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

Erik A. van der Biezen*, Jongho Sun†, Mark J. Coleman*, Mervyn J. Bibb, and Jonathan D. G. Jones*§

*The Sainsbury Laboratory and †Department of Genetics, John Innes Centre, Norwich NR4 7UH, United Kingdom

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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 domains of the NB-ARC1–518, NB-ARC161–518, and ARC226–531 were made by fusing the cognate Avr product (10). RPP5 is a member of a subclass with similarity to the effector domains of the NB-LRR genes in the corresponding sites of the vector pADB42 (CLONTECH) and library plasmid DNA was isolated by using Tip500 columns (Qiagen, Chatsworth, CA). For the two hybrid cDNA library, mRNA was isolated (Amersham Pharmacia) from healthy leaves of 4-week-old wild-type and papd4 mutant Ler 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)γ-primed, size-selected (>0.8–

Materials and Methods

Yeast Two-Hybrid Plasmids and Library. The Matchmaker LexA Two-Hybrid system was used (CLONTECH). The plasmids pJK101 and pRFH1 were kindly provided by R. Brent, Massachusetts General Hospital, Boston. RPP5 cDNA fragments were obtained by reverse transcription–PCR from Arabidopsis Landsberg erecta (Ler). The RPP5 baits TIR1–223, TIR-NB-ARC1–518, NB-ARC161–518, and ARC226–531 were made by fusing (EcoRI/BamHI) 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.

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; pppGpp, 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)].

§To whom reprint requests should be addressed. E-mail: jonathan.jones@bbsrc.ac.uk.

†Present address: School of Biological Sciences, University of East Anglia, Norwich NR4 7NW, United Kingdom.

‡To whom reprint requests should be addressed. E-mail: jonathan.jones@bbsrc.ac.uk.

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The RPP5 bait constructs, empty vectors, and several unrelated baits and prey (e.g., pLexLam, pRFAHIM1, pLexS3, 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-ARC1-518 together 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-ARC161-518 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 (Ndel/XbaI) 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 (NdeI/NcoI) and the 1.6-kb 5′ portion (NdeI/XbaI) were cloned in the thiostrepton-inducible expression vector pJ8600 (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. 1A), 19 colonies were identified that conferred 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. 1B).

In a second screen, the NB-ARC bait (Fig. 1A) identified 15 clones. DNA sequence analysis showed that 12 cDNA inserts were identical to the RSH1–18 DNA sequence analysis showed that 12 cDNA inserts were identical to the RSH1–18 DNA sequence. A second screen, the NB-ARC bait (Fig. 1B) identified 19 colonies were analyzed for activation of the LEU2 and lacZ reporters. The RSH1–18 bait was used to screen 3748 (3.748 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.

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In a second screen, the NB-ARC bait (Fig. 1A) identified 15 clones. DNA sequence analysis showed that 12 cDNA inserts were identical to the RSH1–18 DNA sequence 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).

**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, and S. coelicolor RelA.

**At-RSH1 Gene Structure and Predicted Functional Domains.** DNA blot analysis (2X SSC, 50°C) showed that At-RSH1 is a single-copy gene in several Arabidopsis ecotypes. The Col-0 At-RSH1 gene 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×
SSC, 65°C) revealed a single transcript of 3.2 kb (Fig. 2). 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. 2B). The 3' end of At-RSH1 corresponds to two expression 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. 1B) 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. 1B); it contains a hydrophilic solvent-exposed region of ≈160 aa (residues 634–793) that may function as the RPP5-interacting domain (Fig. 1B). 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 (AA75394 and AA473095), nematode (Z82096), and two additional Arabidopsis sequences (see next section) that appear to be homologous to (parts of) the RelA/SpoT (pppGpp 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, H76177, 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 (pppGpp synthetase domains of At-RSH1 and At-RSH2 and 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. 2A). 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. 2B). The low DNA homology between At-RSH1 and At-RSH2 and 3 and the different positions of the introns in the (pppGpp 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 (pppGpp 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. 1C and 3). The (pppGpp 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 (pppGpp synthetase domains, display little or no homology to the corresponding portions of At-RSH1 or to other proteins. Phylogenetic analysis of the (pppGpp 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 (pppGpp, respectively, and SpoT also is capable of (pppGpp synthesis under conditions of energy limitation. (pppGpp 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 (pppGpp synthesis (27). Moreover, because a relA, spoT mutant lacks (pppGpp phosphorylase activity, induced synthesis of (pppGpp 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 (pppGpp synthetase, the 1.6-kb 5' region of At-RSH1 containing the putative (pppGpp synthetase domain was cloned in the temperature-inducible pT7-5 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 (pppGpp synthesis sufficient to prevent growth of even a spoT– strain on supplemented minimal medium.

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agar. However, growth of the relA mutant was restored on SMG agar under noninducing conditions (30°C; Fig. 5A), 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.

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<sup>2</sup>, spoT<sup>1</sup>) and essentially abolished growth of CF1693 (relA<sup>2</sup>, spoT<sup>2</sup>) (Fig. 5B), consistent with high levels of (p)ppGpp synthesis. Thus, expression at two different levels of the 5<sup>'</sup> 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.
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. 5C).

Expression of At-RSH1 or a 5’ portion containing the (p)p-pGpp 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. 5C). 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)p-pGpp 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 evolutionarily 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 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)p-pGpp induces and represses transcription of genes involved in a wide variety of processes (18).

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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 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’-3’ 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.

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
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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