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Arabidopsis RelA/SpoT homologs implicate (p)ppGpp in plant signaling

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Arabidopsis RPP5 **is a member of a large class of pathogen resistance genes encoding nucleotide-binding sites and leucine-rich repeat domains. Yeast two-hybrid analysis showed that RPP5 specifically interacts with At-RSH1, an *Arabidopsis* RelA/SpoT homolog. In *Escherichia coli*, RelA and SpoT determine the level of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), which are the effector nucleotides of the bacterial stringent response. Functional analysis in *E. coli* and in *Streptomyces coelicolor* A3 (2) showed that *At-RSH1* confers phenotypes associated with (p)ppGpp synthesis. We characterized two additional *Arabidopsis* RelA/SpoT homologs, *At-RSH2* and *At-RSH3. At-RSH* genes may regulate a rapid plant (p)ppGpp-mediated response to pathogens and other stresses.**

Pathogens have developed specialized infection strategies, and plants have evolved systems to rapidly detect attempted pathogen ingress (1). For example, certain pathogenic bacteria transfer effector proteins into the host cytoplasm, where they are thought to enhance virulence by subversion of the host defense machinery and other cellular functions (2). Superimposed on this basal line of host defense, plants have evolved pathogen surveillance systems comprising numerous resistance (R) proteins (3). When viral, bacterial, or fungal virulence factors are detected by the plant's surveillance system, they then become genetically defined as avirulence (Avr) products (4–8). After specific recognition of pathogen Avr products, R proteins rapidly trigger a defense response that is associated with complex cellular metabolic alterations and production of active oxygen species and nitric oxide and typically appears microscopically as host cell death at the site of pathogen ingress (9).

Arabidopsis thaliana ecotypes carrying *RPP5* elicit defense responses after detection of *Peronospora parasitica* strains that carry the cognate Avr product (10). RPP5 is a member of a superfamily of cytoplasmic R proteins that contain nucleotide-binding (NB) sites and leucine-rich repeat (LRR) domains and is grouped further into a subclass with similarity to the effector domain of the *Drosophila* and human Toll and IL-1 receptors (TIR domain) (10). All NB-LRR proteins contain a central region with three conserved motifs predicted to constitute an ATP- or GTP-binding pocket and several other motifs with unknown function (3). This "NB-ARC" (NB-Apaf-1, R proteins, and CED-4) domain or "Ap-ATPase" domain is also present in several structurally related regulators of animal apoptosis, including human Apaf-1 and nematode CED-4 (11, 12), where it functions as a protein–protein interaction module (13–15). Interestingly, *RPP5* family members carry diverged NB-ARC domains, possibly reflecting functional differences such as interactions with different proteins (16). To find host proteins that interact with the NB-ARC domain of RPP5, we used the yeast two-hybrid assay (17) and identified *At-RSH1*.

At-RSH1 is a predicted plasma membrane-anchored cytoplasmic molecule with significant homology to bacterial RelA and SpoT proteins. These enzymes determine the level of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (ppGpp), which are the effector nucleotides of the prokaryotic stringent response (18) and also play a role in antibiotic production and differentiation in *Streptomyces* species (19). Eukaryotic *relA/spoT* homologs have not been described to date. *Arabidopsis* contains three unlinked *relA/spoT* homologs (*At-RSH1*, *2*, and *3*). Here, we show that *At-RSH1* confers several distinct phenotypes associated with (p)ppGpp synthesis in both *Streptomyces coelicolor* A3 (2) and in *Escherichia coli*. This functional characterization of *At-RSH1* is consistent with a role for (p)ppGpp in mediating a stress-induced defense system in plants analogous to the bacterial stringent response.

Materials and Methods

Yeast Two-Hybrid Plasmids and Library. The Matchmaker LexA Two-Hybrid system was used (CLONTECH). The plasmids pJK101 and pRFHM1 were kindly provided by R. Brent, Massachusetts General Hospital, Boston. *RPP5* cDNA fragments were obtained by reverse transcription–PCR from *Arabidopsis* Landsberg *erecta* (L*er*). The RPP5 baits TIR$^{1–223}$, TIR-NB-ARC$^{1–518}$, NB-ARC$^{161–518}$, and ARC$^{226–531}$ were made by fusing (*Eco*RI/*Bam*HI) the cDNA fragments with the DNA-binding domain of the pLexA vector. The pLexA baits with the plant NB-LRR genes *RPP1A* (20), *RPM1* (21), *RPS4* (22), and *N* (23) were kindly provided by M. Botella (Sainsbury Laboratory, Norwich, U.K.), M. Grant (Wye College, Wye, U.K.), W. Gassman and B. Staskawicz (Univ. of California, Berkeley), and M. Dutton and B. Baker (Univ. of California, Berkeley), respectively.

For the two hybrid cDNA library, mRNA was isolated (Amersham Pharmacia) from healthy leaves of 4-week-old wild-type and *pad4* mutant L*er* plants and from leaves of these plants harvested at several time points after infection with *Pseudomonas syringae* pv. *tomato* carrying *AvrRPS4* (22) or *P. parasitica* Noco2 (10). Directional, poly(dT)$_{17}$-primed, size-selected (>0.8-kb) cDNA with *Eco*RI/*Xho*I adapters (Stratagene) was ligated in the corresponding sites of the vector pADB42 (CLONTECH) and used to transform electrocompetent *E. coli* DH10B cells (GIBCO/BRL). Approximately 2.5×10^6 clones were obtained, and library plasmid DNA was isolated by using Tip500 columns (Qiagen, Chatsworth, CA).

Yeast Two-Hybrid Assays. All RPP5 baits repressed the pJK101 *lacZ* reporter in yeast EGY48, indicating that the LexA-RPP5 fusion proteins are expressed and transferred to the nucleus (17).

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Abbreviations: Avr, avirulence; NB, nucleotide-binding; ARC, Apaf-1, R proteins, CED-4; LRR, leucine-rich repeat; R, resistance; ppGpp, guanosine tetraphosphate; pppGppp, guanosine pentaphosphate; SMG medium, minimal agar medium supplemented with Ser, Met, and Gly.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF225702, AF225703, and AF225704 (*At-RSH1*, *At-RSH2*, and *At-RSH3*, respectively)].

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The RPP5 bait constructs, empty vectors, and several unrelated baits and preys (e.g., pLexLam, pRFHM1, pLex53, pB42-T) were tested in all combinations, but no activation of the reporter genes was observed, and hence, no indications were obtained for nonspecific interactions with RPP5 baits. Transformation of EGY48 (p8op-lacZ) carrying the RPP5 bait TIR-NB-ARC$^{1–518}$ with the *Arabidopsis* cDNA library resulted in ≈3.5 million primary transformants. After amplification, ≈38 million colony-forming units (cfu) were analyzed for activation of the *LEU2* and *lacZ* reporters. The NB-ARC$^{161–518}$ bait was used to screen ≈4.5 million EGY48 (p8op-lacZ) library transformants, and ≈45 million cfu were analyzed for activation of the *LEU2* and *lacZ* reporters. Library plasmids were rescued in *E. coli* strain XL-1 Blue MRF′ (Stratagene). Yeast transformations and liquid β-galactosidase assays were done as described in ref. 17 and CLONTECH's Yeast Protocols.

Nucleic Acid Analysis. Recombinant plasmids were made according to standard procedures (17). DNA sequence reactions (Perkin–Elmer) were run on a 377 DNA sequencer (Applied Biosystems). The cDNA sequences were extended by using the 5′ rapid amplification of cDNA ends system (GIBCO/BRL). Other DNA and RNA manipulations were done essentially as described previously (10). DNA sequences and predicted gene products were aligned by using the CLUSTALW algorithm (24), and phylogenetic analysis was done with the neighbor-joining method (25), with 1,000 bootstrap replicates.

Bacterial Expression and Complementation Analysis. For expression in *E. coli*, the 1.6-kb 5′ region (*Nde*I/*Xba*I) of the *At-RSH1* cDNA was cloned in pT7–7 under the control of the heat-inducible T7 RNA polymerase promoter system (26). The *E. coli* wild-type strain CF1648 and its derived mutants, CF1652 (*relA*::*kan*) and CF1693 (*relA*::*kan*, *spoT*::*cam*) (27), were kindly provided by M. Cashel, National Institutes of Health, Bethesda, MD.

For expression in *S. coelicolor*, the full-length *At-RSH1* cDNA (*Nde*I/*Nco*I) and the 1.6-kb 5′ portion (*Nde*I/*Xba*I) were cloned in the thiostrepton-inducible expression vector pIJ8600 (28). Plasmids were transferred to *S. coelicolor* strain M600 as described previously (29). Cloning details are available upon request.

Results

The NB-ARC Domain of RPP5 Interacts with At-RSH1 in Yeast. We used the yeast two-hybrid assay to identify RPP5-interacting protein(s). With the TIR-NB-ARC bait (Fig. 1*A*), 19 colonies were identified that conferred both leucine prototrophy and β-galactosidase activity. The 3′ ends of 10 clones were identical, and the corresponding gene was designated *A. thaliana RelA/SpoT homolog 1* (*At-RSH1*). The different lengths of the inserts indicated the isolation of five independent *At-RSH1* clones (Fig. 1*B*). In a second screen, the NB-ARC bait (Fig. 1*A*) identified 15 clones. DNA sequence analysis showed that 12 cDNA inserts were identical to the RSH1–18/16/20 group identified in the previous screen (Fig. 1*B*).

Retransformation of yeast cells with the TIR-NB-ARC or the NB-ARC baits with four different *At-RSH1* library plasmids showed that the *LEU2* and *lacZ* reporters were consistently activated only in the presence of galactose, indicating that *GAL4* promoter-driven expression of the *At-RSH1* cDNAs is required. In cells with either the TIR-NB-ARC bait or the NB-ARC bait, the four RSH1 library plasmids differentially activated the *LEU2* and *lacZ* reporters in a similar manner (Fig. 1*B*). Furthermore, the TIR bait and the ARC bait did not interact with any RSH1 clone (Fig. 1*A*), indicating that the NB-ARC domain of RPP5 is required and sufficient for interaction with At-RSH1.

We then examined whether At-RSH1 interacts with other

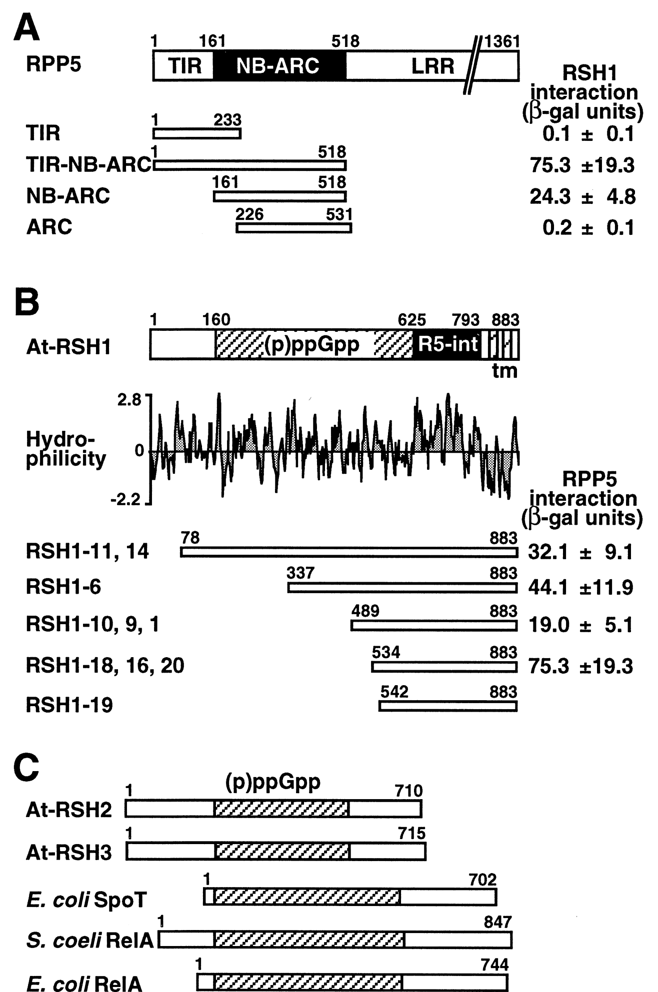

Fig. 1. Overview of RPP5/At-RSH1 interactions and (p)ppGpp synthetase domain-containing proteins. (*A*) Domain structure of RPP5 and interaction of RPP5 bait constructs with At-RSH1 (RSH1–18) in the yeast two-hybrid assay. β-Galactosidase ± SD in Miller units are averages from two replicates with three transformants. (*B*) Domain structure of At-RSH1, hydrophilicity analysis, and the five groups with At-RSH1 clones. R5-int, RPP5 interaction domain; TM, two transmembrane segments. (*C*) Domain structure of At-RSH2 and At-RSH3 with the (p)ppGpp synthetase domains aligned relative to that of At-RSH1, *E. coli* RelA (GenBank J04039) and SpoT (M24503), and *S. coelicolor* RelA (X87267).

NB-LRR proteins. We made an identical bait construct with the expressed *RPP5* homolog from the Columbia ecotype (Col-0), *RPP5-ColF* (87% identity, 92% similarity), of unknown function (16). In addition, we obtained four other comparable NB-ARC domain-containing baits derived from the *Arabidopsis RPP1A* (20), *RPM1* (21), and *RPS4* genes (22) and the tobacco *N* gene (23). Based on the pJK101 repression assay (17), all fusion proteins expressed from these NB-LRR baits were transferred to the nucleus. However, none of these baits activated the reporter genes upon coexpression with any of the RSH1 clones, indicating that At-RSH1 specifically interacts with RPP5 bait constructs.

***At-RSH1* Gene Structure and Predicted Functional Domains.** DNA blot analysis (2× SSC, 50°C) showed that *At-RSH1* is a single-copy gene in several *Arabidopsis* ecotypes. The Col-0 *At-RSH1* genome sequence (GenBank accession no. AF075597) showed that *At-RSH1* resides at 14 cM from the top of chromosome 4. Hybridization of *At-RSH1* to poly(A)-enriched leaf RNA (0.2×

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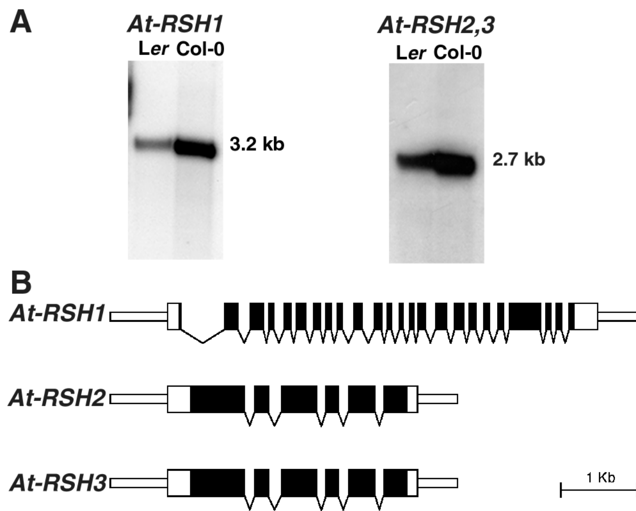

Fig. 2. *At-RSH1*, *2*, *and 3* transcript analysis and gene structures. (*A*) Poly(A) RNA gel blot with leaf RNA isolated from the *Arabidopsis* Landsberg *erecta* (L*er*) and Columbia (Col-0) ecotypes, hybridized to radiolabeled cDNA sequences corresponding to *At-RSH1* and *At-RSH2*; transcript sizes indicated in kilobases. (*B*) Intron/exon structures of *At-RSH1*, *2*, and *3*. Wide rectangles indicate transcribed regions, and solid rectangles constitute coding sequence. Narrow rectangles indicate 5′ and 3′ sequences, and intron positions are marked by arrowheads.

SSC, 65°C) revealed a single transcript of 3.2 kb (Fig. 2*A*). No induction of the *At-RSH1* transcript was observed in 10 mg of total RNA isolated from leaves harvested at several time points after infection with *P. syringae* DC3000, *P. syringae* DC3000 carrying *AvrRPS4* (22), forceps wounding, or treatment with 0.5 mM salicylic acid or 1 mM methyl jasmonate. A full length *At-RSH1* cDNA (3,137 bp) was obtained by 5′ rapid amplification of cDNA ends and encodes a 2,649-bp ORF. Alignment of the *At-RSH1* gene (5,729 bp) with the corresponding cDNA revealed 23 introns (Fig. 2*B*). The 3′ end of *At-RSH1* corresponds to two expressed sequence tags (ESTs) (GenBank accession nos. Z34756 and Z34769).

At-RSH1 codes for an 883-aa residue protein of 98.6 kDa. Secondary structure and hydropathy analysis (Fig. 1*B*) suggested two C-terminal transmembrane segments at residues 811–827 and 848–864. Topology predictions indicated that At-RSH1 is cytoplasmically localized but anchored at the plasma membrane. The C-terminal portion of At-RSH1 is sufficient for interaction with RPP5 (Fig. 1*B*); it contains a hydrophilic solvent-exposed region of ≈160 aa (residues 634–793) that may function as the RPP5-interacting domain (Fig. 1*B*). Database searches using TBLASTN (30) failed to reveal any sequences with significant homology to this RPP5-interaction domain. The central portion of At-RSH1 (residues 160–625) shows a high level of similarity to the central regions (≈450 residues) of bacterial RelA and SpoT proteins (≈30% identity, ≈58% similarity; Figs. 1 *B* and *C* and 3). Database searches using At-RSH1 identified uncharacterized amino acid sequences derived from rice (GenBank accession no. D48993), human (THC205397), mouse (AA475394 and AA473095), nematode (Z82096), and two additional *Arabidopsis* sequences (see next section) that appear to be homologous to (parts of) the RelA/SpoT (p)ppGpp synthetase domain.

Two Other *Arabidopsis* RelA/SpoT Homologs: *At-RSH2* and *At-RSH3*. Two unlinked and expressed *Arabidopsis* genes with significant homology to *At-RSH1* were identified by using TBLASTN (30). We designated these genes *At-RSH2* on chromosome 3 (GenBank accession nos. AB019229; ESTs N38487, W43725, H76717, AA713029) and *At-RSH3* on chromosome 1 (AC006577; EST W43807). *At-RSH2* and *At-RSH3* are highly similar (75% overall nucleotide identity) but share little DNA homology with *At-RSH1*; even the regions encoding the putative (p)ppGpp synthetase domains of *At-RSH1* and *AtRSH2* and *3* share only ≈38% nucleotide identity, whereas these domains of *At-RSH2* and *At-RSH3* share 84% nucleotide identity. DNA gel blot analysis (2× SSC, 50°C) using *At-RSH2* EST N38487 as probe revealed both *At-RSH2* and *At-RSH3* fragments. Hybridization to poly(A)-enriched leaf RNA (0.2× SSC, 65°C) showed a single transcript of ≈2.7 kb (Fig. 2*A*). Full-length *At-RSH2* and partial *At-RSH3* cDNAs were obtained by 5′ rapid amplification of cDNA ends. The *At-RSH2* cDNA is 2,605 bp in length with an ORF of 2,130 bp. The *At-RSH2* and *At-RSH3* genes have five introns at identical positions (Fig. 2*B*). The low DNA homology between *At-RSH1* and *At-RSH2* and *3* and the different positions of the introns in the (p)ppGpp synthetase domains indicate an ancient divergence or an independent origin.

At-RSH2 and *At-RSH3* encode 710- and 715-aa residues, respectively, with a molecular size of ≈80 kDa. Their central (p)ppGpp synthetase domains (318 residues) share 90% identity (94% similarity) and are 147 residues (32%) shorter than the same domain of At-RSH1 (465 residues; Figs. 1*C* and 3). The (p)ppGpp synthetase domains of At-RSH2 and At-RSH3 are markedly more similar to bacterial RelA/SpoT (≈46% identity, ≈66% similarity) than is At-RSH1 (≈30% identity, ≈58% similarity) and share 38% identity and 59% similarity with the corresponding region of At-RSH1 (Fig. 3). At-RSH2 and At-RSH3 do not contain predicted transmembrane-spanning regions, cleavable signal peptides, or hydrophilic C-terminal regions and are predicted to be located in the cytoplasm. The N- and C-terminal portions of At-RSH2 and At-RSH3, excluding the (p)ppGpp synthetase domains, display little or no homology to the corresponding portions of At-RSH1 or to other proteins. Phylogenetic analysis of the (p)ppGpp synthetase domains of At-RSH1, 2, and 3 together with a wide range of bacterial RelA/SpoT proteins grouped the *Arabidopsis* sequences with homologs from a number of intracellular pathogens (Fig. 4), perhaps indicative of lateral gene transfer early in evolution.

***At-RSH1* Restores Growth of an *E. coli relA* Mutant but Not of a *relA, spoT* Double Mutant.** RelA and SpoT play central roles in the bacterial stringent response, allowing prompt physiological responses to rapidly changing environmental conditions (18). The primary functions of RelA and SpoT are to synthesize and to degrade (p)ppGpp, respectively, and SpoT also is capable of (p)ppGpp synthesis under conditions of energy limitation. (p)ppGpp functions to regulate the transcription of a large number of genes, both positively and negatively. *E. coli* relA mutants are unable to grow on minimal agar medium supplemented with the amino acids Ser, Met, and Gly (SMG medium), a phenotype that is complemented through engineered (p)ppGpp synthesis (27). Moreover, because a *relA, spoT* mutant lacks (p)ppGpp phosphohydrolase activity, induced synthesis of (p)ppGpp in the double mutant abolishes growth, presumably through cessation of rRNA and tRNA synthesis, a primary characteristic of the stringent response (18).

To examine whether *Arabidopsis At-RSH1* might function as a (p)ppGpp synthetase, the 1.6-kb 5′ region of *At-RSH1* containing the putative (p)ppGpp synthetase domain was cloned in the temperature-inducible pT7–7 vector (26) and introduced into *E. coli* CF1648 (*relA*$^+$, *spoT*$^+$), CF1652 (*relA*$^-$, *spoT*$^+$), and CF1693 (*relA*$^-$, *spoT*$^-$). None of the *At-RSH1*-containing strains grew on SMG agar under inducing conditions (37°C and 42°C), indicating that high-level expression of *At-RSH1* was toxic, potentially reflecting levels of (p)ppGpp synthesis sufficient to prevent growth of even a *spoT*$^+$ strain on supplemented minimal medium

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At-RSH1 AFEAHHGQKRRSGEPFIIHPVAVARILGELELDWESIVAGLLHDTVEDTNFITFEKIEEEFGA 219
At-RSH2 AEKAHRGQMRASRDPYLQHCVETAMLLANIGANSTVVVAGLLHDTVDDS-FMSYDYILRNFGA 279
Ec-SpoT ARDAHEGQTRSSGEPYITHPVAVACILAEMKLDYETLMAALLHDVIEDT-PATYQDMEQLFGK  91
Sc-RelA AERWHRGQKRKSGDPYITHPLAVTTILAELGMDPATLMAGLLHDTVEDT-EYGLEDLRRDFGD 195
Ec-RelA AYCLQQTQGHPDASLLLWRGVEMVEILSTLSMDIDTLRAALLFPLADAN-VVSEDVLRESVGK 101

At-RSH1 TVRHIVEGETKVSKLGKLKCKTESETIQDVKADDLRQMFLAMTDEVRVIIVKLADRLHNMRTL 282
At-RSH2 GVADLVEGVSKLSQLSKLA-RENNTACKTVEADRLHPMFLAMA-DARAVLIKLADRLHNMKTL 340
Ec-SpoT SVAELVEGVSKLDKL-KFR-DK------KEAQAENFRKMIMAMVQDIRVILIKLADRTHNMRTL 147
Sc-RelA VVTLLVDGVTKLDKV-KFG-E--------AAQAETVRKMVVAMAKDPRVLVIKLADRLHNMRTM 249
Ec-RelA SVVNLIHGVRDMAAIRQLK-ATHTDSVSSEQVDNVRRMLLAMVDDFRCVVIKLAERIAHLREV 163

At-RSH1 CHMPPHKQSSIAGETLQVFAPLAKLLGMYSIKSELENLSFMYVSAEDYDRVTSRIANLYKEHE 345
At-RSH2 YALSPVKQQRFAKETLEIFAPLANCLGISTWKVQLENLCFKHLYPNQHNEMSTMLEDSFD--E 401
Ec-SpoT GSLRPDKRRRIARETLEIYSPLAHRLGIHHIKTELEELGFEALYPNRYRVIKEVVKAARGNRK 210
Sc-RelA RYLKREKQEKKARETLEIYAPLAHRLGMNTIKWELEDLAFAILYPKMYDEIVRLVAERAPKRD 312
Ec-RelA KDAPEDERVLAAKECTNIYAPLANRLGIGQLKWELEDYCFRYLHPTEYKRIAKLLHERRLDRE 226

At-RSH1 KELTEANRILVKKIEDDQFLDLVTVNTDV-RSVCKETYSIYKAALKSKGSINDYNQIAQLRIV 407
At-RSH2 AMITS----AIEKLDQA--LKKAGISYHVLCGRHKSLYSIYSKMLKKKLTVDEIHDIHGLRLI 458
Ec-SpoT EMIQK----ILSEIEGR--LQEAGIPCRV-SGREKHLYSIYCKMVLKEQRFHSIMDIYAFRVI 266
Sc-RelA EYLAV----VTDEVQQD--LRAARIKATV-TGRPKHYYSVYQKMIVRGRDFAEIYDLVGIRVL 368
Ec-RelA HYIEE----FVGHLRAE--MKAEGVKAEVY-GRPKHIYSIWRKMQKKNLAFDELFDVRAVRIV 282

At-RSH1 VKPKPSVGVGPLCSPQQICYHVLGLVHEIWKPIPRTVKDYIATPKPNGYQSLHTTVIPFLYES 470
At-RSH2 V------------DNEGDCYKALGVVHSLWSEVPGKLKDYITHPKFNGYQSLHTVVMD---NG 506
Ec-SpoT V------------NDSDTCYRVLGQMHSLYKPRPGRVKDYIAIPKANGYQSLHTSMIG---PH 314
Sc-RelA V------------DTVRDCYAALGTVHARWNPVPGRFKDYIAMPKFNMYQSLHTTVIG---PG 416
Ec-RelA A------------ERLQDCYAALGIVHTHYRHLPDEKDDYVANPKPNGYQSIHTVVLG---PG 330

At-RSH1 MFRLEVQIRTEEMDLIAERGIAVYYNGKSLSTGLVGNAVPL[GRNSRGKT]GCLNNADFALRVGW 533
At-RSH2 TVPLEVQIRTQEMHLQAEFGFAAHWRYKE                                      535
Ec-SpoT GVPVEVQIRTEDMDQMAEMGVAAHWAYKEHGETSTT------[--------]------AQIRAQRW 358
Sc-RelA GKPVELQIRTFDMHRRAEYGIAAHWKYKQEAVAGASKVRTD[APKSSGKS]----KDDHLNDMAW 475
Ec-RelA GKTVEIQIRTKQMHEDAELGVAAHWKYKEGAAAGGAR-----[--------]-----SGHEDRIAW 376

At-RSH1 LNAIREWQEEFVGNMSSREFVDTITRDLLGSRVFVFTPKGEIKNLPKGATVVDYAYLIHTEIG 596
Ec-SpoT MQSLLELQQS---AGSSFEFIESVKSDLFPDEIYVFTPEGRIVELPAGATPVDFAYAVHTDIG 418
Sc-RelA LRQLLDWQKE---TEDPGEFLESLRFDLSRNEVFVFTPKGDVIALPAGATPVDFAYAVHTEVG 535
Ec-RelA LRKLIAWQEE---MADSGEMLDEVRSQVFDDRVYVFTPKGDVVDLPAGSTPLDFAYHIHSDVG 436

At-RSH1 NKMVAAKVNGNLVSPTHVLENAEVVEIVTYNALSSKSAFQRHKQWLQHAKTRSARHKI 654
Ec-SpoT HACVGARVDRQPYPLSQPLTSGQTVEIIT--APGARPNAAWL----NFVVSSKARAKI 470
Sc-RelA HRTIGARVNGRLVPLESTLDNGDLVEVFTSKAAGAGPSRDWL----GFVKSPRARNKI 589
Ec-RelA HRCIGAKIGGRIVPFTYQLQMGDQIEIITQKQP--NPSRDWLNPNLGYVTTSRGRSKI 492
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Fig. 3. Sequence relationships between the (p)ppGpp synthetase domains of At-RSH1 and At-RSH2, and those of *E. coli* (Ec) RelA and SpoT and *S. coelicolor* (Sc) RelA. At-RSH1 and *S. coelicolor* RelA contain ATP/GTP-binding site motifs (P-loop; boxed) that are absent in other RelA/SpoT proteins.

agar. However, growth of the *relA* mutant was restored on SMG agar under noninducing conditions (30°C; Fig. 5*A*), consistent with a low level of expression of *At-RSH1* from the T7 promoter that would give rise to sufficient (p)ppGpp to suppress the SMG phenotype, but not to prevent growth (growth of *E. coli* on SMG medium requires only low steady-state levels of (p)ppGpp). Growth of the double mutant was not restored on SMG agar at 30°C (not shown), consistent with the inability of the *relA*, *spoT* double mutant to degrade *At-RSH1*-derived (p)ppGpp.

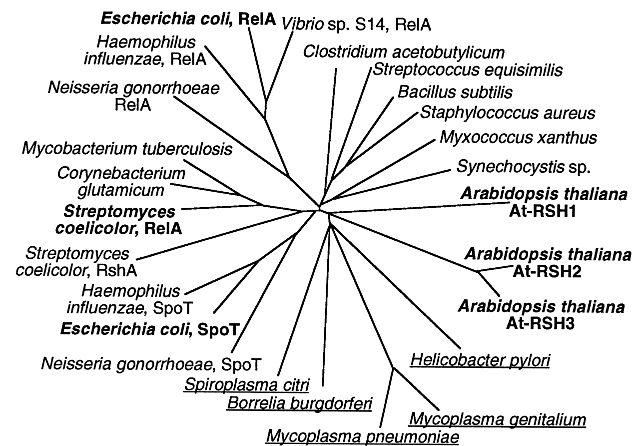

Fig. 4. Phylogenetic analysis of the (p)ppGpp synthetase domains of members of the RelA/SpoT family. The At-RSH1, 2, and 3 homologs and the *E. coli* and *S. coelicolor* RelA/SpoT proteins are shown in boldface type. At-RSH1, 2, and 3 cluster with RelA/SpoT proteins from intracellular pathogens (underlined).

There was no difference in growth rate between the *At-RSH1*-containing derivatives and their vector controls when the strains were grown in rich L-broth under noninducing conditions (30°C); any basal level of expression of *At-RSH1* under these conditions presumably produces insufficient (p)ppGpp to impair growth in this nutrient-rich liquid medium. In contrast, induction of the truncated *At-RSH1* at 42°C in rapidly dividing L-broth cultures reduced the growth rate of CF1652 ($relA^-$, $spoT^+$) and essentially abolished growth of CF1693 ($relA^-$, $spoT^-$) (Fig. 5*B*), consistent with high levels of (p)ppGpp synthesis. Thus, expression at two different levels of the 5′ portion of *At-RSH1* containing the (p)ppGpp synthetase domain in *E. coli* confers two distinct phenotypes associated with (p)ppGpp synthesis: restoration of growth of a *relA* mutant on minimal SMG agar when expressed at a low level, and abolition of growth of a *relA*, *spoT* double mutant in rich L-broth when expressed at a high level.

***At-RSH1* Confers Phenotypes Associated with (p)ppGpp Synthesis in *S. coelicolor*.** To analyze further the potential (p)ppGpp synthetase activity of *At-RSH1*, it was expressed in *S. coelicolor*. *S.*

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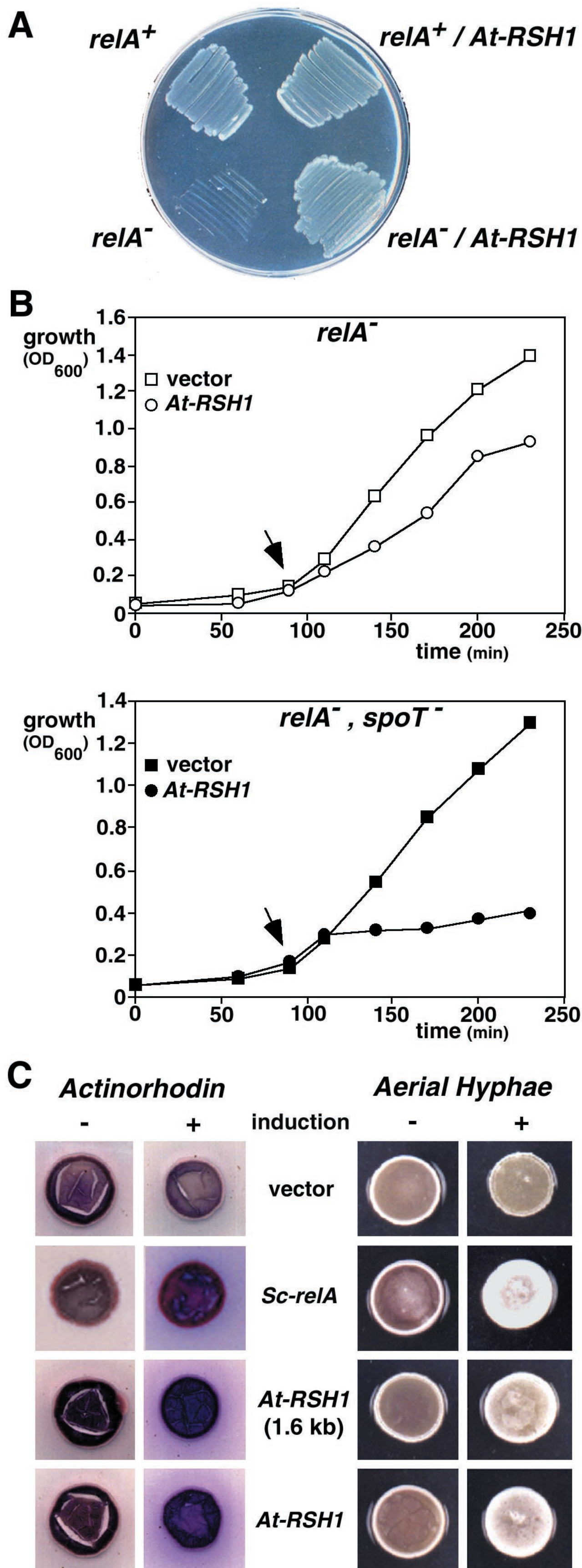

Fig. 5. Functional analysis of *At-RSH1* in bacteria. (*A*) *At-RSH1* complements an *E. coli relA*⁻ mutant. The 1.6-kb 5′ region of *At-RSH1* restores growth of the *E. coli relA* mutant (CF1652) on SMG medium at 30°C. (*B*) *At-RSH1* expression is lethal in an *E. coli relA*⁻, *spoT*⁻ double mutant. The 1.6-kb 5′ region of *At-RSH1* was cloned in the temperature-inducible pT7–7 vector (26). Expression was induced by rapidly transferring rich L-broth cultures from 30°C to 42°C (denoted by arrows). Induced expression of *At-RSH1* (1.6 kb) in the *E. coli relA* mutant (CF1652) slightly reduces growth rate (*Upper*), whereas in the *E. coli relA*, *spoT* double mutant (CF1693) growth is abolished (*Lower*). (*C*) *At-RSH1* expression in *S. coelicolor* M600. *At-RSH1* was expressed from the thiostrepton-inducible *tipA* promoter of pIJ8600 (28). Spores of each strain were dropped on SMMS medium (28) and allowed to dry. Twelve microliters of 1 mg/ml thiostrepton in 2% DMSO was added to induce *tipA* expression (+), and 12 μl of 2% DMSO was added to the control cultures (−). The plates were incubated at 30°C for 4 days. Induced expression of the *S. coelicolor relA* gene and the full-length and 1.6-kb 5′ region of *At-RSH1* results in precocious antibiotic production (the blue pigment, actinorhodin) and precocious aerial hyphae formation, giving a white appearance to the mycelium.

coelicolor relA null mutants do not produce (p)ppGpp (A. Hesketh and M.J.B., unpublished data); consequently, they are deficient in antibiotic production and show delayed morphological differentiation, i.e., delayed formation of aerial hyphae and spores (19, 29). Induced expression of the endogenous *relA* gene in the *relA*⁺ *S. coelicolor* M600 strain provokes the precocious production of the blue-pigmented antibiotic actinorhodin and of aerial hyphae (Fig. 5*C*).

Expression of *At-RSH1* or a 5′ portion containing the (p)ppGpp synthetase domain in M600 using the thiostrepton-inducible pIJ8600 vector (28) yielded phenotypes that were indistinguishable from those observed upon induction of the endogenous *relA* gene (Fig. 5*C*). Thus, expression of *At-RSH1* in *S. coelicolor* again induces two distinct phenotypes associated with (p)ppGpp synthesis: precocious antibiotic production and the early onset of morphological differentiation.

Discussion

We identified an *Arabidopsis* RelA/SpoT homolog, *At-RSH1*, that specifically interacts with the NB-ARC domain of RPP5 in yeast. We showed that *At-RSH1* confers disparate phenotypes associated with (p)ppGpp synthetase activity in both *S. coelicolor* and *E. coli*. In addition to *At-RSH1*, we characterized two other *Arabidopsis* RelA/SpoT homologs, *At-RSH2* and *At-RSH3*. The *At-RSH* genes are the first eukaryotic homologs of bacterial *relA* or *spoT* genes described to date. RelA and SpoT play a central role in the bacterial stringent response (18). Both enzymes are made constitutively and are allosterically activated under sudden nutritional and environmental stress conditions such as amino acid, carbon, nitrogen, or phosphate starvation, as well as upon abrupt increases in temperature and osmolarity. RelA and, under certain conditions, SpoT transfer pyrophosphate groups from ATP to the 3′ positions of GDP and GTP, resulting in the rapid accumulation of ppGpp and pppGpp. In bacteria, (p)ppGpp induces and represses transcription of genes involved in a wide variety of processes (18).

The striking amino acid similarity and the ability of *At-RSH1* to complement such disparate phenotypes in two evolutionary distinct bacteria strongly suggest that At-RSH1 is capable of (p)ppGpp synthesis. Although several nucleotide derivatives such as cAMP, cGMP, and cADP-ribose have been implicated in plants as intracellular secondary signaling molecules (31, 32), ppGpp and pppGpp have not been described unambiguously in plants or other eukaryotes. By analogy to its role in bacteria, it is conceivable that (p)ppGpp functions in plants as a rapidly activated transcription cofactor. Rapid cellular stress responses are required in plants subject to pathogen infection and after wounding, sudden drought or flooding conditions, or osmotic shock. Stress-induced (p)ppGpp-mediated transcriptional repression of genes involved in normal cellular metabolic processes could rapidly prevent loss of compounds and energy and would

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nutritionally deprive invading pathogens. In addition, (p)ppGpp-mediated transcriptional activation may rapidly induce stress- and defense-related genes and compounds.

The yeast two-hybrid interaction between At-RSH1 and RPP5 is intriguing, but a function for *At-RSH1* has not yet been established *in planta*. We have postulated that NB-LRR proteins may "guard" host proteins for interference from pathogen Avr products (33). In line with this model is the idea that RPP5 has evolved to specifically recognize the physical association of *P. parasitica* (a)virulence factors with At-RSH1 and subsequently activate defense mechanisms. The C-terminal LRR domain of RPP5 could, like the WD40 repeats in Apaf-1 (34), ensure that activation of the protein complex is signal-dependent. This model implies that plants do not adapt to pathogens producing a virtually unlimited number of Avr effectors, but rather produce a restricted number of R proteins that guard a finite number of host targets.

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