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Physiological and Molecular Plant Pathology 65 (2004) 257–267

PMPP

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Identification and functional analysis of Arabidopsis proteins that interact with resistance gene product RPS2 in yeast

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Accepted 16 February 2005

Abstract

A yeast-two-hybrid screen was used to investigate the interaction controlled by resistance gene *RPS2* of *Arabidopsis thaliana* and avirulence gene *avrRpt2* of *Pseudomonas syringae*. Twelve Arabidopsis proteins that physically interact with RPS2 in yeast were identified. Four of these were studied further including a calmodulin, a putative ankyrin/tetratricopeptide repeat-containing protein, a protein with a nuclear localization domain, and a protein of unknown function. In yeast, all four interacted with RPS2 and not with a related resistance protein, RPM1. Three of the four interacted solely with N-terminal domains of RPS2 and not with the RPS2 LRR domain. Interactions were not impacted by RPS2 nucleotide binding site mutations. When disease resistance was tested in Arabidopsis, plant lines carrying T-DNA insertions that disrupt the genes for the ankyrin/tetratricopeptide repeat protein or the protein of unknown function exhibited reduced resistance to *P. syringae* pv. *tomato* strain DC3000 expressing *avrRpt2*. The RPS2 interacting proteins identified in this screen are new targets for studies of plant defense signal transduction.

Keywords: Plant disease resistance; RPS2; avrRpt2; NB-LRR; Coiled-coil; Nucleotide binding site; Leucine-rich repeat; Po-1; At2g27030; At3g04710; At2g03440; At3g48760; At1g79550; At1g56340; At4g16660; At4g24190; At3g48990; At5g57290; BX829211; At1g10760

1. Introduction

Plants carry resistance (*R*) genes whose products detect attack by pathogens and activate downstream defense responses [1–4]. *R* gene products allow specific recognition of avirulence (Avr) factors from pathogens. In the absence of the cognate *R* gene these Avr factors often enhance pathogen fitness by contributing to host colonization, in some cases directly disabling plant defense components. As such, Avr factors are pathogen effector molecules. However, if the plant carries an *R* gene product with specificity for a particular Avr product that the pathogen expresses, then strong defenses are triggered. Much has been learned about *R* gene structure and evolution [4,5].

Recent studies have increased our understanding of the mechanistic function of *R* gene products. The simplest model, the ligand-receptor model in which the avirulence effector directly interacts with a receptor R protein, has experimental support in some plant-pathogen systems [6–9]. In other systems a 'guard' model is followed [10–12]. Briefly, R proteins function to guard the plant cellular targets of pathogen effectors (Avr proteins), and the R proteins trigger defenses upon recognition that these targets are under pathogen attack. The guard model is consistent with the informally reported failure of many groups to observe direct interaction between R proteins and Avr effectors, and new experimental support for this model is accumulating [13–16]. Interestingly, two different R proteins monitor the status of one apparent 'guardee' protein, the membrane-associated RIN4 [3,13–15]. Phosphorylation of RIN4 upon exposure to the *Pseudomonas syringae* effector proteins AvrRpm1 and AvrB is likely to be

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doi:10.1016/j.pmpp.2005.02.006

the trigger for RPM1-mediated defenses [13]. RPS2, the subject of the present study, also interacts with RIN4. The bacterial effector protein AvrRpt2 that is recognized by RPS2 eliminates RIN4 in an RPS2-independent manner [14,15]. *rin4* null mutants, which are seedling lethal, could be rescued by crossing to *rps2* mutants, suggesting that ectopic activation of RPS2 by the absence of RIN4 caused seedling death [14]. The above suggests a model in which RPS2 detects the AvrRpt2-mediated disappearance of RIN4, triggering signaling that leads to defense.

The Arabidopsis resistance gene *RPS2* belongs to the most abundant structural class of resistance genes, the NB-LRR (nucleotide binding site, leucine rich repeat) class [4,17,18]. RPS2 carries an N-terminal coiled-coil domain, followed by a NB site and other conserved domains, and then degenerate LRRs at its C terminus. Many elements of NB-LRR primary structure are conserved beyond plant R gene products, and are also found in mammalian innate immune system or cell death-mediating proteins [19–21]. Mutational analysis indicates that the NB domain and an N-terminal leucine zipper motif are essential for RPS2 function, and that RPS2 does not have any large domain that is dispensable for resistance function [22]. However, the N-terminal CC-NB portion of RPS2 (no LRRs) can activate defenses in the absence of AvrRpt2 if it is overexpressed [22]. LRR domains are generally thought to be responsible for specificity of pathogen recognition [23–26]. However, the RPS2 LRR domain has been shown to also play a role in the interaction with other host factors [27]. A functional HA-tagged RPS2 allele behaves as a membrane-associated protein [15]. RPS2 and other NB-LRR R proteins may serve as molecular scaffolds that mediate the interaction of multiple partner proteins [21,28].

The *P. syringae* effector protein AvrRpt2, in addition to triggering *RPS2*-dependent gene-for-gene resistance, contributes to pathogen virulence [29,30]. AvrRpt2 is translocated into plant cells by a type III (*hrp*) secretion apparatus. Within the plant cell, AvrRpt2 is cleaved by a plant factor, releasing a C-terminal product that is sufficient to elicit an RPS2-dependent response [31]. AvrRpt2 is likely to function as a cysteine protease [32], although recent evidence indicates that it may have additional functions [30]. Functional HA-tagged AvrRpt2 stably expressed in transgenic Arabidopsis is membrane-associated [15].

Separate evidence for interactions between RPS2, AvrRpt2 and other proteins comes from work where AvrRpt2 and RPS2 were transiently expressed in Arabidopsis leaf mesophyll protoplasts and shown to co-immunoprecipitate in a complex with at least one unidentified plant protein of approximately 75 kDa. This or other proteins could be necessary for Avr-R complex formation or be involved in defense signal transduction [33]. At least one chaperone protein (HSP90.1) contributes to proper RPS2 expression and/or function [34], and RAR1 and possibly Sgt1a/b also play significant roles in mediating proper RPS2 function [35–38], most likely by mediating RPS2 protein localization or turnover. The RPS2/RIN4/AvrRpt2 interaction apparently describes RPS2 function before and during recognition of AvrRpt2 [14,15]. However, additional proteins are likely to exist that physically interact with RPS2 at different stages of its function (see for example [21,28]). In particular, the proteins with which RPS2 and other NB-LRR proteins directly communicate to activate defenses after pathogen recognition remain undetermined.

In this study, we report the identification, via a modified yeast-two-hybrid assay, of twelve Arabidopsis proteins that interact with RPS2 in yeast. For four of these newly identified interactors, the yeast assay was further used to characterize interactions with RPS2 sub-segments, full-length RPS2 carrying NB domain mutations, and RPS2 from the Po-1 ecotype, as well as RPM1 and AvrRpt2. Plant studies were initiated with tests of disease resistance in T-DNA insertion lines. The proteins identified in this study are strong candidates for future research.

2. Materials and Methods

2.1. cDNA preparation

Arabidopsis thaliana Col-0, were grown for 5 weeks under 9 h days at 20 °C. Total RNA was extracted from healthy rosette leaves for 'the non-infected cDNA library'. For the 'infected leaf library', bacterial suspensions (1×10^5 cfu/ml of *Pseudomonas syringae* pv. tomato DC3000 carrying *AvrRpt2* in 10 mM $MgCl_2$) were vacuum infiltrated into plants and equal amounts of inoculated leaves were collected at 6, 12, 18, and 24 h after inoculation and then pooled to make the cDNA library. Total RNA was extracted with Tri Reagent (Sigma, St Louis, MO) following the manufacturer's instructions. Total RNA preparations from each time point were combined together for the construction of the 'infected cDNA library'. The CytoTrap XR library construction kit (Stratagene, La Jolla, CA) was used for subsequent mRNA isolation and cDNA library construction.

2.2. Cloning of RPS2 variants and p413CYC1::avrRpt2, p413CYC1::avrRpm1, pSos::RPS2 and pSos::RPM1

Because *RPS2* does not have introns in its genomic sequence, *RPS2* variants, namely *N-RPS2*, *C-RPS2* and Po-*RPS2*, were amplified directly from genomic DNA of Arabidopsis ecotypes Col-0 (*N-RPS2* and *C-RPS2*) or Po-1 (Po-*RPS2*) using primers with engineered restriction enzyme sites. Full-length Po-1 *RPS2* was amplified with primers RPS2-fwd1-4s2 and RPS2-rvs2s4, N terminus RPS2 with primers RPS2-fwd1-4s2 and N-RPS2r and C terminus RPS2 with primers C-RPS2-fwd and RPS2rvs2s4 (Table 1). PCR was performed with the high fidelity DNA polymerase HF2 according to manufacturer's

Table 1
List of primers

Primer	Sequence
RPS2-fwd1-4s2 (*Sal* I)	5′ CGAGAAAT*GTCGAC*AAATGGATTTCATCTCATCTCTTA 3′[a]
RPS2-rvs2s4 (*Sac* I)	5′ CTTAGCTCT*GAGCTC*AATTTGGAACAAAGCGCGGTAAAT 3′
N-RPS2r (*Sac* I)	5′ GCTTGTC*GAGCTC*TTTCTGCTTTAGGAGCTTCAGT 3′
C-RPS2-fwd (*Sal* I)	5′ GCATGGG*GTCGAC*TGAAGCTCCTAAAGCAGAAAACTG 3′
RPS2f14s2 (*Sal* I)	5′ CGAGAAAT*GTCGAC*AATGGATTTCATCTCATCTCTTAT 3′
strightRPS2r2s4 (*Sac* I)	5′ CTTAGCTCT*GAGCTC*AATTTGGAACAAAGCGCGGTAAAT 3′
avrRpt2fs2 (*Sal* I)	5′ CTCCCCGA*GTCGAC*TGATGAAAATTGCTCCAGTTGC 3′
avrRpt2rs1 (*Sal* I)	5′ AGATACCC*GTCGAC*TTAGCGGTAGAGCATTGCGTGT 3′
AvrRpm1-5′ (*Bam*H I)	5′ CC*GGATCC*TTTTTATGGGCTGTGTATCGAG 3′
AvrRpm1-3′ (*Eco*R I)	5′ AC*GAATTC*TTTTTGGTAGCCTTATCTTAAAAGTCA 3′
RPM1-5′ (*Mlu* I)	5′ GCC*ACGCGT*CATGGCTTCGGCTACTGTTGA 3′
RPM1-3′ (*Not* I)	5′ TAGAGCTC*GCGGCCGC*CTAAGATGAGAGG 3′

[a] Engineered restriction enzyme sites are underlined in primer sequence.

recommendations (Advantage-HF2 PCR kit, Clontech, Palo Alto, CA). PCR products were purified using the Ultraclean 15 kit (Mo Bio Laboratories) and cloned as blunt fragments into the vector pCR-Blunt II-TOPO or into pCR-Blunt (Invitrogen, Carlsbad, CA). Subsequently, *RPS2* variants were excised from these vectors by digesting with *Sal* I and *Sac* I and cloned into the yeast vector pSos (Stratagene, La Jolla, CA) digested with the same enzymes. RPS2 NB mutant alleles G182A and T189A were created by a PCR based site-directed mutagenesis [39]. A *BamH* I fragment of 939 bp containing the G182A or the T189A mutation was exchanged with the wild-type fragment of RPS2 and directionally cloned into the yeast vector pSos at the *Sal* I *Sac* I sites.

AvrRpt2 was amplified by PCR using primers avrRpt2fs1 and avrRpt2rs1 (Table 1) and initially cloned into pET29b(+). *AvrRpt2* DNA from the pET29(b)::*avrRpt2* construct was then digested with *Sal* I, and this *Sal* I fragment was ligated to the *Sal* I-digested and CIAP-treated p413CYC1. The correct orientation of p413CYC1::*avrRpt2* was confirmed by diagnostic restriction digestions.

AvrRpm1 was amplified by PCR with primers avrRpm1-5′ and avrRpm1-3′ (Table 1) using the Expand Long Template PCR System (Roche, Penzberg, Germany). The PCR product was digested with *Bam*H I and *Eco*R I, and ligated to *Bam*H I/*Eco*R I digested p413CYC1.

For the pSos::*RPS2* construct, *RPS2* was PCR amplified with *Pfu* (Stratagene, La Jolla, CA) using the primers RPS2f14s2 and RPS2r2s4 (Table 1) and p4104 DNA as a template. The PCR products were digested with *Sal* I and *Sac* I, and then ligated to the *Sal* I–*Sac* I digested pSos. The bait construct was confirmed by restriction mapping and expression was confirmed by Western blot analysis.

RPM1 was amplified by PCR with primers RPM1-5′ and RPM1-3′ (Table 1) using the Expand Long Template PCR System (Roche, Penzberg, Germany) with Columbia-0 DNA as a template. The PCR product was digested with *Mlu* I and *Not* I before ligation to *Mlu* I–*Not* I digested pSos. Expression was confirmed by Western blot analysis.

2.3. Analysis of protein interaction

The different constructs (e.g. pSos with variant *RPS2*, pMyr with an RPI and p413CYC1 with or without *avrRpt2*) were transformed into cdc25H yeast cells and protein interactions were tested as described in the Stratagene Cytotrap™ XR library construction kit manual (Stratagene, La Jolla, CA). To summarize, cdc25H yeast simultaneously carrying all three plasmids were selected by growth in media lacking uridine, leucine and histidine. To test for interactions, three or four independent transformed strains were plated on selective media containing galactose/raffinose or glucose as a carbon source. Identical galactose/raffinose and glucose plates were incubated at 25 and 37 °C. At 25 °C, the permissive temperature, cdc25H should grow on either carbon substrate. At 37 °C, the restrictive temperature, cdc25H cells grown on galactose/raffinose media are not expected to be viable unless the *cdc25-2* mutation is complemented by the interaction between bait (expressed from pSos) and prey (expressed from pMyr) fusion proteins. In all experiments, dependence of the interaction on prey expression was tested by confirming absence of growth on glucose plates at 37 °C; prey expression from pMyr is under control of the glucose-repressible GAL promoter. Glucose plates incubated at 37 °C are also an important control for other spurious sources of complementation such as reversion of the temperature-sensitive *cdc25-2* allele. Interactions were generally detected as yeast cell growth after incubation for 4 days at 37 °C on galactose/raffinose plates. The positive (pSos MAFB and pMyr MAFB) and negative (pSos Coll and pMyr Lamin C) control plasmids for two-hybrid interaction were supplied in the Stratagene Cytotrap XR library construction kit. Unless specifically noted, qualitative stability of yeast growth results was observed across repeat experiments despite some quantitative differences between experiments that may have arisen due to variations involving growth media batch, plate dryness, plate pre-warming, incubator temperature fluctuation, and the physiological status and precise density of the cells when plated.

2.4. Sequence analysis

Sequences of RPI clones were compared with the NCBI public database using the BLAST and/or BLASTX algorithm [40,41] Information about each clone was also obtained from the TAIR, TIGR, MIPS and PROSITE databases (www.arabidopsis.org; [42–44]).

2.5. Immunoblot analysis

Extraction of yeast total protein was as described in Stratagene's Cytotrap XR library construction kit manual Samples were run on a 4% SDS-PAGE gel. After transfer to a nitrocellulose membrane, standard Western blot procedures were followed (ECL, Amersham Pharmacia Biotech, UK). Primary anti-human Sos antibodies (Transduction Laboratories, Lexington, NJ) were used at 1:250 dilution. Horse-radish peroxidase labeled anti-mouse immunoglobulin G antibody was used at a 1:2,000 dilution to detect primary antibodies via chemiluminescence (ECL, Amersham Pharmacia Biotech, UK). Anti-AvrRpt2 antibodies [30,32] were supplied courtesy of B. Kunkel and B. Staskawicz.

2.6. Arabidopsis insertional mutants

For the four RPS2 interactors that received further study, Arabidopsis lines carrying T-DNA insertions that disrupt the coding region were identified by reference to the SIGnAL T-DNA Express database, identification of homozygous T-DNA insertion lines by the suggested PCR methods, and confirmation of locus homozygosity by probing of genomic DNA blots ([77]; http://signal.salk.edu/cgi-bin/tdnaexpress). The relevant starter lines were Salk 027181, Salk 123077, Salk 066554, and Salk 111768, which are all in the Col background. Plants were grown at 22 °C under 9 h daylength and to test for resistance to *P. syringae* pv. *tomato* strain DC3000 carrying pVSP61 (no *avr* gene) or pV288 (*avrRpt2*$^{+}$), vacuum infiltration was used to introduce into leaf mesophyll tissue 5×10^{4} cfu/ml suspensions of bacteria in 10 mM $MgCl_2$ +50 μl/L Silwet L-77. Leaf *P. syringae* population sizes were determined three days after inoculation by dilution plating ground leaf material on NYGA media containing cycloheximide (50 mg/L) and kanamycin (50 mg/L). Each plated sample contained discs removed from two plants (four leaf discs per sample); for each plant/strain combination four independent samples were tested in each of three temporally independent experiments. Pairwise T-tests comparing Salk line and wild-type Col-0 control were performed on combined data from the three independent experiments (ANOVA using a generalized linear model confirmed that experiment-to-experiment variation was not significant).

3. Results

3.1. Identification of RPS2 interactors in a modified CytoTrap yeast-two-hybrid screen

We employed a modified yeast-two-hybrid system (CytoTrap™ system from Stratagene) to identify Arabidopsis proteins that interact with RPS2. The CytoTrap system detects bait–prey interactions that occur in the cytoplasm. Prey proteins are fused to a short myristylation signal that fosters membrane association. Bait proteins are fused to a Sos (GEF; guanyl nucleotide exchange factor) protein. Bait–prey interactions bring Sos into proximity with a separate membrane-associated Ras protein, activating Ras signaling and thereby rescuing the heat sensitive phenotype of yeast strains carrying the *cdc25-2* mutation [45]. Our initial screen for RPS2-interactors was also performed in the presence of an AvrRpt2 expression plasmid (see Section 2).

Two different Arabidopsis cDNA libraries were constructed in the prey vector (pMyr); one was from healthy leaves and the other from leaves sampled at four time points after infection with *P. syringae* pv. *tomato* DC3000 carrying *avrRpt2*. DNA from each cDNA library was transformed into the yeast strain cdc25H carrying both pSos::*RPS2* (the bait construct) and p413CYC1::*avrRpt2*. In the absence of natural reversions of the *cdc25H* temperature-sensitive mutation, yeast growth at the restrictive temperature (37 °C) on selective media (CM-dropout media without Leu, Ura, and His) containing galactose is indicative of interaction between the bait and prey fusion proteins, which leads to complementation of the cdc25H mutant phenotype. A total of approximately 1.1×10^{6} prey plasmids were tested from the two different libraries in four separate experiments, and 43 positive candidates were initially selected. The positive prey plasmids were purified and then re-transformed and tested in positive- and negative-control strains or conditions (see Section 2). Fifteen positive clones were confirmed as true RPS2-interactors in yeast in these re-test experiments. Results from these and subsequent CytoTrap experiments are reported in qualitative terms because qualitative measures of interaction (e.g. relative growth of temperature-sensitive yeast at restrictive temperature) were reproducible, while precisely quantitative measures of relative interaction (e.g. colony-forming units (cfu) at restrictive temperature relative to cfu at permissive temperature) were quite variable and potentially misleading. For the fifteen positive RPS2-interacting clones, DNA sequence analysis revealed three instances of two cDNAs derived from the same gene, hence, twelve different RPS2 interactors were identified in our yeast-three-hybrid screen, and were provisionally named *RPI-1* through *12* (for *RPS2-Interactor*; Table 2). As Table 2 shows, not all cDNA clones were full length.

Comparison of the RPI sequences with public databases suggested biochemical functions for eight out of the twelve RPIs. Three RPIs showed no similarity to proteins of known

Table 2
RPS2 interacting proteins identified in modified yeast-two-hybrid screen

Name	Library	Gene code	Predicted function	cDNA clone
RPI-1	Non-infected (2 clones)	At3g48760	DHHC- type zinc finger motif	Both lack first 48 bp of 5′ UTR; coding sequence complete
RPI-2	Non-infected and infected (2 clones)	At2g27030	Calmodulin-2 (*CAM2*; calmodulin-2/3/5)	Full length
RPI-3	Non-infected (2 clones)	At1g79550	Phosphoglycerate kinase (cytolsolic)	Full length
RPI-4	Non-infected	At3g04710	Ankyrin repeat and tetratricopeptide repeat motifs	Lacks first 24 bp of 5′ UTR; coding sequence complete
RPI-5	Infected	At1g56340	Calreticulin-1 (*CRT1*)	Lacks 5′UTR and first 827 bp of coding sequence
RPI-6	Infected	At4g16660	putative HSP70, HSP110-like	Lacks 5′UTR and first 1533 bp of coding sequence
RPI-7	Infected	At4g24190	HSP-90-like (*SHD*, GRP94-ortholog)	Lacks 5′ UTR and first 1703 bp of coding sequence
RPI-8	Infected	At3g48990	4-coumarate-CoA ligase-like; Acetoacetyl-CoA synthase and AMP binding domains	Lacks 5′UTR and first 1077 bp of coding sequence
RPI-9	Infected	At5g57290	60S acidic ribosomal protein P3 (*RPP3B*)	Lacks 5′UTR and first 178 bp of coding sequence
RPI-10	Infected	T13766, BX829211	Nuclear localization sequence	Gene not annotated (see footnote)
RPI-11	Non-infected	At2g03440	Unknown protein	Lacks first 32 bp of 5′UTR
RPI-12	Non-infected	At1g10760	starch excess protein (*SEX1*)	Lacks 5′ UTR and first 2715 bp of coding sequence

An At gene code number has not yet been assigned for RPI-10, but Genbank cDNA accession BX829211 and EST T13766 match our RPI-10 cDNA sequence, which is encoded between At4g04610 and At4g04620 in the Arabidopsis genome.

function. Two separate clones of RPI-1 (unknown function) and RPI-3 (resembles phosphoglycerate kinase) were identified from the non-infected library. RPI-2 (calmodulin-2) was also identified two times, once each from the 'non-infected' and 'infected' cDNA libraries. The remaining clones were identified once in our screen. We noted that three putative ER-localized chaperone proteins were isolated from the cDNA library made from infected leaves. RPI-6 is HSP110-like, RPI-7 resembles a HSP90 protein [43], and RPI-5 is a calreticulin, a calcium binding protein that also acts as a chaperone. Other functional domains were predicted in other RPIs, including ankryin repeats and tetratricopeptide repeats (RPI-4), and a nuclear localization signal (RPI-10).

3.2. Functional analysis of RPS2 interaction with RPIs

RPIs 2, 4, 10 and 11 were chosen for further study because they were apparent full-length cDNA clones that exhibited particularly reliable interaction with RPS2, and contained interesting predicted domains. To determine elements of RPS2 that are required for the observed interaction with RPIs, we examined yeast interaction phenotypes using different RPS2 constructs. The tested RPS2 constructs (Fig. 1) included full-length RPS2, an N-terminal segment of RPS2, a C-terminal segment of RPS2, full-length RPS2 NB domain mutants where the glycine at position 182 is mutated to an alanine (G182A) or the threonine at position 189 is mutated to an alanine (T189A), and the full-length RPS2 allele from the Arabidopsis ecotype Po-1. All constructs were based on the Col-0 allele of RPS2 except for the full-length Po-1 RPS2. The N-terminal construct contained the coiled-coil domain, nucleotide binding site and associated conserved domains (e.g. [11,20]). The RPS2 C-terminal segment encompassed the LRR domain. The RPS2 NB domain alleles carry mutations in the putative nucleoside triphosphate-binding domain (G182A and T189A); these mutations were shown to cause a loss of resistance function in planta [39]. The Po-1 *RPS2* allele is non-functional in Po-1 but does function in a Col-0 background; the amino acids responsible for the non-functionality of Po-1 RPS2 in a Po-1 background have been pinpointed to the LRR domain [27]. In all bait constructs, DNA encoding the C terminus of Sos protein was fused in frame to DNA encoding the N terminus of the RPS2 construct (see Section 2). Expression of RPS2 baits in yeast

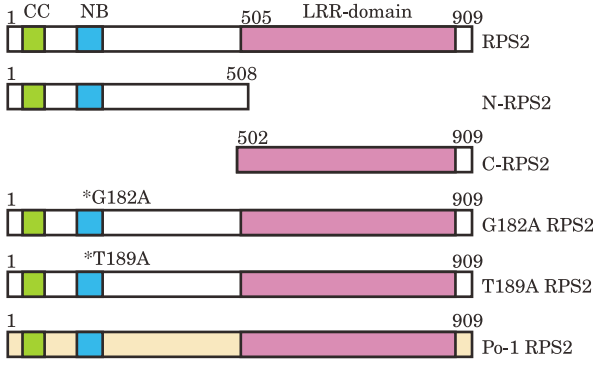

Fig. 1. Schematic of bait constructs of RPS2 variants. Full-length RPS2 contains a coiled-coil (CC) domain, a nucleotide-binding site (NB) and a leucine-rich repeat (LRR). The N-RPS2 construct contains the CC, NB and other conserved domains commonly found prior to the LRR domain of NB-LRR R proteins. The C-RPS2 construct encompasses the LRR-domain and C terminus. G182A and T189A RPS2 constructs contain mutations in the NB domain. Po-1 RPS2 differs from Col-0 RPS2 by 11 amino acids distributed throughout the protein [27].

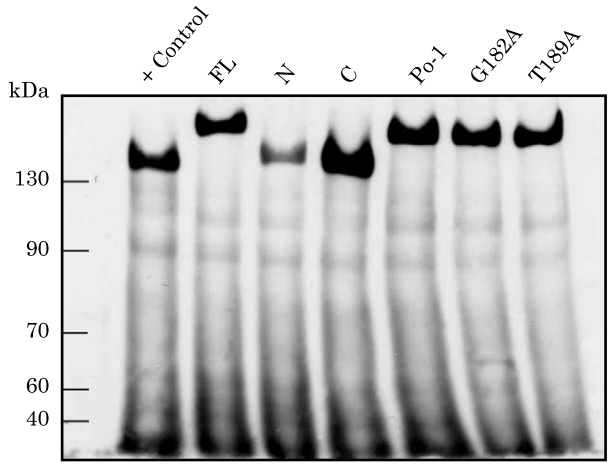

Fig. 2. Evidence that RPS2 bait proteins were expressed in transformed cdc25H yeast cells. Protein extracts from cdc25H yeast triply transformed with pSos::RPS2 variants (bait), pMyr::RPI2 (prey) and p413CYC::AvrRpt2 (non-fusion protein) were run on SDS-PAGE gels. RPS2 variants were full-length (FL), N terminus RPS2 (N), C terminus RPS2 (C), Po-1 RPS2 (Po-1), G182A RPS2 (G182A) and T189A RPS2 (T189A). Proteins were transferred to a nitrocellulose membrane, and probed with anti-Sos antibodies. '+Control' is the MAFB bait construct supplied by Strategene.

was confirmed by Western blot with anti-Sos antibodies (Fig. 2).

Interactions were tested by moving an RPS2 bait (as described above) and an RPI prey (RPI-2, RPI-4, RPI-10 or RPI-11) into cdc25H yeast that had previously been transformed with p413CYC1::AvrRpt2. As expected, wild-type full-length RPS2 once again showed interaction with all RPIs (Fig. 3). The N-terminal segment of RPS2 also

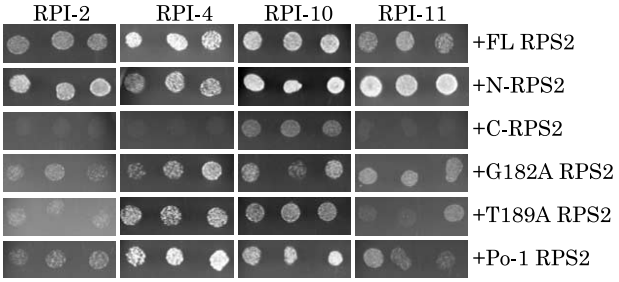

Fig. 3. Evidence for physical interaction of RPI fusion proteins with some but not all RPS2 baits. Yeast two-hybrid assays are shown after 4 days of growth at the restrictive temperature (37 °C) on selective -Leu, -Ura, -His dropout media containing galactose. Each panel shows the growth of 3 independent transformants of identical genotype; all spots contained similar quantities of yeast at the time of plating. Yeast strains carrying *avrRpt2* were transformed with RPI (RPI-2, RPI-4, RPI-10 or RPI-11) and RPS2 variant clones as noted (F.L. RPS2 is full-length, N-RPS2 contains the N-terminus, C-RPS2 contains the C terminus, and G182A RPS2 mutant, T189A RPS2 mutant or the Po-1 RPS2 allele as described in text). Additional controls (not shown) gave results as expected: replicate plates containing glucose and incubated at 37 °C showed no growth, and glucose and galactose plates incubated at the permissive temperature (25 °C) showed normal growth for all strains. Note also that in repeat experiments, cell growth was stronger (closer to FL-RPS2 control) for RPI-2 or RPI-11 strains carrying T189A-RPS2 or Po-1 RPS2.

interacted with all tested RPIs. Interestingly, although the LRR region of RPS2 interacted with RPI-10, this region did not interact with RPI-2, RPI-4 and RPI-11. Full-length RPS2 carrying NB domain mutations G182A or T189A both interacted with all four RPIs. The Po-1 RPS2 also interacted with all four tested RPIs. In replicated experiments (Fig. 3 and data not shown) the strength of interaction of the different RPS2 constructs with a given RPI, as reflected by yeast cell density upon plating at restrictive temperature, was not reproducibly different except in the case of C-RPS2 interactions with RPI-2, RPI-4 and RPI-11. Hence for RPI-2 (calmodulin), RPI-4 (ankyrin and tetratricopeptide repeats) and RPI-11 (unknown), although structural shifts in RPS2 caused by changes in nucleotide binding or in LRR configuration may still modulate the ability of RPS2 to interact with these proteins in Arabidopsis, interaction with RPS2 apparently occurs primarily via the N-terminal regions of RPS2 and can occur independent of a functional NB domain. RPI-10 may bind a folded RPS2 via contacts with domains in both the N- and C-portions of RPS2.

3.3. No effect of AvrRpt2 and AvrRpm1 on RPI interaction with RPS2 in yeast

Yeast strains lacking an *avrRpt2* construct but carrying the full-length RPS2 bait and an RPI prey (RPI-2, RPI-4, RPI-10 or RPI-11) were tested to determine if AvrRpt2 was required for the interaction of RPIs with RPS2. RPS2 showed interaction with all four RPIs in the absence of AvrRpt2 (Fig. 4). Although growth for strains containing RPI-10 or RPI-11 was weaker than for strains containing RPI-2 or RPI-4, growth (RPS2/RPI-dependent complementation of the temperature-sensitive yeast phenotype) was consistently seen for all these strains in three temporally independent repetitions of the experiment.

Further experiments tested for an effect of AvrRpm1 on the interaction of RPS2 with RPIs 2, 4, 10 and 11. Yeast strains were transformed with the full-length RPS2 bait, the RPI prey (RPI-2, RPI-4, RPI-10 or RPI-11) and either AvrRpm1 expressed as a non-fusion protein from the vector p413CYC1, or the empty p413CYC1 vector. All of these strains grew well under selective conditions and any given bait–prey combination grew similarly regardless of the presence or absence of AvrRpm1, showing that the interaction between RPS2 and RPIs 2, 4, 10 and 11 is not affected by AvrRpm1. These experiments also reaffirmed that the four RPIs interact with RPS2 in yeast in the absence of AvrRpt2.

3.4. Direct interaction of AvrRpt2 with RPS2 or RPIs not observed

Direct interaction between RPS2 as bait and AvrRpt2 or AvrRpt2 in its plant-associated cleaved form (c-AvrRpt2) as prey was also tested. Using standard CytoTrap system protocols, no interaction between AvrRpt2 and RPS2 was

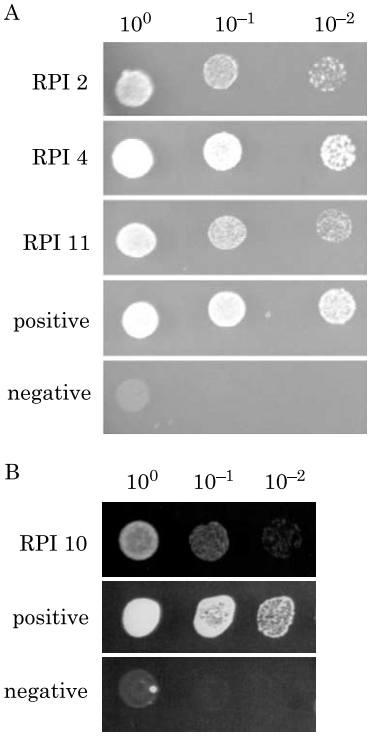

Fig. 4. RPI interaction with RPS2 in the absence of AvrRpt2. With the exception of the 'positive' and 'negative' strains, all strains expressed RPS2 from the bait construct as a fusion with the hSOS peptide, and expressed RPI 2, RPI 4, RPI 10 or RPI 11 from the prey construct under control of the GAL1 promoter. The positive and negative controls expressed MAFB from the bait construct, and MAFB or LaminC from the prey construct, as described in the CytoTrap manual (Stratagene, La Jolla, CA). A dilution series (10^0, 10^{-1}, 10^{-2}) was plated on galactose-containing media at 36 °C and growth was recorded after four days. Results for strains carrying RPI 2, RPI 4, and RPI 11 are shown in (A), with the positive and negative controls, and results from a separate experiment, for the strain carrying RPI 10, are shown in (B), with the positive and negative controls from that experiment.

observed. AvrRpt2 prey expression from the pMyr vector was confirmed by Western blot analysis using anti-AvrRpt2 antibodies; for example, the expected band for an AvrRpt2 fusion protein of approximately 30 kDa (AvrRpt2 is ~28 kDa) was observed in yeast cells carrying pMyr-::AvrRpt2 but not in untransformed cells. RPS2 expression as a bait from the vector pSos had previously been confirmed (Fig. 2). The observed lack of direct interaction is consistent with previous findings and current models of RPS2 and AvrRpt2 activity [12,33], including earlier interaction tests we had performed with *GAL4*-based and *lexA*-based yeast two-hybrid systems [46,47] where we also never observed interaction between an RPS2 bait and an AvrRpt2 prey (Zabala, Yu and Bent, unpublished data).

We also conducted yeast two-hybrid assays for direct interaction between AvrRpt2 or c-AvrRpt2 as a bait and RPI-2, RPI-4, RPI-10 and RPI-11 as preys. Positive control protein interaction constructs allowed yeast growth at 37 °C but the AvrRpt2-RPI combinations did not, suggesting that AvrRpt2 does not directly interact with these RPI proteins.

3.5. Interaction with RPIs: specific for RPS2 and not RPM1

We tested for interaction between the NB-LRR resistance protein RPM1 and RPI-2, RPI-4, RPI-10 or RPI-11 in the presence or absence of the cognate avirulence peptide AvrRpm1. Interactions were tested in yeast triply transformed with an RPM1 bait, the RPI prey (RPI-2, RPI-4, RPI-10 or RPI-11) and either AvrRpm1 expressed as a non-fusion protein from the vector p413CYC1, or the empty p413CYC1 vector. No interaction was observed (there was no growth at 37 °C) for strains carrying RPM1 and any of the four RPIs, regardless of the presence or absence of AvrRpm1, while simultaneously tested positive and negative controls (described in previous sections) performed as expected. These data indicate that RPIs 2, 4, 10 and 11 do not bind plant NB-LRR proteins in a non-specific manner, but instead preferentially interact with RPS2.

3.6. Reduced RPS2-mediated resistance in plant lines mutated for RPI-4 and RPI-11

Plant-based analysis of RPI-2, RPI-4, RPI-10 and RPI-11 was initiated by isolation of insertional gene knockout Arabidopsis lines homozygous for T-DNA insertions in the genes encoding these four RPS2 interactors. Along with wild-type Col-0 as a control, these plant lines were tested for their ability to restrict growth of virulent *P. syringae* pv. *tomato* strain DC3000 or avirulent DC3000 (*avrRpt2*$^+$). Fig. 5 shows leaf bacterial populations three days after bacterial inoculation. Col-0 plants express *RPS2* and as expected, Col-0 restricted growth of the DC3000 (*avrRpt2*$^+$) strain to a much greater extent than DC3000 lacking *avrRpt2*. The growth of DC3000 (*avrRpt2*$^+$) was

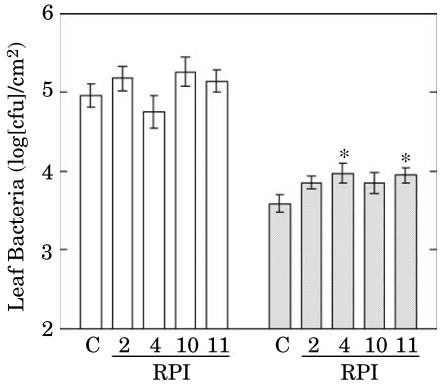

Fig. 5. Growth of virulent and avirulent *P. syringae* pv. *tomato* in RPI knockout Arabidopsis lines. Leaf bacteria population sizes were determined three days after inoculation of leaf rosettes with strain DC3000 not carrying an added *avr* gene (open bars) or with DC3000 expressing *avrRpt2* (shaded bars). Plant lines were wild-type Col-0 (C) or Col lines homozygous for an insertional T-DNA disruption of the gene for the designated RPI. Pooled data from three independent experiments ($n=12$) are shown as mean ± standard error of the mean; the asterisks (*) indicate that leaf population sizes of DC3000 (*avrRpt2*$^+$) on the RPI-4 and RPI-11 knockout lines were significantly different from those on Col-0 (paired T-tests, $P<0.05$). All other comparisons to the corresponding Col-0 control were not significant.

statistically indistinguishable between Col-0 and the RPI-2 and RPI-10 knockout lines. However, the RPI-4 and RPI-11 knockout lines were partially but significantly impaired in their ability to restrict growth of DC3000 (*avrRpt2*$^{+}$), relative to wild-type Col-0 (Fig. 5). It is also important to note that growth of the virulent DC3000 strain (not carrying *avrRpt2*) was similar in Col-0 and all of the RPI knockout lines (Fig. 5).

4. Discussion

This paper describes yeast-two-hybrid and three-hybrid assays that were used to identify Arabidopsis proteins that can interact with the resistance protein RPS2. The original *GAL4*-based yeast two-hybrid assay and the popular *lexA*-based yeast-two-hybrid assay both monitor protein interactions that occur in the cell nucleus [46,47]. We used the CytoTrap system to study interactions that occur in the cytoplasm, the most likely location for RPS2 and AvrRpt2 interaction in plant cells [15]. This system is well suited for qualitative monitoring of protein–protein interactions. A total of 12 different genes, provisionally named RPI 1–12, were identified in a screen of cDNA libraries made from non-infected leaves and from leaves inoculated with *P. syringae* expressing *avrRpt2*. Interactors identified from the pre-infection cDNA library are encoded by genes that are expressed in the absence of pathogen infection. One can expect that many components of the disease resistance signal transduction pathway are already present in plant cells before the pathogen attack—for example, RPS2 is constitutively expressed [17,18]. Other defense mediators, including postulated components that turn off signaling and/or re-set the pathogen detection system, may be inducible and therefore more readily identifiable in libraries made from infected leaves.

It was not feasible to conduct all of the studies described above with all twelve candidate RPIs. RPIs 2, 4, 10 and 11 were chosen for further studies because they were full-length clones that exhibited reproducibly strong interaction with RPS2, and because of the potential interest of their postulated functional domains. For example, RPI-2 encodes a calmodulin [48]. Calmodulins couple intracellular Ca^{2+} signals to a variety of cellular responses, and Ca^{2+} has been implicated repeatedly as a mediator of defense signal transduction [49–54]. This calmodulin may be brought into proximity with other proteins by RPS2, positively or negatively modulating their association with protein complexes and/or their signal transduction activities. We identified cDNAs for the same calmodulin from two different cDNA libraries. The derived amino acid sequences of RPI-4 and RPI-10 did not match particular proteins of known function in database searches, but did reveal known motifs. RPI-4 has apparent ankyrin repeat protein-protein interaction domains, and tetratricopeptide repeats, which target proteins (including defense-associated Sgt1) for interaction with chaperones that hold proteins in complexes and/or foster movement of those proteins to different cellular locations [55–57]. Ankyrin repeats have also been identified in NPR1/NIM1, a key mediator of salicylic acid-based plant defense transcriptional regulation, and in RFXANK, which interacts with the mammalian immune system NB-LRR protein CIITA and directly modulates MHC gene expression [58,59]. RPI-10 is predicted to contain a bipartite nuclear localization sequence [60], and may serve to communicate signals between the cytoplasm and the nucleus. RPI-11 showed similarity to a protein of unknown function, and was an especially strong RPS2-interactor.

The remaining RPIs that we identified also merit brief discussion. RPI-1 shows no similarity to proteins of known function, however, search against the PROSITE motif database revealed that it has a DHHC-type zinc finger motif [43,61].

RPI-3, an apparent cytosolic phosphoglycerate kinase (PGK), was identified in two independent screenings of the non-infected library. Although this enzyme is most commonly known for its function in glycolysis, recent studies in a mammalian system showed that PGK is secreted by tumor cells and that activates the serine proteinase plasmin in the stimulation of angiogenesis [62,63]. Separately, phosphoglycerate kinase has been shown to serve as an activator of Sendai virus transcription initiation complex even when the protein is mutated to lose phosphoglycerate kinase activity [64].

RPI-5 is calreticulin-1 (*CRT1*), a Ca^{2+}-modulated chaperone that is ER-localized [65]. RPI-6 is a putative HSP110-like chaperone and RPI-7 bears HSP90-family signatures (PROSITE motif). The RPI-6 and RPI-7 predicted proteins also have canonical endoplasmic reticulum (ER) targeting signals at their C-termini (HDEL and KDEL, respectively). It is possible that in vivo, when RPS2 is synthesized or as part of a turnover reaction, it is trafficked through the ER and interacts with RPI-5, RPI-6 and/or RPI-7. RPS2 is apparently membrane-localized [15]. It is noteworthy that Hsp90 chaperones have recently been implicated in the function of a number of R genes [66–68]. RPI-7 was recently reported as important to the folding and/or complex formation of CLAVATA proteins [69]. In other plants, Calreticulin and BiP (an HSP70-family protein that associates with calreticulin) exhibit interesting pathogen-modulated changes in expression level [70–72]. We note that all three chaperones found as RPS2-interactors in the present study were isolated from the cDNA library of infected leaves. However, as for the other RPIs, extensive study will be required to assess defense-associated roles, and at present it cannot be ruled out that the interaction of RPS2 with RPI-5, RPI-6 and RPI-7 is merely due to the innate nature of chaperones to bind to other proteins.

RPI-8 shows similarity to 4-coumarate-CoA ligases. Expression of two other Arabidopsis 4-coumarate-CoA ligase genes, *AtCL1* and *AtCL2*, was shown to be strongly

induced in an incompatible interaction with *Peronospora parasitica* [73].

RPI-9 is a ribosomal protein. RPI-12 has been previously identified as the SEX1 protein [74]. The *sex1* mutant (for starch excess) is defective in starch mobilization and shows high starch content in leaves after long dark periods. SEX1 has a plastid-targeting sequence and there is some data to support its localization inside of Arabidopsis chloroplasts [74].

The four RPIs that were further tested, RPI-2, 4, 10 and 11, all exhibited specificity for RPS2 in that they failed to interact with RPM1, another Arabidopsis CC-NB-LRR R protein. In our experiments where different domains of RPS2 were tested for interaction with RPIs, we observed that the N-terminal segment of RPS2 mediated interaction with all RPIs tested. The LRR region interacted only with RPI-10 (Fig. 3). RPI-10 may interact with a folded form of RPS2 through contacts with both the N- and C- portions. Failure to observe C-RPS2 interaction with RPI-2, 4 and 11 could conceivably have arisen if there was poor expression of C-RPS2 at 37 °C, but the positive interaction result for C-RPS2 and RPI-10 (Fig. 3) suggests that sufficient C-RPS2 was present in these assays.

The N-terminal segment of RPS2 that interacts with RPIs 2, 4, 10 and 11 carries the CC, NB and other conserved domains. We tested whether RPS2 alleles carrying mutations in the NB domain affected the interaction with RPIs. The NB domain is able to bind and hydrolyze nucleotides and it is thought that these activities may influence the interaction between R proteins and other components of the disease resistance pathway to modulate signal transduction [75,76]. However, definitive understanding of how the NB domain participates in NB-LRR R protein signal transduction is still lacking. When expressed *in planta*, the G182A RPS2 allele showed complete loss-of-function in both HR and inability to restrict growth of *avrRpt2* expressing bacteria [39]. The *in planta* phenotype of the T189A RPS2 mutation is milder than for the G182A RPS2 allele, with nearly complete loss of HR upon challenge with *P. syringae* expressing *avrRpt2* but intermediate levels of pathogen growth [39]. In our yeast-three-hybrid assays, no qualitative difference between wild-type RPS2 and the NB RPS2 mutants were found. Therefore, although these NB mutations may affect overall RPS2 disease resistance signaling, they do not in yeast affect RPS2 interaction with RPIs 2, 4, 10 or 11.

Po-1 and Col-0 RPS2 differ by 11 amino acids. The Po-1 *RPS2* allele is able to function in a Col-0 background but not in Po-1 (i.e. the Po-1 product can function with other Col-0 host proteins to respond to *P. syringae* expressing *avrRpt2*). The amino acid differences responsible for this functional polymorphism have been narrowed down to the six amino acid polymorphisms in the LRR domain [27]. While the Col-0 allele of *RPS2* functions well in Po-1 or Col-0, the severity or penetrance of the HR mediated by Po-1 *RPS2* in Col-0 is low relative to that generated by Col-0 *RPS2* [27]. To test the hypothesis that the Po-1 RPS2 product only interacts poorly with one or more of the RPIs, possibly accounting for the reduced response to AvrRpt2, Po-1 RPS2 was included in our studies. We did not observe any qualitative differences in the Po-1 RPS2 interactions with the RPIs, relative to Col-0 RPS2. However, the RPIs we tested interact strongly with the N-terminal domain of RPS2, and the N-terminal domain of Po-1 RPS2 is fully functional in either Po-1 or Col-0 plants when present as a hybrid Po/Col RPS2 protein [27].

In our yeast-two-hybrid assays no direct interaction between AvrRpt2 and RPS2 or the RPIs was observed. The results with AvrRpt2 and RPS2 are consistent with our current understanding of the guard model for RPS2 action, in which RPS2 detects AvrRpt2 due to the AvrRpt2-induced disappearance of RIN4 rather than directly [14,15]; see also [33]. In the present study we also explored the possibility that AvrRpt2 directly binds RPIs, but no interaction was observed in yeast. One or more of the RPIs that we identified may be the target of a pathogen effector other than AvrRpt2, and be monitored by RPS2. As another alternative, some of the RPIs may be immediate downstream targets in the defense signaling activated by RPS2, or may foster formation of the different macromolecular complexes in which RPS2 engages. Macroscopic analyses of RPI gene knockout plants would not differentiate among these possibilities, but further understanding of RPI interactions with RPS2 could come from immunoprecipitation studies out of plant cells. Such studies are planned but are beyond the scope of the present study.

We did, however, initiate plant-based studies by testing the disease resistance phenotypes of plant lines that carry an insertional disruption in the genes encoding RPI-2, 4, 10 or 11. Across three separate experiments, significant disruption of resistance against *P. syringae* pv. *tomato* strain DC3000 (*avrRpt2*$^{+}$) was observed in Arabidopsis RPI-4 and RPI-11 knockout lines. *RPS2*/*avrRpt2*-dependent resistance was reduced rather than eliminated, but the results support the hypothesis that RPI-4 (ankyrin/tetratricopeptide repeat-containing protein) and RPI-11 (unknown protein) contribute to the RPS2 defense signal transduction apparatus. This promising result motivates further study of RPI-4 and RPI-11, which will initially include pursuit of homozygous lines for a second independently derived mutation for each gene. Such mutations have more recently become available. For RPI-2 (calmodulin, *CAM2*), tests we performed with a second independent insertional gene disruption plant line (data not shown) again revealed no difference in *avrRpt2*-specific resistance compared to Col-0, further solidifying that result. However, this negative result with RPI-2 does not necessarily indicate that calmodulins play no role in RPS2-mediated resistance, because Arabidopsis expresses multiple similar calmodulin isoforms that may exhibit significant functional redundancy [78]. Other RPS2 interactors (Table 1) have received little attention. Further experiments involving gene knockout and gene

overexpression lines, and co-immunoprecipitation from plant extracts, are therefore underway not only for RPI-4 (ankyrin/tetratricopeptide repeat protein) and RPI-11 (unknown protein), but also for the other RPS2-interacting proteins identified in this study.

Acknowledgements

We thank Ryan Kipp and Kendra Hogan for their substantial contribution to the completion of these experiments. We also thank Dr Christine Pfund for insightful discussions, I-ching Yu for her contributions during the earliest stages of this project, and B. Kunkel and B. Staskawicz for their generous gifts of anti-AvrRpt2 antibodies. This work was supported by NIH GM53595 and USDA-NRI 2003-35319-13863. B.F.Q. is supported by CNPq 4700881/2003-8 and IFS C/3576-1.

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