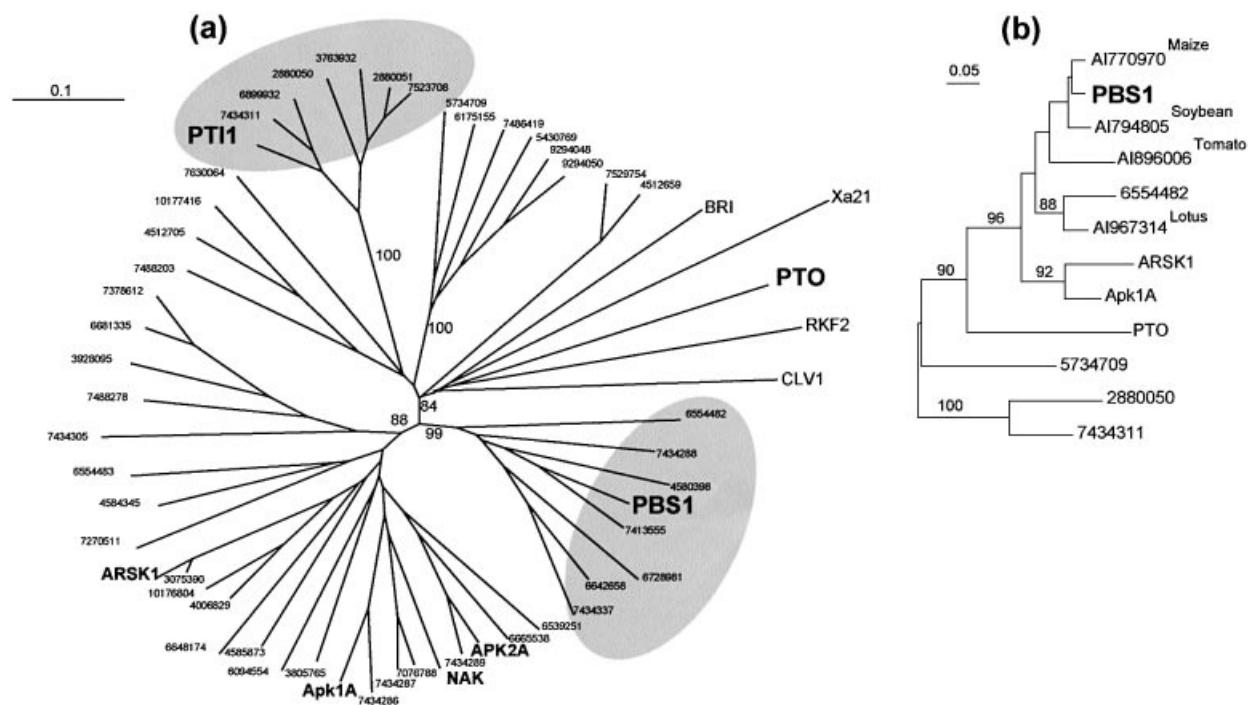


Figure 4. Phylogenetic relationship between PBS1 and other plant protein kinases.

(a) An unrooted phylogenetic tree showing the relationship of PBS1 to the 50 most similar kinases in the *Arabidopsis* genome. The tomato Pto and Pti1 kinases, along with several transmembrane receptor kinases were included for reference (see text). This tree was constructed using the neighbor-joining method of CLUSTAL W (Thompson *et al.*, 1997), and is based on the full kinase domain of each protein (see Experimental procedures). Numbers at internal branch points represent bootstrap support expressed as a percent of 1000 replicates. Numbers at branch tips represent the GenPept indexes in the GenBank protein database. Names indicate kinases that have been at least partially characterized. A bar for calibration of phylogenetic distances is provided at the left corner. The PBS1-like and Pti1-like subfamilies are marked by a gray ellipse.

(b) An unrooted tree showing the relationship of *PBS1* to similar kinases from other plant species. This tree is based on a subregion of each kinase extending from the H/Y-RD triplet in subdomain VI to the APE triplet in subdomain VIII (Figure 2). Numbers on internal branches indicate bootstrap support expressed as a percent of 1000 replicates. Only values above 80% are shown. Superscripted names indicate the plant species of origin for each kinase. Numbers without superscripts are *Arabidopsis* kinases and are extracted from specific branches of the tree shown in part (a) to allow comparison of the two trees.

Experimental procedures). Purified PBS1 protein was incubated with [γ - 32 P]ATP in a kinase assay buffer (see Experimental procedures) and then run on a denaturing polyacrylamide gel and autoradiographed. A single strongly labeled band of the expected 50 kDa size was observed (Figure 5a). To exclude the possibility that contaminating protein kinases were present in the purified PBS1 protein preparation, we overexpressed the pTYB2 empty vector and tested equivalent fractions in the kinase assay. Because this control contains very little protein (Figure 5b), we also conducted autophosphorylation assays with this fraction, to which we added either myelin basic protein (a common substrate for many protein kinases), or maltose binding protein. None of these reactions produced detectable bands on the autoradiograph (Figure 5a). These data indicate that the kinase activity observed in the purified PBS1 preparation was because of the PBS1 protein itself, and not a contaminating kinase.

Discussion

We have now cloned and characterized two *Arabidopsis* genes, *PBS1* and *RPS5*, that are required for specific recognition of the bacterial protein AvrPphB. *RPS5* encodes an NBS-LRR protein typical of plant *R*-gene products (Warren *et al.*, 1998), thus it is tempting to speculate that the LRR region of *RPS5* participates directly in AvrPphB binding. However, the discovery that *PBS1* encodes a functional protein kinase with similarity to the tomato Pto kinase suggests that *PBS1* might fulfil this role. Thus far we have failed to detect interactions between AvrPphB and *PBS1*, AvrPphB and *RPS5*, or *PBS1* and *RPS5* using yeast two-hybrid approaches, thus it is not yet clear whether *PBS1*, *RPS5*, both, or neither interact directly with AvrPphB.

The *pbs1-2* mutation caused a glycine to arginine substitution at a highly conserved position in subdomain VIII of *PBS1*. This position corresponds to the first residue

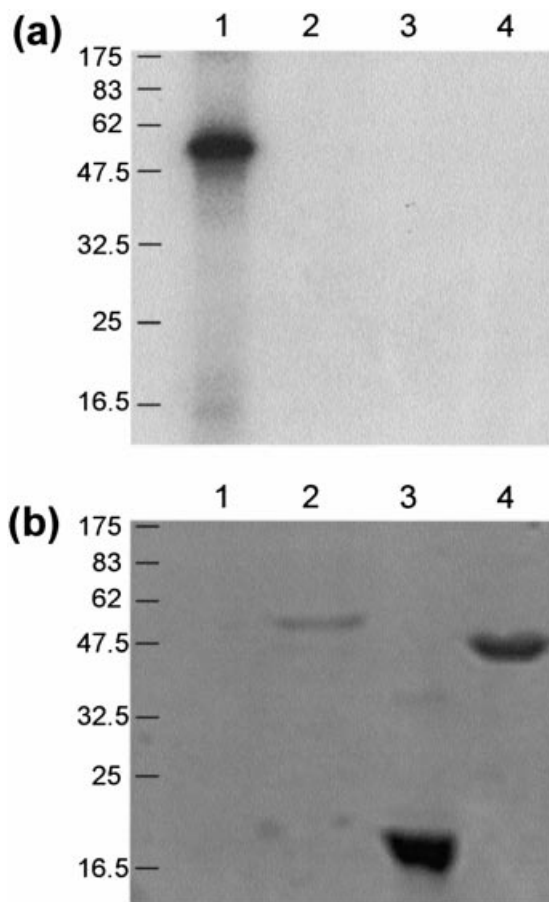


Figure 5. PBS1 displays autophosphorylation activity.

(a) Autophosphorylation assay. Approximately 150 ng of purified PBS1 was incubated with [γ - 32 P]-ATP and then separated on a SDS polyacrylamide gel (lane 1). As a control, a sample purified in the same way, but derived from *E. coli* expressing the empty vector pTYB2, was assayed for kinase activity (lane 2). Lanes 3 and 4 show kinase assays containing the same pTYB2 fraction plus 10 μ g myelin basic protein (lane 3) or 4 μ g maltose binding protein (lane 4). Kinase activity was observed only in the PBS1-containing sample.

(b) Coomassie-stained gel. To establish the purity of the PBS1 protein preparation, and to demonstrate that protein was present in lanes 3 and 4 of the autophosphorylation assay, a parallel gel was run and stained with Coomassie Blue. Lane 1, pTYB2 vector only control. Lane 2, c. 1.5 μ g of purified PBS1. Lane 3, pTYB2 vector control plus 10 μ g myelin basic protein. Lane 4, pTYB2 vector control plus 4 μ g maltose binding protein.

of the $P + 1$ loop (Figure 2), which is a region identified in the crystal structures of various protein kinases and is located at the end of the activation segment (Johnson *et al.*, 1996). The latter is defined as the region spanning conserved sequences DFG in subdomain VII through APE in subdomain VIII (Figure 2). The activation segment is known to regulate substrate binding and/or catalytic activity in various protein kinases (Johnson *et al.*, 1996). Mutations in the $P + 1$ loop of the tomato Pto kinase disrupt binding to AvrPto in a yeast two-hybrid assay (Frederick *et al.*, 1998; Rathjen *et al.*, 1999). In addition,

substitution of a conserved tyrosine residue with aspartate in the $P + 1$ loop of Pto (Y207D) causes Pto to activate an HR-like response when transiently expressed in tomato leaves in the absence of AvrPto (Rathjen *et al.*, 1999). These data implicate the activation segment of Pto as a key regulatory component of the Pto-mediated HR. We created a mutation in PBS1 (Y256D) analogous to the Y207D mutation of Pto and assayed it for activation of an HR-like response in wild-type Col-0 Arabidopsis leaves. No HR activity was observed using the transient assay described in Figure 3(a) (data not shown), suggesting that PBS1 and Pto may differ in how they regulate the hypersensitive response.

A potential myristoylation motif is present at the N-terminus of PBS1 (Figure 2). Interestingly, RPS5 and AvrPphB also contain myristoylation motifs. AvrPphB is processed in *P. syringae* (Puri *et al.*, 1997), and after processing, the myristoylation motif is exposed in the larger 28 kDa portion. This fragment, when transiently expressed in leaves of wild-type Col-0, causes cell death (data not shown). Myristoylation motifs have also been identified in the *P. syringae* Avr proteins AvrB, AvrRpm1 and AvrPto and seem to be necessary for induction of a hypersensitive reaction (Nimchuk *et al.* 2000; Shan *et al.*, 2000b). This suggests that many Avr proteins may associate with cellular membranes where recognition of Avr factors by plant receptors may occur. In agreement with such a model the *R*-gene product RPM1 is plasma membrane localized (Boyes *et al.*, 1998). At least one other component of the RPS5 and RPM1 signal transduction pathway might be associated with membranes, NDR1 (Century *et al.*, 1996). These data suggest that recognition complexes may associate with the plant cell plasma membrane.

Besides the kinase domain, PBS1 contains short N- and C-terminal extensions. The sequence of the C-terminal region seems to be unique, while the sequence of the N-terminus shows weak but significant similarity to other kinases, including the tomato Pti1 kinase. The PBS1 N-terminal and C-terminal extensions show no similarity to Pto, suggesting that PBS1 and Pto may serve different functions.

As suggested by their similarity in the N-termini, PBS1 is significantly more similar to Pti1 than to Pto. Overexpression of *Pti1* in tobacco enhances the hypersensitive response induced by *P. syringae* strains expressing *avrPto*, suggesting that *Pti1* plays a role in *Pto*-mediated resistance (Zhou *et al.*, 1995). However, it is not known if *Pti1* is specific for *Pto*-mediated resistance or if it plays a more general role. The epistatic relationship between *Pti1* and *Prf*, which is required for *Pto*-mediate resistance (Salmeron *et al.*, 1996), also is not clear. Since Pti1 interacts with Pto, it is possible that Pti1 operates upstream of Prf or on a separate pathway. One possibility is that Pti1 and

PBS1 both represent signal transduction components that function downstream of pathogen recognition.

An alternative role for PBS1 could be to regulate expression of *RPS5*, or to modify the RPS5 protein. RT-PCR analysis revealed that *RPS5* is expressed in *pbs1* mutants, which suggests that *PBS1* does not control expression of *RPS5* (data not shown). We have not yet been able to detect RPS5 protein in wild-type Arabidopsis plants, thus have no data on protein levels or the possible phosphorylation status of RPS5 in *pbs1* mutants. RPS5 does have several potential phosphorylation sites based on predictions by the NetPhos 2.0 program (<http://www.cbs.dtu.dk/services/NetPhos/>). These residues are localized throughout the RPS5 protein sequence and among them are 5 serines occupying positions 732–738 in the LRR domain. The hypothesis that PBS1 may phosphorylate RPS5 will be tested directly once purified PBS1 and RPS5 proteins become available.

A third potential role for PBS1 may be as a virulence target of AvrPphB, and as a ligand for RPS5. Recently it has been proposed that Pto functions as virulence target for AvrPto and that Prf (an NBS-LRR protein) senses a resulting change of Pto conformation or activity, thereby inducing an HR (Van der Biezen and Jones, 1998). By analogy, AvrPphB may target PBS1 in its role as a virulence factor, with RPS5 functioning to 'guard' PBS1.

If the guard hypothesis were correct, it would imply that each NBS-LRR protein should have a partner 'target' protein that it guards. However, these target proteins need not be functionally or structurally related to each other. Our phylogenetic analysis of plant protein kinases suggests that *PBS1* and *Pto* are distantly related (Figure 4), and thus likely perform different functions. This relationship is more consistent with the guard model than a model in which PBS1 and Pto both function as specific receptors for pathogen avr proteins. The guard model would also explain how a single NBS-LRR protein, RPM1, can mediate recognition to two dissimilar Avr proteins, AvrB and AvrRpm1 (Grant *et al.*, 1995); RPM1 would only have to guard a single plant protein that was targeted by both AvrB and AvrRpm1. It should be pointed out in this context, that if the guard model applies to the Prf and Pto, then Prf must in fact guard at least two proteins, as it is also required for cell death responses initiated by the Fen kinase (Salmeron *et al.*, 1994). Furthermore, if Pto is the target of avrPto, it cannot be the only target, as AvrPto enhances the virulence of *P. syringae* pv. *tomato* strain T1 on tomato plants that lack Pto (Chang *et al.*, 2000; Shan *et al.*, 2000a).

If PBS1 is in fact a target of AvrPphB, then it must have a function separate from *RPS5*-mediated pathogen recognition. What this function may be is unclear, however, as *pbs1* mutants display no obvious phenotype in the absence of pathogens, or in response to pathogens

carrying avr genes other than *avrPphB* (Warren *et al.*, 1999).

One potential function of PBS1 and other 'targets' may be the regulation of defense responses. In support of this hypothesis, some *P. syringae* avr genes are known to suppress defense responses in susceptible plant hosts (White *et al.*, 2000). For example, the *avrPphF* gene of *P. syringae* pv. *phaseolicola* suppresses the HR induced by *P. syringae* strain RW60 on bean cultivar Tendergreen, enabling this strain to infect Tendergreen (Tsiamis *et al.*, 2000). Also consistent with this hypothesis is the observation that overexpression of *Pto* leads to a constitutive disease resistance phenotype (Tang *et al.*, 1999; Tobias *et al.*, 1999). We are currently testing whether *PBS1* will display similar properties when overexpressed.

As stated above, one prediction of the guard hypothesis is that most *R* genes should require a second gene (the 'guardee') for recognition of pathogen virulence factors. However, there are few examples in the literature of two plant genes being required for recognition of a single pathogen Avr protein. Other than *PBS1/RPS5* and *Pto/Prf*, the only other example of which we are aware is recognition of the Avr2 peptide from the fungus *Cladosporium fulvum* by tomato, which requires the *Cf2* and *Rcr3* genes (Dixon *et al.*, 2000). *Cf2* encodes a putative transmembrane extracellular receptor consisting almost entirely of LRRs (Dixon *et al.*, 1996), while *Rcr3* has not yet been identified. Although *Cf2* belongs to a different class of resistance genes than *RPS5* and *Prf*, it is formally possible that *Rcr3* represents the target of Avr2, and that *Cf2* functions to guard *Rcr3* (Dixon *et al.*, 2000). Another possible example of a 'guardee' protein is the TIP protein of Arabidopsis. This protein has recently been shown to interact in a yeast two-hybrid assay with the coat protein of turnip crinkle virus (TCV; Ren *et al.*, 2000). Significantly, amino acid substitutions within the TCV coat protein that disrupt this interaction in yeast enable TCV to infect the resistant Arabidopsis accession Di-17, suggesting that this interaction may be required for recognition of TCV by Di-17. Resistance to TCV in Di-17 is mediated by an NBS-LRR R gene designated *HRT* (Cooley *et al.*, 2000), thus both *HRT* and *TIP* may be required for recognition of TCV; however, mutations in *TIP* have not yet been described. Although not characterized at a molecular level, there are several reports in the older literature in which resistance to a specific pathogen was obtained after crossing two susceptible parents (Singh and McIntosh, 1984), implying that the parents lacked different genes required for recognition of the specific pathogen.

With the identification of *PBS1* we can now take advantage of the many tools developed for Arabidopsis to test the hypotheses proposed above. For example, if PBS1 has a function independent of AvrPphB recognition, we may be able to identify the pathway(s) regulated by *PBS1* using

cDNA or oligonucleotide microarrays. As a complementary approach, we are pursuing yeast two-hybrid approaches to identify *Arabidopsis* proteins that interact with *PBS1*, which may also provide insight into the pathways regulated by *PBS1*. Ultimately, a clear understanding of how plant *R* gene products mediate recognition of pathogen molecules should facilitate development of more effective disease control strategies in plants.

Experimental procedures

Plant growth conditions and bacterial inoculations

Cultivation of *Arabidopsis* and susceptibility tests were performed as described by Warren *et al.* (1999).

Development of molecular markers

Fragments from Ws-0 and Col-0 *Arabidopsis* ecotypes corresponding to ends of several BAC clones lying between CAPS markers CHS and P104a were amplified by PCR, and then sequenced and compared. Single nucleotide polymorphisms (SNPs) were found in the Sp6 end of BAC clone F5H2 (accession number AL082738) and the *NotI* arm of TAC clone MBJ5 (<http://www.kazusa.or.jp/kaos/>). These SNPs were converted into cleaved amplified polymorphic sequence (dCAPS) markers, as described (Michaels and Amasino, 1997; Neff *et al.*, 1997). Primers used in amplification reactions and restriction enzymes used in subsequent digestion reactions were as follows: for the MBJ5/*NotI* arm: mbj5F-5'-CTATCTACGTATAAGCATGTGC-3', mbj5R-5'-AGAAAGACGAGTCGTCGATG-3', mbjSPE - 5'-TTGAATCTTAAGTTGTATGTGACTAG-3', *SpeI*; for the F5H2/Sp6 arm: f5h2sp6F-5'-CAAATCAACTGTCTGCTCAC-3', f5h2sp6R-5'-AGAGAGTTTCTGAATGCATG, f5h2sp6EcoNI - 5'-TCTGAAAAGTTA-CTCACCTATG-3', Primers designed to amplify the Sp6 end of BAC clone T27G13 were as follows: T27Sp6F-5'-TCG-CCTGTCGTTGAACAATG-3', T27Sp6-5'-GAGGCTTAGATTGGAACATG-3'.

Race-PCR

5'-RACE-PCR was performed using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA, USA) according to the manufacturer's protocol. Amplified fragments were cloned into the pGEM-T Easy vector (PROMEGA, Madison, WI, USA) and several independent clones were sequenced.

Construction of cosmid libraries

Bacterial artificial chromosome clones (BAC) F7J12 and F5H2, obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH, USA), were purified using Tip-20 columns (Qiagen, Valencia, CA, USA) and partially digested with *Sau3A*I. Size selection was accomplished by electrophoresis through 1% agarose. DNA fragments >8 kb were purified by digesting the agarose block with beta-agarase (New England Biolabs, Beverly, MA, USA) according to manufacturer's instructions and precipitated with isopropanol. DNA was ligated into the binary cosmid vector pCLD04541 (Bent *et al.*, 1994), packaged

in vitro with GigapackIII Gold (Stratagene, La Jolla, CA, USA) and transfected into *Escherichia coli* XL1-BlueMR.

Southern hybridization

Genomic DNA was purified using a DNAeasy kit from Qiagen. About 1 µg of DNA was digested with *HindIII*, *DraI* and *BglII* and separated on a 0.9% agarose gel. DNA was blotted to a nitrocellulose membrane and hybridized to a [³²P]-dATP-labeled PCR product derived from the Sp6 end of BAC clone T27G13. The blot was washed twice for 15 min in 2 × SSC, 0.1% SDS; once in 0.3 × SSC, 0.1% SDS; twice in 0.15 × SSC, 0.1% SDS and autoradiographed for 2 days.

Assembly of cosmid contigs

Overlapping cosmid clones were identified by PCR-screening libraries with specific primer pairs. To obtain sequences for primer design, BAC F7J12 was restricted in separate digests with *BglII*, *EcoRI* and *HindIII* and fragments cloned into pBluescript SK + (Stratagene). Several clones with inserts 400–2000 bp in size were sequenced using a T3 primer. A second set of primers was designed using known IGF BAC end sequences overlapping the F7J12 BAC (http://www.mpimp-golm.mpg.de/101/mpi_mp_map/bac.html). Physical gaps between sets of overlapping cosmids were filled by hybridization using cosmid ends as probes and by PCR with primers designed to cosmid end sequences.

DNA sequencing

All sequencing reactions were performed using Dye or BigDye Termination Kits (Applied Biosystems, Foster City, CA, USA) and separated on an ABI 377 sequencer. The *PBS1* gene was sequenced directly from cosmids and the sequence confirmed by sequencing PCR fragments amplified using genomic DNA as template. The cDNA sequence was completed by sequencing EST clone 130C10T7 (accession number T45087) and several 5'-RACE clones. Cosmid end sequences were obtained using T3 and M13forward primers.

Transient expression assays

The putative coding region of *PBS1* was amplified directly from total cDNA using tailed oligonucleotides. Sequences of the primers were as follows: PBS1-F 5'-TACCGTCGACAACAATG GTTGGTTCTCTGGTTTGA-3'; PBS1-R 5'-ACTGACTAGTGACAA-TAAATGAGAGTCTTGAG-3'. The PBS1-F primer included a *Sall* recognition site and consensus translation initiation sequence around the ATG codon (Lutcke *et al.*, 1987). PBS1-R contained a *SpeI* recognition site. The amplified fragment was digested with *Sall* and *SpeI* and cloned into pTA7002 (Aoyama and Chua, 1997) digested with *XhoI* and *SpeI*, transformed into *E. coli* DH10B (Life Technologies, Rockville, MD, USA) and then retransformed into *Agrobacterium tumefaciens* strain GV3101.

Agrobacterium containing pTA7002/PBS1 were grown in 2 ml of LB liquid medium with 10 µg ml⁻¹ gentamycin and 50 µg ml⁻¹ kanamycin overnight at 30°C and prepared for inoculations as described by Nimchuk *et al.* (2000). The density of the culture was corrected to 0.4 and bacteria were infiltrated into leaves of 5-week-old *Arabidopsis* plants using a needleless syringe. After 48 h, plants were sprayed with a 50-µM dexamethasone (Sigma,

St. Louis, MO, USA) solution in water. Leaves were visually scored for collapse of tissue 1 and 2 days later.

Introduction of the *pbs1-2* mutation into the *PBS1* cDNA

A mutation that changed glycine 252 into arginine (recreating the *pbs1-2* allele) was introduced using a two-step recombinational PCR method (Higuchi, 1990). The whole *PBS1* cDNA was then amplified using the same tailed primers that were used in amplifying the wild-type *PBS1* cDNA.

Stable plant transformation

Arabidopsis plants were transformed according to Bechtold *et al.* (1993) as modified by Clough and Bent (1998). Transformants were selected on 0.5x Murashige and Skoog agar plates with 50 µg ml⁻¹ hygromycin (pTA7002) or 50 µg ml⁻¹ kanamycin (pCLD04541). The cosmid used for stable complementation of the *pbs1-1* mutant (cosmid A1 in Figure 1b) contained a 17-kb insert. Comparison of the end sequences from this insert to the recently completed genomic sequence from this region (BAC clone T19L5) revealed that the A1 clone contained only one other predicted gene, the senescence associated gene SAG29.

Protein sequence alignments and construction of phylogenetic trees

Arabidopsis protein kinase sequences were extracted from The Arabidopsis Information Resource database (<http://www.arabidopsis.org/>) using the BlastP algorithm (Altschul *et al.*, 1997). Protein alignments were prepared by Clustal X and manually corrected (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbor-joining method and was based on the part of alignment that contained the kinase domains. The resulting tree was subjected to 1000 rounds of bootstrap analysis and visualized as unrooted using the TreeView program (Page, 1996). Kinase domains were identified by searching kinase sequences against the Profile database (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html).

Purification of *PBS1* protein

PBS1 protein was expressed in *E. coli* and purified using the IMPACTTM-CN expression system (New England Biolabs, Beverly, MA, USA). In brief, we cloned the *PBS1* open reading frame into the vector pTYB2. This created a fusion between the C-terminus of *PBS1* and a chitin-binding domain. At the junction of these two domains is an intein, which enables the chitin-binding domain to be removed by an inducible self-cleavage reaction (Chong *et al.*, 1997). To clone *PBS1* into the pTYB2 vector, the *PBS1* open reading frame was first amplified by PCR using a cDNA template and the following primers: 5'-AGCTCATATGGGTTGTTTCTCGT-GTTTTGATTCG-3' and 5'-AGCTGAATCCCGGTACTGTTGCTC-TCTG-AAG-3'. The PCR product was digested with *NdeI* and *EcoRI* restriction enzymes and ligated into *NdeI/EcoRI* digested vector. The resulting clone was checked by DNA sequencing and then expressed in *E. coli* strain ER2566 (New England Biolabs) according to the manufacturer's directions. Cells were lysed using a French press and the lysate was clarified by centrifugation at 20 000 × g. The supernatant was loaded onto a column containing chitin beads (New England Biolabs) and then washed with 25 ml of column buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl

and 1.0 mM EDTA). Self-cleavage of the fusion protein was induced by incubating the column overnight in column buffer containing 40 mM DTT. The *PBS1* protein was then eluted in column buffer and the concentration of elution fractions checked by SDS polyacrylamide gel electrophoresis and Coomassie Blue staining. Note that this system yields purified protein that lacks the affinity tag.

Autophosphorylation assays

These assays were performed essentially as described by Sessa *et al.* (1998). Approximately 150 ng of purified *PBS1* protein was incubated in 20 µl of kinase buffer (50 mM Tris-HCl, pH 7.0, 20 mM MnCl₂, 40 µM ATP, 1 mM DTT) supplemented with 5 µCi [γ -³²P]ATP (4500 Ci mmol⁻¹; ICN) for 15 min at room temperature. The reactions were stopped by adding SDS sample buffer, and then separated on a 10% polyacrylamide gel. Control reactions contained an equivalent column fraction derived from *E. coli* expressing the pTYB2 empty vector, and either 10 µg of myelin basic protein (Sigma), or 1 µg of maltose binding protein (New England Biolabs).

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GenBank accession number AF314176 (PBS1cDNA sequence).