Inducible Expression, Enzymatic Activity, and Origin of Higher Plant Homologues of Bacterial RelA/SpoT Stress Proteins in *Nicotiana tabacum*

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All living cells possess adaptive responses to environmental stress that are essential to ensuring cell survival. For motile organisms, this can culminate in avoidance or attractive behavior, but for sessile organisms such as plants, stress adaptation is a process of success or failure within the confines of a given environment. Nearly all bacterial species possess a highly evolved system for stress adaptation, known as the “stringent response.” This ancient and ubiquitous regulatory response is mediated by production of a second messenger of general stress, the nucleotide guanosine-3′,5′-(bis)pyrophosphate (ppGpp), which mediates reprogramming of the global transcriptional output of the cell. Accumulation of ppGpp is stress-induced through the enzymatic activation of the well known bacterial ppGpp synthetases, RelA and SpoT. We have recently discovered homologues of bacterial relA/spoT genes in the model plant *Nicotiana tabacum*. We hypothesize that these homologues (designated RSH genes for RelA/SpoT homologues) serve a stress-adaptive function in plants analogous with their function in bacteria. In support of this hypothesis, we find 1) inducibility of tobacco RSH gene expression following treatment with jasmonic acid; 2) bona fide ppGpp synthesis activity of purified recombinant Nt-RSH2 protein, and 3) a wide spread distribution of RSH gene expression in the plant kingdom. Phylogenetic analyses identifies a distinct phylogenetic branch for the plant RSH proteins with two subgroups and supports ancient symbiosis and nuclear gene transfer as a possible origin for these stress response genes in plants. In addition, we find that Nt-RSH2 protein co-purifies with chloroplasts in subcellular fractionation experiments. Taken together, our findings imply a direct mode of action of these ppGpp synthetases with regard to plant physiology, namely regulation of chloroplast gene expression in response to plant defense signals.

The relA and spoT genes in bacteria encode enzymes that synthesize the unusual nucleotide guanosine-3′,5′-(bis)pyrophosphate (ppGpp), which is a second messenger of the so-called “stringent response” to nutrient deprivation and environmental stress. ppGpp is the intracellular effecter of the stringent response, which acts by binding directly to and inducing allosteric modification of the bacterial RNA polymerase (RNAP) (1). This results in global reprogramming of the bacterium’s transcriptional activity. There is a general inhibition of transcription and halting of the production of components of the protein synthesis apparatus in order to conserve energy. Simultaneously, there is an induction of stress genes to ensure proper cell adaptation and survival (2). Until recently, it was believed that the stringent response was limited to the bacterial domain of the prokaryote kingdom; however, plant homologues to these bacterial stress enzymes were recently identified (3, 4). In *Arabidopsis thaliana*, a relA/spoT homologue *At-RSH1* was discovered in a yeast two-hybrid system using a disease resistance protein as bait (3). Two additional *Arabidopsis* homologues, *At-RSH2* and *At-RSH3*, were subsequently identified upon completion of sequencing of the *Arabidopsis* genome (3). In another study, rice RSH genes were recovered from a jasmonic acid (JA)-treated subtractive cDNA library (4). Here we report the identification of a relA/spoT homologue in the tobacco plant, *Nicotiana tabacum*, which we designate Nt-RSH2 (NCBI accession number AA346377). This gene contains a 2154-bp open reading frame with 78% identity to *At-RSH2* and *At-RSH3*, two of the three RelA/SpoT homologues present in *Arabidopsis*, and very low similarity (55%) to the third *Arabidopsis* protein At-RSH1. Tobacco Nt-RSH2 expressed at low levels in bacteria has been found to be active for synthesis of ppGpp synthesis based on two criteria: 1) genetic complementation of a bacterial relA mutant and 2) toxicity to hosts strains lacking ppGpp-degrading activity. This is similar to the behavior of the *Arabidopsis* homologues (3). In addition, and distinct from all previous studies, we directly demonstrate ppGpp and guanosine 3′(2′)-diphosphate 5′-triphosphate (ppGpp) (a precursor) synthetase activity of the purified Nt-RSH2 protein by *in vitro* biochemical assays. Northern analysis has confirmed the presence of basal level Nt-RSH2 transcripts in tobacco, and cross-species hybridization gives evidence of *RSH1* transcripts in a wide range of plant species. Treatment of tobacco plants with JA or EtOH induced a rapid and persistent increase in accumulation of Nt-RSH2 transcripts and Nt-RSH2 protein. In addition, Nt-RSH2 protein abundance is elevated in tobacco plants with JA or EtOH induced a rapid and persistent increase in accumulation of ppGpp (a precursor) synthetase activity of the purified Nt-RSH2 protein by *in vitro* biochemical assays. Northern analysis has confirmed the presence of basal level Nt-RSH2 transcripts in tobacco, and cross-species hybridization gives evidence of *RSH1* transcripts in a wide range of plant species. Treatment of tobacco plants with JA or EtOH induced a rapid and persistent increase in accumulation of Nt-RSH2 transcripts and Nt-RSH2 protein. In addition, Nt-RSH2 protein abundance is elevated in response to infection with a bacterial pathogen. Taken to-
gether, these findings suggest a central role for Nt-RSH2 in response to biotrophic pathogens and environmental stress. Phylogenetic analysis suggests an ancient symbiotic origin for RSH genes in plants, most likely inherited by horizontal transfer from the chloroplast into the nucleus. Furthermore, we have localized Nt-RSH2 protein to the chloroplasts of the tobacco plant, consistent with the horizontal transfer hypothesis and implicating Nt-RSH2 as a regulator of plastid gene expression in analogy with the role of RelA and SpoT in the bacterial cell.

EXPERIMENTAL PROCEDURES

Plant Material, Growth Conditions, and Treatments—N. tabacum (strain SR1) plants used for isolation of total RNA and for experimental treatments were germinated and grown under sterile conditions on MS medium (Duchefa) solidified with 0.8% agar in analogy with the role of RelA and SpoT in the bacterial cell. For standardization, membranes were rehybridized with a 250-bp PCR product encompassing the full-length Nt-RSH2 gene, with an added NdeI site overlapping the AUG start codon and an in frame Xhol site just prior to the natural UAG stop codons. This PCR fragment was cleaved with NdeI and BamHI and gel-purified, and then ligated into corresponding NdeI and Xhol sites of the overexpression vector pET21b (Novagen, Inc.).

Tagged Expression Constructs and Protein Purification—Nt-
RSH2 was amplified by PCR using the forward primer, GGAGATC-
CATATGGCGGCTTCCGAGATACGC (NdeI site underlined) and the reverse primer, CCCCGCTCGAGAATTCGCGGACAGCTCT (Xhol site underlined) from pNt-RSH2. This produced a 1947-bp PCR product encompassing the 5'-end of the Nt-RSH2 gene, with an added NdeI site corresponding to amino acid positions 430–550 of the RSH2 sequence. Amplifications using the forward primer plus the two reverse primers, respectively, to facilitate induce T7 RNAP expression. Nt-RSH2 overexpression was accomplished by the pET21b(Nt-RSH2) construct (9). After transformation, the strain BL21(DE3) was grown in 2 liters of Superbroth (1% yeast extract, 1.6% tryptone, and 0.5% NaCl) with 0.2% glucose and 0.1 mM IPTG. Growth was monitored turbidometrically at 600 nm, at an absorbance of 0.5, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce T7 RNA polymerase expression. Nt-RSH2 expression was allowed to proceed for 3 h after the addition of isopropyl-β-D-thiogalactopyranoside. Cells were harvested by centrifugation and stored as cell pellets at −75 °C. 7.0 g (dry weight) of cells were resuspended in 30 ml of lysis buffer (50 mM sodium phosphate, pH 7.0, 250 mM NaCl, 10 mM imidazole, 0.1% Triton X-100, 5 mM β-mercaptoethanol). The complete EDTA-free protease inhibitor mixture (Roche Applied Science) was added to the lysis buffer following the manufacturer’s recommendations. The cell suspension was incubated for 30 min on ice. Cells in suspension were disrupted by mechanical agitation. The cellular lysate was centrifuged at 12,000 × g and, insoluble (pellet) and soluble protein fractions were checked for the presence of Nt-RSH2 protein by SDS-PAGE and Coomassie Blue staining. Approximately 95% of Nt-RSH2 protein was in the insoluble pellet fraction.

The insoluble pellet was resuspended in 25 ml of lysis buffer plus 4 mM guanidine HCl, with vigorous overnight rotation on a rocking platform. The resuspended protein pellet was then diluted 1:1 with 25 ml of lysis buffer lacking guanidine HCl before the addition of 1 ml of a 50% slurry of Ni2+-nitrilotriacetic acid-agarose (Qiagen). The protein-Ni2+
-nitrilotriacetic acid-agarose slurry was left muting for an additional 4 h at 4 °C prior to loading on a 1-cm diameter × 20-cm length column with a stoopkock fused capillary. The suspension was allowed to drip through with stopcock full open until all of the Ni2+-agarose resin was collected. Wash solution (50 mM sodium phosphate, pH 7.0, 250 mM NaCl, 35 mM imidazole, 0.1% Triton X-100, 5 mM β-mercaptoethanol, complete EDTA-free protease inhibitor mixture) was run through the column until no protein was detected in the elution. The His-tagged Nt-RSH2 was then eluted using a wash buffer containing 10 mM β-
mercaptoethanol, and 1 ml of wash buffer plus 0.5 M imidazole, and each fraction was checked for the presence of protein until no eluting protein could be detected. Protein-containing fractions were pooled, −15 ml total, and precipitated with 0.28 g/ml ammonium sulfate incubated at 4 °C overnight. The precipitate was harvested by centrifugation at 12,900 × g for 30 min. The pellet was resuspended in 300 μl of 100 mM Tris-HCl, pH 7.4, 250 mM NaCl, and dialyzed overnight against 2 liters of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM β-mercaptoethanol, 50% glycerol at 4 °C, with two changes. Final protein concentration was 1 mg/ml. SDS-PAGE analysis indicated greater than 95% purity. A histidine-tagged version of the...
E. coli RelA protein cloned into the identical pET21b vector was purified in parallel with the Nt-RSH2 (His-tagged) protein using identical procedures.

**ppGpp Synthesis Assays**—Purified Nt-RSH2 protein was assayed for ppGpp and pppGpp synthesis activity as described for bacterial RelA enzyme (10). Reaction buffer consisted of 50 mM HEPES, 250 mM NaCl, 2 mM EDTA, 1 mM β-mercaptoethanol, 14 mM MgSO₄, 8 mM ATP (0.2 μCi reaction [γ-32P]ATP), 6 mM GTP, and/or 6 mM GDP, as indicated in the legend to Fig. 2. Reactions were initiated by the addition of different amounts of Nt-RSH2 protein: 1, 2, 5, and 10 μg as indicated in a final volume of 20 μL. In control reactions, different concentrations of purified E. coli RelA protein (0.5, 1, 2.5, and 5 μg) were added instead of Nt-RSH2. Reactions were allowed to proceed at room temperature for 4 h, and reactions were terminated and mixed with 6 μL of 3 M formic acid. The entire sample was then spotted in two applications onto the origin of a 20 × 20-cm 100-μm pore polyethyleneimine-cellulose flexible TLC sheets (Selecto Scientific). After drying, reaction products were resolved by thin layer chromatography using either 1.5 M potassium phosphate, pH 3.4, or 1.75 M sodium phosphate buffer, pH 3.4, for mobile phase, as indicated. Once the solvent front had reached 4 cm from the top, the TLC sheets were dried for 1 h at room temperature and autoradiographed for 4–5 h. We note the consistent presence in commercial radioactive [γ-32P]ATP of a radioactive contaminant that serendipitously migrated to a position located between the migration of ppGpp and pppGpp. We found this contaminant to be present in all commercially available sources of [γ-32P]ATP that we tested, and the amount of radioactive contaminant appeared to vary from batch to batch even from the same commercial vendor. For example, as seen in Fig. 2A, the amount of contaminant in the first lane is considerably less than the amount of contaminant seen in Fig. 2B, and in fact these two experiments were performed with differing batches of radioactive [γ-32P]ATP both from the same vendor.

**Phylogenetics**—We obtained Rel-like proteins from a BLAST search using Nicotiana as well as all Rel-like sequences from different genera of plants, bacteria, and cyanobacteria, with inclusion of the most divergent sequences from the major clades (11). This yielded 47 sequences (see supplementary table for accession numbers and full names) with eight of these from plants and six from cyanobacteria. Sequences were imported into ClustalX and aligned using the default parameters and the Gonnet 250 matrix (12). An alignment of the conserved core (405 amino acids), which is available from Treebase (available on the World Wide Web at www.treebase.org; New York University, Buffalo, NY), was used for phylogenetic analyses.

Phylogenetic analyses were conducted in PAUP* 4.0 (13) and Mr. Bayes 3.0 (14, 15). To estimate phylogenetic uncertainty, we used a Bayesian statistical method with Markov chain Monte Carlo sampling (14). The Markov chain Monte Carlo method samples trees from the universe of possible trees in proportion to their probability given a model of evolution. We used the JTT model (16) with 50,000 generations and five parallel runs with random starting trees and four chains. We removed the first 3000 trees (after graphical inspection) to account for variance due to convergence of the parameters on the Markov chains. The remaining trees were exposed to 50% majority rule consensus tree analysis in PAUP. The proportion of trees containing a clade represents its posterior probability or the probability of being correct given the data and model of evolution.

**Nt-RSH2 Antiserum Production**—Using the His-tagged purified Nt-RSH2 protein described above we contracted production of rabbit antiserum (Harlan Bioproducts for Science, Inc.). Prior to rabbit inoculation, five preimmune antisera were assayed by Western analysis for the absence of RelA/SpoT cross-reactivity using purified E. coli RelA and SpoT proteins and isolating antisera with bacterial cross-reacting antigens. A single preimmune antiserum was found to be free of cross-reactivity to bacterial RelA and SpoT proteins, and the rabbit from which this antiserum was tested was used for Nt-RSH2 immunization, followed by three booster immunizations performed at 1-month intervals for a 3-month period, followed by production and final bleeds 1 month after the third booster immunization.

**Fractionation and Analysis of Purified Tobacco Chloroplast with Nt-RSH2 Antiserum**—Intact tobacco chloroplasts were isolated from leaf tissue and purified on Percoll gradients as described (17). Soluble and insoluble plastid protein fractions were resuspended in 1 mL each of lysis buffer. Samples (20, 10, 5, and 2.5 μL) of these two fractions were mixed with sample buffer and fractionated by SDS-PAGE. Proteins were transferred to activated polyvinylidene difluoride membrane and probed by conventional Western blot analysis with rabbit Nt-RSH2 antiserum followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase. Nt-RSH2 antibodies were visualized by chemiluminescence using the Renaissance Enhanced Luminal Kit (Pierce/Elmer Life Sciences) and exposure to x-ray film.

**RESULTS**

**Degenerate PCR and Identification of the Nt-RSH2 Gene**—For initial isolation, two internal relA/spot homologous fragments of the expected size were PCR-amplified from tobacco leaf RNA, using degenerate oligonucleotide primers that were based on a region of conserved protein sequences of the E. coli relA and spoT genes and the Arabidopsis At-RSH1, At-RSH2, and At-RSH3 (3). After confirmation of relA/spot homologous DNA sequences, these PCR fragments were used to probe a commercial tobacco leaf Zap cDNA library. Five rescued plasmids containing relA/spot homologues were isolated. Two of these contained identical sequences bearing a full-length open reading frame and untranslated flanking regions and were designated Nt-RSH2. The remaining cDNAs bore partial sequences that differed slightly and may represent separate Nt-RSH genes.

The Nt-RSH2 mRNA (accession number AY346377) is 2551 bp in length and contains an open reading frame for a 718-amino acid polypeptide that spans nt positions 276–2430. The predicted Nt-RSH2 protein is highly similar (79%) along its entire length to the At-RSH2 and At-RSH3 proteins (698 and 695 amino acids, respectively) of Arabidopsis (3) (Fig. 1). Nt-RSH2 protein shows much lower similarity to E. coli RelA and SpoT proteins, with only 50 and 55% overall similarity, respectively, over the 327 amino acids that span the central ppGpp synthetase domain. This cross-species similarity is approximately the same as that found between the two cytoplasmic Arabidopsis At-RSH proteins and E. coli RelA and SpoT proteins (3). Similarity of Nt-RSH2 to the Arabidopsis At-RSH1 is considerably less, 55% over the 351 central amino acids. Nt-RSH2 shows highest similarity to At-RSH2 and At-RSH3 of Arabidopsis (Fig. 1) and is more divergent from At-RSH1. Based on this observation, and on an overview of RSH sequences present in GenBank, we propose the classification of plant RSH genes into two distinct groupings, RSH1 and RSH2. The RSH1 group would contain genes such as At-RSH1 (and Nt-RSH1, recently entered into GenBank as accession number AB095098), which lack a plastid transit sequence and appear to be membrane-associated. The RSH2 group would contain proteins similar to Nt-RSH2, At-RSH2, and At-RSH3, which possess characteristics of soluble, plastid-localized proteins.

Several aspects of the full-length cDNA indicate that Nt-RSH2 is a true nuclear-encoded plant gene and did not originate from a bacterial contaminant or from organellar transcripts. First, a computer search confirmed that the tobacco chloroplast genome (accession number NC_001879) does not contain any Nt-RSH sequences. The Nt-RSH2 cDNA also has a long 5′-untranslated region of 276 nucleotides and 3′-untranslated region of 316 nucleotides, which is very atypical for bacterial or organellar mRNAs. The Nt-RSH2 5′-untranslated region is highly AU-rich, another characteristic typical of nuclear encoded plant mRNAs (18). The initiation codon is in good context (AUGGC) for efficient translation initiation in plants (19). Taken together, these results suggest that Nt-RSH2, At-RSH2, and At-RSH3 all represent a highly conserved group of...
soluble nuclear encoded RelA/SpoT-like proteins, distinct from
the putative membrane-associated Arabidopsis At-RSH1 pro-
tein and distinct from the bacterial RelA and SpoT proteins.
The N-terminal portion of Nt-RSH2 has several features
associated with plastid transit sequences (20). These include 1)
the presence of an amino-terminal MA dipeptide, 2) no charged
residues in the amino-terminal 15 amino acids, and 3) an
abundance of serine residues (27%) and few Asp (2.9%), Glu
(0%), or Tyr (4.3%) residues within the first 90 amino acids.
The TargetP version 1.0 program (available on the World
Wide Web at www.cbs.dtu.dk/services/TargetP/) (21, 22) predicts
that Nt-RSH2 has a 0.712 probability of containing an N-
terminal chloroplast transit sequence, with a possible cleavage
site at amino acid 93. The At-RSH2 and At-RSH3 proteins gave
similar results with this program and probably also possess
plastid transit peptides. Nt-RSH2, At-RSH2, and At-RSH3
share several conserved regions within their N-terminal por-
tions (positions 1–107 in Fig. 1) that are not found in At-RSH1
or in the SpoT protein from E. coli. These latter two proteins
were not predicted to bear chloroplast transit sequences.

Cross-species Complementation of E. coli with NtRSH2—
The Nt-RSH2 cDNA borne on the phagemid pNt-RSH2 is fortu-
itously oriented such that expression of Nt-RSH2 is possible in
E. coli by virtue of the presence of the E. coli lac promoter
upstream of the Nt-RSH2 gene. Translation in E. coli is possible
via an “in frame” fusion of Nt-RSH2 to the Lac α-peptide,
which overlaps the multiple cloning site of the pBluescript
SK+ vector. Taking advantage of these circumstances, the
pNt-RSH2 plasmid was checked for phenotypic complementa-
tion in the E. coli strain, CF1651 (9), which is deleted for the
relA gene. This relA null strain displays no obvious phenotypic
manifestations of the loss of relA, except with respect to growth
on specialized selective growth media (2). For example, relA
null mutants cannot grow on minimal salt glucose medium
supplemented with the amino acids serine, methionine, and
glycine (SMG media). Inability of relA mutants to grow on SMG
medium is due to the phenomenon that this particular combi-
nation of amino acids invokes a metabolic imbalance that re-
sults in isoleucine starvation due to suppression of expression
of the isoleucine biosynthetic operon, ilvIH. Induction of high
level accumulation of ppGpp by RelA following isoleucine star-
vation induced by growth on SMG medium in turn induces high
level expression of ilvIH, leading the cell to overcome SMG-
duced isoleucine starvation. In the absence of the RelA strin-
gent factor, growth on SMG medium is restricted due to inabil-
ity to accumulate high levels of ppGpp needed to prompt

![Figure 1](attachment:image.png)

**Fig. 1.** Sequence homology between plant and bacterial RelA/SpoT proteins. Amino acid sequence alignment showing the relationship between plant RSH proteins and the E. coli SpoT protein, NtRSH2, deduced RelA/SpoT homologue from N. tabacum, AtRSH1, AtRSH3, and
AtRSH3, the Arabidopsis homologues (3). EcoSpoT, E. coli SpoT. Identical residues are indicated by black blocking, and similar residues are shown
by gray boxing. The boxed region indicates the G/A LLPD SpoT region, indicative of the HD box of metallophosphatases.
adequate high level expression of *ilvIH* (23). To check for *relA* phenotypic complementation in *E. coli*, the *relA* null strain CF1651 was transfected with the pNt-RSH2 plasmid and checked for growth on SMG medium, alongside the parental strain CF1651 (negative control) and grandparental “wild-type” *E. coli* strain MG1655 (*relA* + *spoT* + ; positive control), as well as a pBluescript SK + vector transfectant (vector control). After overnight growth at 37 °C, growth was scored by visual inspection for two consecutive days (Table I). Obvious growth (indicative of adaptation) of the *E. coli* relA mutant strains bearing the tobacco *Nt-RSH2* gene on a multicopy plasmid on SMG medium was apparent after 1 day of incubation at 37 °C. In comparison, growth of even the wild-type *E. coli* strain was not apparent until 2 days incubation. Thus, it appears that in the presence of the plasmid-borne tobacco *Nt-RSH2* gene, unregulated elevated levels of ppGpp are present, which allow for “preadaptation” and a more rapid apparent response to isoleucine starvation due to elevated ppGpp levels leading to elevated constitutive expression of *ilvIH*. Consistent with this interpretation, we noted that wild-type *E. coli* strains bearing the pNT-RSH2 plasmid display a slow growth phenotype (data not shown), similar to what is observed for *E. coli* spoT mutants that accumulate high constitutive intracellular ppGpp levels (2).

Additional evidence for the ability of Nt-RSH2 to synthesize ppGpp in vivo was found when we attempted to transflect relA and spoT double-deleted *E. coli* strain, CF1693 (9), with pNT-RSH2. We were unable to obtain any stable transfectants of CF1693 with pNT-RSH2, whereas parallel transfections with equal amounts of the vector plasmid pBluescript SK + resulted in confluent growth (>10,000 colonies) on selective plates. We suspect that the reason no transfectants were obtained with pNT-RSH2 is due to the fact that the deletion of *spoT* together with relA, while it deletes both ppGpp degradase activities in the *E. coli* cell, also deletes the only ppGpp degradation activity contained in the dual function SpoT protein. Therefore, the introduction of a sole ppGpp synthetase activity into the relA spoT double-deleted strain leads to a situation of “runaway” accumulation of ppGpp, due to a lack of ability to turn over ppGpp, with subsequent toxic effects on cell growth and loss of cell viability (2). This probably explains our failure to obtain stable CF1693/pNt-RSH2 transfectants and is consistent with our hypothesis that Nt-RSH2 is a *bona fide* ppGpp synthetase.

**In Vitro ppGpp Synthesis by Nt-RSH2 Protein**—To directly determine the ability of the Nt-RSH2 protein to synthesize ppGpp, we cloned the tobacco Nt-RSH2 gene and the *E. coli* RelA gene into a T7 promoter-based overexpression plasmid system, fusing them at their C-terminal coding region with a hexahistidine tag for conventional Ni²⁺-agarose purification. Both His-tagged proteins were purified in parallel using identical protocols. We had previously found that the presence of a C-terminal hexahistidine tag on the *E. coli* RelA protein fortuitously activates it for constitutive ppGpp synthetase activity independently of ribosomes.² Thus, the His-

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² R. M. Givens, M.-H. Lin, D. J. Taylor, U. Mechold, J. O. Berry, and V. J. Hernandez, unpublished observation.

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### Table I

**RelA complementation on SMG selective growth medium**

| Strain                  | Growth on SMG medium |
|-------------------------|----------------------|
|                         | Day 1 | Day 2 |
| MG1655 (*relA* + *spoT*)| −     | +++  |
| CF1651 (Δ*relA*)        | −     | −    |
| CF1651/pNtRSH2          | ++++  | ++++ |
| CF1651/pBluescript SK   | −     | −    |

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**Fig. 2. In vitro activities of His-tagged purified tobacco RSH2 protein.** C-terminal histidine-tagged Nt-RSH2 protein was overexpressed in *E. coli* and purified to homogeneity. In vitro (p)ppGpp synthesis assays were performed as described under “Experimental Procedures” unless otherwise indicated. A, comparison of ppGpp and pppGpp synthetic activities in the presence of GTP and GDP as pyrophosphate acceptors between *E. coli* RelA (ECO:RelA) and *N. tabacum* RSH2 protein (TBCO:RSH2) with increasing amounts of protein as indicated; the positions of migration of the expected products are indicated. B, kinetics of (p)ppGpp accumulation in the presence of GTP and GDP as pyrophosphate acceptors with the Nt-RSH2 protein (TBCO:RSH2) over a 23-h period. Note the presence of a radioactive contaminant present in the no protein control samples (lanes 1 and 9) with similar mobility to ppGpp. C, reactions were performed in the presence of increasing concentrations of only GDP or GTP or both (central lane) as pyrophosphotransfer acceptor leading to the accumulation of only ppGpp or pppGpp, respectively. Samples were chromatographed using 1.75 mM NaH₂PO₄, pH 3.4, as mobile phase to better resolve products from contaminants. Note the different mobility of radioactive contaminants (lanes 1 and 9) compared with ppGpp and pppGpp.

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Some batches of [γ-¹⁵⁻⁵⁵]ATP used in these studies bore radioactive contaminants that fortuitously chromatographed to a position close to that of ppGpp or pppGpp (observed clearly in Fig. 2B, no protein control). However, alternative chromatographic conditions (Fig. 2C) confirm that the radioactive contaminant is distinct from *bona fide* ppGpp or pppGpp. Levels of radioactivity at the ppGpp and pppGpp positions increased in manner that requires the addition of RSH2 protein in Fig. 2,
plants (probe and then stripped and rehybridized with an 18 S rDNA probe. Amounts of RNA from untreated or untreated various treatments. Control C5S rRNA for each sample. Levels are shown relative to untreated control plants.

...metal-dependent phosphohydrolases (11). This domain is indicative of metal phosphatases, which define a superfamily of ppGpp and pppGpp, respectively, as assigned. Observed radioactive spots probably correspond to the products of either GTP or GDP alone, giving only the possibility of either pppGpp, respectively, reactions were performed in the presence of either GTP or GDP alone, giving only the possibility of either ppGpp, respectively, as assigned.

The kinetics of (p)ppGpp accumulation (Fig. 2B) indicate a slow but steady increase in (p)ppGpp accumulation over a 23-h period. In addition, it appears that ppGpp synthesis occurs more rapidly and for a longer period of linearity than pppGpp (Fig. 2B), accounting most likely for the higher abundance of ppGpp over pppGpp apparent in Fig. 2A. To confirm the identity of the radioactive spots observed in Fig. 2A as ppGpp and pppGpp, respectively, reactions were performed in the presence of either GTP or GDP alone, giving only the possibility of either ppGpp or pppGpp synthesis, respectively. Samples were run together by ascending TLC along with a single reaction sample containing both GTP and GDP as potential pyrophosphate transfer acceptors (Fig. 2C). This result clearly shows that the observed radioactive spots probably correspond to the products ppGpp and pppGpp, respectively, as assigned.

The Nt-RSH2 protein sequence contains the highly conserved HD domain at amino acid residue 268 (Fig. 1, black box), indicative of metal phosphatases, which define a superfamily of metal-dependent phosphohydrolases (11). This domain is strongly associated with the SpoT-like proteins that can degrade as well as synthesize ppGpp (11). In the case of the E. coli SpoT protein, the ppGpp hydrolase activity carries out the following reaction: (p)ppGpp → GT(D)P + PPi. We made several attempts to assess the ability of purified Nt-RSH2 to degrade (p)ppGpp but failed to obtained definitive results. Experiments to assay for (p)ppGpp turnover remain ongoing and will be reported elsewhere.

Inducible Nt-RSH2 Gene Expression in N. tabacum—Northern analysis was performed to determine whether the tobacco Nt-RSH2 is transcribed in intact nonstressed plants and whether Nt-RSH2 mRNA levels are affected by known inducers (JA or SA) of plant defense response pathways (24, 25).

To identify and quantify any possible induction of Nt-RSH2 mRNA accumulation by JA, a series of treatments were conducted (Fig. 3). Three separate “Plantcon” growth boxes containing control untreated plants (Fig. 3A, C1, C2, and C3; 5 plants/box), three boxes containing 0.5% EtOH-treated plants (Fig. 3A, E1, E2, and E3; 5 plants/box), and three boxes containing JA-treated plants each (Fig. 3A, J1, J2, and J3; 5 plants/box) were used. For each treatment, leaves from all five plants grown together in a given box were pooled for RNA extraction 24 h after treatment. In all three untreated control groups, the Nt-RSH2 probe hybridized to a transcript of ~2.5 kb in size. In
the three experimental groups treated with JA for 24 h, there is a 3–4-fold increase in levels of \textit{Nt-RSH2} mRNA relative to the untreated control plants (Fig. 3A; compare \textit{lunes} \textit{C}_1, \textit{C}_2, and \textit{C}_3 with \textit{lunes} \textit{J}_1, \textit{J}_2, and \textit{J}_3). Fig. 3B shows the average levels of JA induction for \textit{Nt-RSH2} mRNA and, furthermore, that these levels were similar for each of the three repeats. These findings are in contrast to those of a similar study with the At-\textit{RSH} genes of \textit{Arabidopsis}, where no induction by JA was observed (3).

It is noteworthy that exposure of tobacco plants to ethanol (0.5% final concentration in medium, to control for the ethanol used to solubilize JA) for 1 day was in itself sufficient to induce \textit{Nt-RSH2} mRNA accumulation ~2-fold 24 h after treatment (Fig. 3, A and B). Thus, \textit{Nt-RSH2} mRNA levels appear to also increase in response to EtOH exposure, a condition associated with stress conditions such as dehydration, osmotic shock, and hypoxia (29–31).

In a separate experiment (Fig. 3C), exposure of the tobacco plants to SA appeared to have no effect on levels of \textit{Nt-RSH2} mRNA, relative to levels already present in untreated tobacco plants (Fig. 3C; compare control lane and lane SA), although plants grown at the same time and under the same conditions did respond to treatment with JA (lane JA). This is consistent with the lack of any SA response reported for At-\textit{RSH} genes of \textit{Arabidopsis} (3).

Taken together, it appears that the \textit{Nt-RSH2} transcript accumulates constitutively in nonstressed plants. Treatment of these plants with SA, a known inducer of plant defense responses (25, 27), had no observable effect on the accumulation of these transcripts. JA, another signaling compound that plays a role in defense and senescence (24, 25, 27, 28), was associated with a consistent 3–4-fold increase in levels of \textit{Nt-RSH2} mRNA accumulation (Fig. 3, B and C). This increase occurred as early as 8 h of treatment and persisted through 48 h (data not shown). The inducibility of mRNA induction for both the JA and EtOH treatments, relative to nontreated controls, were quantitatively reproducible in independently grown groups of plants (Fig. 3A; see error bars from average of all experiments in Fig. 3B). Thus, variables such as potential differences in evaporation of EtOH, placement in the growth chamber, or other unknown factors, had little or no influence on our findings. In addition, using immunoanalysis, we found that in parallel with the 3–4-fold increase in \textit{Nt-RSH2} mRNA levels, \textit{Nt-RSH2} protein synthesis rates were increased 3–4-fold following JA treatment (data not shown). Thus, increases in mRNA levels leads directly to equal increases in \textit{Nt-RSH2} protein levels following JA treatment.

\textbf{RSH Genes Are Expressed in a Variety of Plant Species}—To gain insight about the distribution and expression of \textit{RSH} genes throughout the plant kingdom, Northern analysis was performed using total RNA isolated from a taxonomic cross-section of green plants. These included \textit{Chara longifolia} (\textit{lane C}1), a eukaryotic algae considered to be closely related to the line that gave rise to higher plants (31); \textit{Arabidopsis}, a \textit{C}_2 dicot (\textit{lane A}); \textit{Flaviera bidentis}, a \textit{C}_3 dicot (\textit{lane Fb}); and \textit{Zea mays}, a \textit{C}_4 monocot (\textit{lane Zm}). Hybridization was conducted using a PCR probe corresponding to a conserved region of the \textit{At-RSH2} and \textit{At-RSH2} genes (this 159-bp region is ~83% conserved at the nucleotide level) and amplified from \textit{Arabidopsis} RNA (Fig. 3D, \textit{At-RSH} probe). This probe gave somewhat fainter bands with heterologous plant mRNAs than with the \textit{Arabidopsis} sample as expected, despite approximately equal loading and transfer of the samples (as indicated by hybridization to the 18 S rRNA probe (Fig. 3D, 18S rRNA)). This most likely reflects differences in DNA sequences of \textit{RSH} genes between widely divergent species rather than relative differences in transcript abundance. Hybridization with the \textit{At-RSH} PCR probe produced faint images of bands in \textit{lunes} \textit{Cl}, \textit{Fb}, \textit{Zm}, and \textit{Nt}. All of the higher plant species showed a hybridization band of approximately the same size (2.6–2.7 kb), as expected based on previous reports (3) and cDNA sequences reported in the data base. A larger band (~3.0 kb) was observed in the \textit{C. longifolia} lane. Interestingly, an \textit{RSH} cDNA and corresponding transcript identified from the eukaryotic algae \textit{Chlamydomonas reinhardtii} was also found to be larger than those of the higher plant \textit{Nt-RSH2}, \textit{At-RSH2}, and \textit{At-RSH3} genes (32). Based on this diverse sampling, we expect that RelA/SpoT homologues are widespread and show constitutive basal expression at the level of mRNA accumulation throughout the plant kingdom.

\textbf{Phylogenetic Analysis of Plant \textit{RSH} and Bacterial RelA/SpoT Proteins}—Since it appears that the plant RelA/SpoT homologues may constitute a separate branch of the RelA/SpoT superfamily, we conducted phylogenetic analysis to test this possibility. Consistent with this notion, an initial BLAST analysis with the \textit{Nt-RSH2} protein sequence against the translated non-redundant data base gives highest alignment similarity scores to plant RelA/SpoT homologue or evolutionarily closely related organisms. For example, the highest scoring alignments are in the following order: red pepper \textit{Capsicum annuum} \textit{RSH} gene, \textit{Arabidopsis} \textit{AtRSH2} gene and \textit{AtRSH3} genes, and \textit{Synechocystis} sp. (blue-green biosynthetic cyanobacteria) RelA/SpoT.

Phylogenetic analysis revealed that the plant \textit{RSH} genes appear to constitute two separate branches within the RelA/SpoT superfamily. The optimal tree (Fig. 4) grouped the plant sequences together in a clade with \textit{Deinococcus}, which in turn was grouped with a monophyletic cyanobactera clade. Posterior probabilities indicated moderate support for a plant/cyanobactera clade but strong support for the plant clade and for the dicots containing RSH2-like proteins. \textit{Nicotiana} grouped strongly with the \textit{Capsicum} sequence.

\textbf{Localization of \textit{Nt-RSH2} Protein}—To follow up on the prediction above that the \textit{Nt-RSH2} protein appears to bear a chloroplast-targeting sequence at its N terminus, we assayed by Western analysis for the presence of \textit{Nt-RSH2} protein in tobacco chloroplast using subcellular fractionation. Intact tobacco chloroplast were purified on Percoll gradients as described under “Experimental Procedures.” Immunoreactivity with anti-\textit{Nt-RSH2} antisemur was highly specific for the chloroplast-enriched fractions of tobacco leaf tissue (data not shown). These purifiedoplasts were further fractionated into soluble and insoluble fractions following osmotic lysis and assayed by Western analysis for the presence of \textit{Nt-RSH2} protein (Fig. 5). As observed in Fig. 5, all \textit{Nt-RSH2} reactivity was found to occur only in the insoluble chloroplast fraction, which includes membranes, membrane-associated ribosomes, and other insoluble constituents. In addition, the chloroplast reactivity corresponding to \textit{Nt-RSH2} is smaller than the predicted size of full-length \textit{Nt-RSH2} of 718 amino acid residues. This is probably accounted for by removal of the N-terminal 93 amino acids during chloroplast translocalization.

\textbf{Induction of \textit{Nt-RSH2} Protein by Microbial Infection}—To further investigate the JA-associated increase in \textit{Nt-RSH2} gene expression observed in Fig. 3, and given the fact that JA functions as a messenger that is involved in the activation of some plant stress and defense systems (25), we examined \textit{Nt-RSH2} induction following microbial infection of \textit{N. tabacum}. Tobacco leaves were spot-infected with the bacterial pathogen \textit{E. cartovora cartovora}, as described under “Experimental Procedures.” 20 h postinfection, leaf discs were harvested from infected areas, and protein samples were prepared. When these samples were assayed by Western analyses, levels of \textit{Nt-RSH2} protein were found to increase from barely detectable amounts...
to clearly observable levels (~10-fold) by 20 h postinfection (data not shown). These results are the first direct evidence that a plant RSH protein is induced in response to pathogen infection. Taken together with our findings of JA-inducible Nt-RSH2 gene expression, our results strongly suggest that plant RSH proteins play an active role in a plant-defense response and in plant-pathogen interactions.

**DISCUSSION**

Nt-RSH2 is a plant homologue of the bacterial stress genes relA and spoT in the higher plant Nicotiana tabacum. Similar homologues have very recently been identified in a small number of other plant species, including the higher plants Arabidopsis (3), rice (4), C. annuum (AY043214.1), and the eukaryotic algae Chlamydomonas reinhardtii (32). By Northern analysis, we have found that transcripts that have homology with and similar size as Nt-RSH2 accumulate in a divergent sampling of higher plant species, including two other dicots; a monocot, C3, and C4 plant species; and a eukaryotic algae. The occurrence of these homologues or their transcripts in a diverse cross-section of plant species indicates a high probability that relA/spoT homologues are present and expressed in most, if not all, higher plant species.

Distinct from the RSH genes of Arabidopsis (3), we observed a rapid and persistent JA-induced increase in the accumulation of Nt-RSH2 mRNA in tobacco (~3–4-fold) and in parallel with an equal increase in Nt-RSH2 protein abundance. Similar to findings in Arabidopsis, Nt-RSH2 transcript levels did not increase in response to treatment with SA. In addition, we observed ~10-fold induction of Nt-RSH2 protein levels in response to infection with a bacterial pathogen. These findings indicate that Nt-RSH2 gene expression is responsive to at least some defense and stress-related pathways in tobacco but may be induced in response to different signals in Arabidopsis. The distinct SA-induced defense pathway (25) does not appear to be associated with modulation of these transcripts in either plant. In agreement with findings presented here, a recent report identified a relA/spoT homologue as one of a group of genes inducible by JA in the monocot Oryza sativa (4). Based on these observations, we suspect that signals or molecular processes associated with regulation of RSH gene expression will show some variation among different plant species.

Direct biochemical as well as genetic evidence indicates that the Nt-RSH2 protein is a fully functional ppGpp synthetase, in analogy to the bacterial RelA enzyme (Fig. 2). The purified Nt-RSH2 enzyme was found to be capable of *bona fide* ppGpp synthesis in a highly purified *in vitro* system and is reported here for the first time for any plant RSH protein. These find-
ings support a role for the unique signal-transducing nucleotide, ppGpp, in the regulation of plant gene expression.

Nt-RSH2, At-RSH2, and At-RSH3 (Fig. 1) all have properties that identify them as likely plastid-targeted soluble proteins. Our subcellular fractionation experiments (Fig. 5) provide additional evidence that the Nt-RSH2 protein is localized to the chloroplast. Taken together with recent findings that an RSH protein from the single cell eukaryotic algae *C. reinhardtii* localizes to algal chloroplasts in vitro (32), these observations provide insights as to the potential function of RSH protein in plants. We hypothesize that in plant chloroplasts, as in bacteria, Nt-RSH2 produces ppGpp as a regulatory signal in response to plant stress. This unique nucleotide then binds to and modifies chloroplast RNA polymerase, thus reprogramming its transcriptional output. In support of this hypothesis, it has been noted that the chloroplast RNA polymerase is highly homologous to *E. coli* RNA polymerase (33), where ppGpp is proposed to directly bind and regulate transcription by allosteric modification of *E. coli* RNA polymerase (1). It is notable that Nt-RSH2 co-fractionates exclusively with the insoluble fraction of the chloroplast extracts, since this fraction contains the majority of translating plastid ribosomes (34). This result is consistent with the interaction of Nt-RSH2 with chloroplast ribosomes, again in analogy with the operation of bacterial RelA proteins that are ribosome-associated. However, in *vitro* we have demonstrated ppGpp/ ppGpp synthetic activity without additional or accessory protein factors (Fig. 2). Thus, it may be that Nt-RSH2 operates independently of ribosomes and fortuitously co-fractionates with the insoluble chloroplast extract fraction.

The structural and enzymatic similarities between the prokaryotic RelA/SpoT proteins and their plant homologues and our finding that Nt-RSH2 expression is inducible by JA suggest a stress-signaling role for these proteins in plants. The fact that the *Arabidopsis* At-RSH1 was isolated based on its interactions with RPP5, a known plant R-protein, implies a pathogenesis-related role for At-RSH1. An RSH gene from the halophyte *Suaeda japonica* (Sj-RSH) conferred enhanced salt tolerance when expressed in *E. coli* and in yeast, suggesting that plant RSH proteins might be capable of activating conserved stress-response genes in these organisms (35). Our findings that NtRSH protein levels are increased dramatically in response to infection of plants with the bacterial pathogen *E. carotovora* give stronger support to this hypothesis.

Little is known about the regulation of chloroplast genes in response to JA treatment or pathogen attack. JA, which increases during senescence and in response to necrotrophic pathogens, is known to repress plastid gene expression at the levels of transcription and translation (36, 37). Changes in chloroplast function, viability, and morphology are also characteristics associated with programmed cell death during senescence and in response to disease (38). Chlorophyll breakdown products that originate in the chloroplasts during these events have been implicated in the modulation of cell death and disease resistance (39, 40). Thus, one possible function for RSH genes in plants may be to alter the expression of chloroplast-encoded genes in order to prepare the plastid for a role in stress response, disease resistance, or senescence.

Of the major groups on the tree of life, only bacteria and plants possess RelA/SpoT proteins. Our results (Fig. 4) suggest that a lateral gene transfer from bacteria to plants occurred early in plant evolution, because representative RSH proteins from the other major plant groups (monocots, eudicots, and uncellular green alga) form a single evolutionary group. A possible route for lateral transfer is from the primordial chloroplast, which is generally considered to have originated from an endosymbiotic cyanobacterium (41). Our findings that higher plant

***REFERENCES***

1. Touloukhonov, I. I., Shulgina, I., and Hernandez, V. J. (2001) *J. Biol. Chem.* 276, 1220–1225
2. Cashel, M., Gentry, D. R., Hernandez, V. J., and Dinella (1996) in *Esche- ritchia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., ed) 2nd Ed., pp. 1456–1496, American Society for Microbiology Press, Washington, D.C.
3. van der Biezen, E. A., Sun, J. H., Coleman, M. J., Bibb, M. J., and Jones, J. D. G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3747–3752
4. Xiong, L. Z., Lee, M. W., Qi, M., and Yang, Y. N. (2001) *Mol. Plant-Microbe Interact.* 14, 655–692
5. McCormae, D., Boinek, J. J., Rispensger, V. C., and Berry, J. O. (1997) *Plant Physiol.* 114, 801–815
6. Berry, J. O., Nikolai, B. J., Carr, J. P., and Klessig, D. F. (1985) *Mol. Cell. Biol.* 5, 2238–2244
7. Reed, K. C., and Mann, D. A. (1985) *Nucleic Acids Res.* 13, 7207–7221
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 7.39–7.52, Cold Spring Harbor, NY
9. Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1991) *J. Biol. Chem.* 266, 5890–5900
10. Cashel, M. (1993) *Methods Mol. Genet.* 3, 341–356
11. Mitterhuber, G. (2001) *J. Mol. Microbiol. Biotechnol.* 3, 585–600
12. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* 25, 4876–4882
13. Swidford, D. L. (2000) *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4.0b4a, Sinauer, Sunderland, MA
14. Huelsenbeck, J. P., Rannala, B., and Masly, J. P. (2000) *Science* 290, 2349–2350
15. Huelsenbeck, J. P., and Ronquist, F. (2001) *Bioinformatics* 17, 754–755
16. Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992) *Comput. Appl. Biosci.* 8, 275–282
17. Hira, T., and Sugiyama, M. (1996) *EMBO J.* 15, 1687–1695
18. Gallie, D. R. (1995) *Annu. Rev. Plant Physiol.* 44, 77–105
19. Lutcke, H. A., Chow, K. C., Nickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987) *EMBO J.* 6, 41–48
20. Vonheijne, G., Steppuhn, J., and Herrmann, B. G. (1989) *Eur. J. Biochem.* 180, 535–545

"RelA/SpoT Homologous Proteins in N. tabacum"
