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Report

Nuclear Accumulation of the *Arabidopsis* Immune Receptor RPS4 Is Necessary for Triggering EDS1-Dependent Defense

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Summary

Recognition of specific pathogen molecules inside the cell by nucleotide-binding domain and leucine-rich repeat (NB-LRR) receptors constitutes an important layer of innate immunity in plants [1]. Receptor activation triggers host cellular reprogramming involving transcriptional potentiation of basal defenses and localized programmed cell death [1–3]. The sites and modes of action of NB-LRR receptors are, however, poorly understood. *Arabidopsis* Toll/Interleukin-1 (TIR) type NB-LRR receptor RPS4 recognizes the bacterial type III effector AvrRps4 [4]. We show that epitope-tagged RPS4 expressed under its native regulatory sequences distributes between endomembranes and nuclei in healthy and AvrRps4-triggered tissues. RPS4 accumulation in the nucleus, mediated by a bipartite nuclear localization sequence (NLS) at its C terminus, is necessary for triggering immunity through authentic activation by AvrRps4 in *Arabidopsis* or as an effector-independent "deregulated" receptor in tobacco. A strikingly conserved feature of TIR-NB-LRR receptors is their recruitment of the nucleocytoplasmic basal-defense regulator EDS1 in resistance to diverse pathogens [5, 6]. We find that EDS1 is an indispensable component of RPS4 signaling and that it functions downstream of RPS4 activation but upstream of RPS4-mediated transcriptional reprogramming in the nucleus.

Results and Discussion

An Epitope-Tagged *RPS4* Transgene Complements *rps4* Loss of Resistance

The *RPS4* gene (At5g45250) in *Arabidopsis* accession Col-0 was cloned previously on the basis of its ability to confer resistance to *P. syringae* pv *tomato* strain DC3000 expressing AvrRps4 (hereafter referred to as *Pst*-AvrRps4) in the susceptible accession, RLD [4]. We characterized two T DNA insertion lines (SALK_057697 and GABI_130F04; designated *rps4-2* and *rps4-3*, respectively) in Col-0. Both lines have insertions in the second exon of *RPS4* encoding the nucleotide binding (NB) domain [4] (Figure S1A in the Supplemental Data available online) that is essential for NB-LRR-receptor function [7] and are transcript null mutants (Figure S1B). When challenged with avirulent *Pst*-AvrRps4, *rps4-2* and *rps4-3* allowed 1–1.5 log units more growth than resistant Col-0 (Figure S2A). Bacterial titers in the *rps4* mutants were not as high as those attained in Col-0 with virulent *Pst* (Figure S2B), indicating that one or more receptors besides RPS4 contribute to AvrRps4 recognition in Col-0. Because the residual recognition was lost in Col-0 *eds1* (Figure S2B), it is likely to be mediated by another *TIR-NB-LRR* gene.

We transformed *rps4-2* with Col-0 genomic *RPS4* that was under control of the *RPS4* promoter and that was C-terminally fused to a double HA-StrepII epitope tag. A stable transgenic line (denoted RPS4-HS) was selected that expressed the RPS4-HA-StrepII fusion protein to detectable levels. Expression of epitope-tagged RPS4 in RPS4-HS recovered *RPS4*-mediated resistance fully (Figure S1A) as was the case for three other independent transgenic lines (data not shown). Growth of virulent *Pst* was not affected in RPS4-HS (Figure S2C), indicating that RPS4-HS does not display constitutive defense. Therefore, the RPS4-HA-StrepII fusion protein in RPS4-HS confers the function of endogenous RPS4.

RPS4 Protein Associates with Endomembranes

We assessed where RPS4 protein accumulates inside healthy plant cells and found that the majority of RPS4 protein fractionated with microsomes, whereas the defense regulator EDS1 remained soluble, as reported previously (Figure 1A) [5]. RPS4 was released from microsomes in buffers containing 0.5M NaCl or 2M urea (Figure 1A), consistent with it not being an integral membrane protein and the absence of putative transmembrane domains in the RPS4 primary amino acid sequence [4]. Aqueous two phase separation of *Arabidopsis* microsomes [8] revealed that RPS4 cofractionated with the ER marker BIP but not the plasma-membrane-attached BON1 protein [9] (Figure 1B). Association with endomembranes is reminiscent of another *Arabidopsis* TIR-NB-LRR receptor, RPP1A [10], but differs from the major pools of the tobacco N and pepper Bs4 TIR-NB-LRR receptors that are soluble and cytoplasmic [11, 12]. Therefore, the TIR domain does not appear to direct these proteins to a common cellular location.

A Subpool of Functional RPS4 Protein Localizes to Nuclei

A putative bipartite NLS in the C-terminal extension domain of RPS4 was predicted by the WoLF PSORT algorithm [13] (Figure 2A). We therefore tested whether a pool of RPS4 localizes to nuclei. Epitope-tagged RPS4 protein was detected in both nuclear and nuclei-depleted

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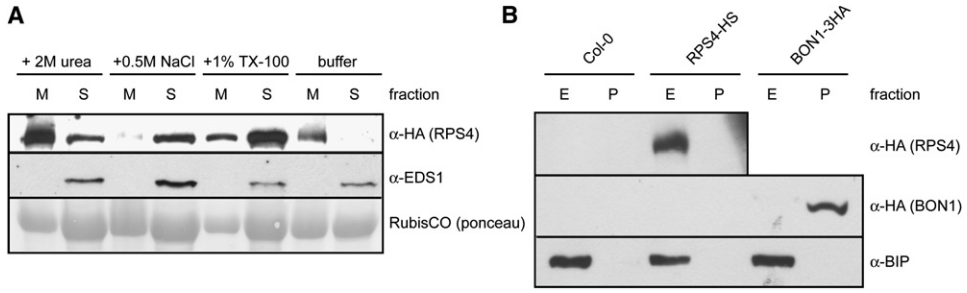

Figure 1. RPS4 Protein Associates with Endomembranes

(A) Equal volumes of the 2000 × g supernatant of crude RPS4-HS extracts were incubated in extraction buffer alone or in the same buffer supplemented with urea, NaCl, or Triton X-100 at the given concentrations. We then subjected samples to ultracentrifugation at 100,000 × g to determine the solubility of RPS4 protein. S, soluble fraction; M, membrane fraction.

(B) Aqueous two-phase separation of *Arabidopsis* microsomes. Endomembrane (E) and plasma membrane (P) fractions were analyzed by western blotting.

fractions from healthy leaf tissues (Figure 2B). By comparing proteins of known subcellular localization in nuclear partitioning experiments, we found a significant nuclear signal only for histone H3 and RPS4 (Figure 2B and Figure S3A). The nuclear fraction was approximately 16-fold concentrated over the nuclei-depleted fraction, and we estimated the proportion of RPS4 accumulating in nuclei to be 6%–10% of total RPS4. EDS1 protein distributes between the cytoplasm and nuclei (Figures S3A and S3B; [5]). Therefore, subpools of RPS4 and EDS1 cofractionate in nuclei. Nuclear accumulation of endogenous RPS4 protein was also seen with an antiserum raised against the RPS4 NB domain (Figure S3C), although a specific RPS4 signal could not be detected in total protein preparations because of nonspecific background (data not shown). The α-RPS4 antiserum detected a double band of ~140 kDa in Col-0 and RPS4-HS nuclear extracts, with the upper band corresponding to full-length RPS4 protein (predicted MW 138 kDa) because it was not present in the *rps4-2* mutant (Figure S3C).

Nuclear Localization of RPS4 Is Needed to Trigger the Immune Response

To elucidate the role of the putative NLS in RPS4, we substituted four lysine residues that constitute the core basic regions of the bipartite NLS with alanines (K1172A, K1173A, K1184A, and K1185A; marked with

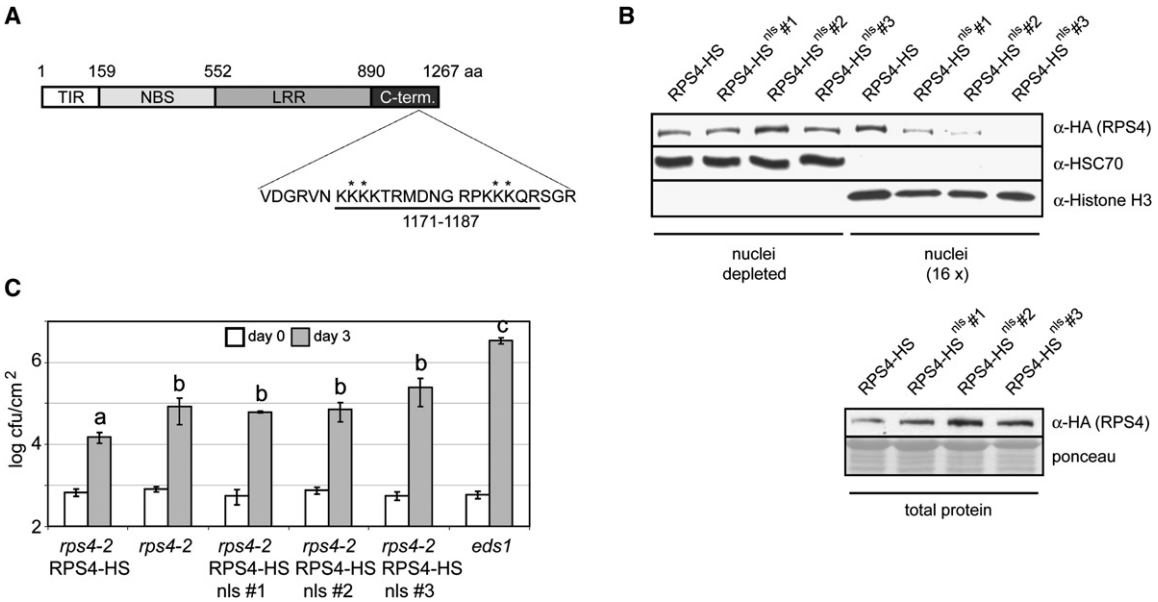

Figure 2. A Subpool of RPS4 Protein Accumulating in Nuclei Is Required for AvrRps4-Triggered Resistance in *Arabidopsis*

(A) Domain structure of RPS4. The position of the predicted bipartite NLS (underlined amino acids) is indicated. Four lysine residues substituted by alanines in site-directed "nls" mutants are marked by asterisks.

(B) Western blot showing total RPS4-HA-StrepII protein levels in line RPS4-HS and three independent transgenic lines expressing the site-directed RPS4^{nls}-HA-StrepII mutant variant (lower panel). As shown in the upper panel, leaf tissue of the same lines was separated into nuclei and nuclei-depleted fractions and probed on a western blot with antibodies as indicated. Depletion of nuclear RPS4 levels in site-directed RPS4^{nls} mutants was consistent in six independent experiments.

(C) *Pst* DC3000 expressing AvrRps4 was infiltrated into leaves of the indicated plants at 4 × 10^5 colony forming units (cfu) per ml. Bacterial growth (cfu/cm^2) was determined 1 hr (white bars) and 3 days (gray bars) after infiltration. Error bars represent SD of four samples. Lowercase letters a, b, and c indicate significant differences between values (t test, p = 0.05).

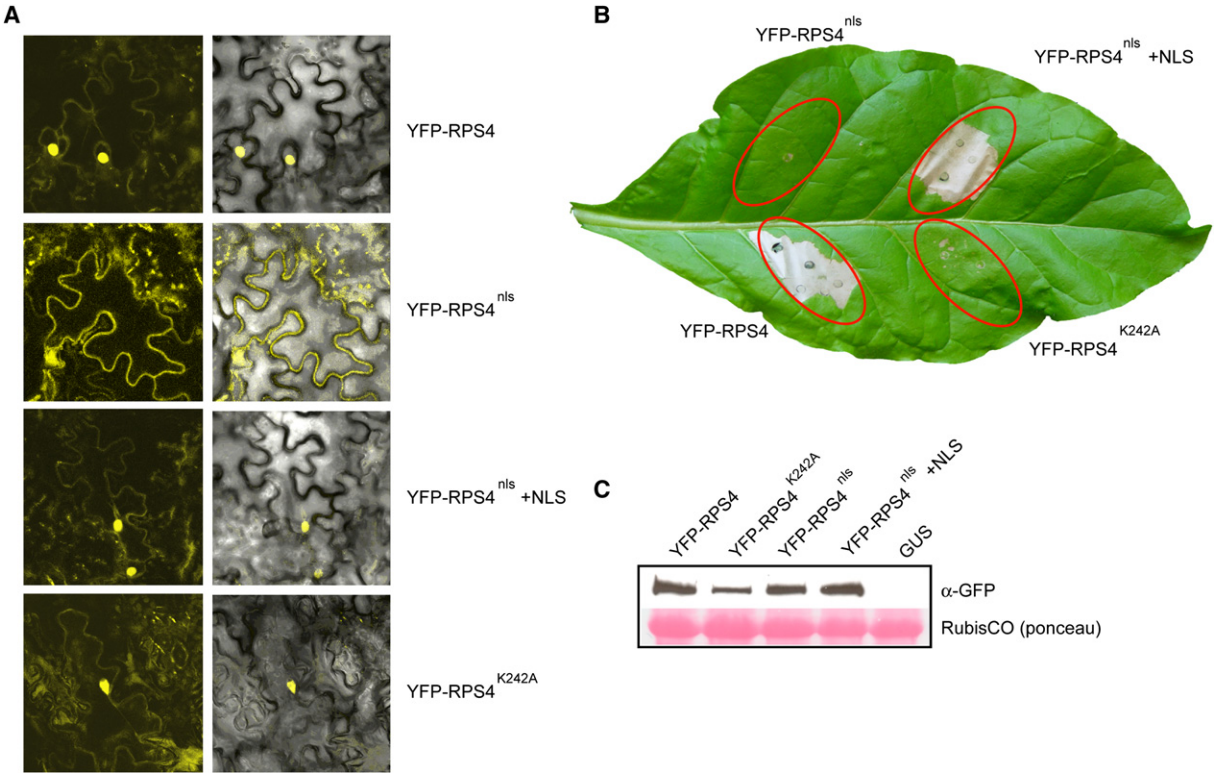

Figure 3. Nuclear Localization of RPS4 Is Necessary for AvrRps4-Independent Cell Death in Tobacco

(A) Confocal images showing the subcellular localization of YFP-RPS4 variants transiently expressed in *N. tabacum* 24 hr after *Agrobacterium* infiltration. Z stacks of cells showing a representative distribution of each YFP-RPS4 variant were taken. No nuclei were detected with the YFP-RPS4^{nls} construct.

(B) Elicitation of AvrRps4-independent HR in *N. tabacum* by YFP-RPS4 variants viewed 3 days after *Agrobacterium* infiltration.

(C) Western blot showing expression of YFP-RPS4 variants tested in (C) 24 hr after *Agrobacterium* infiltration.

asterisks in Figure 2A). We tested the effect of these amino acid exchanges on nuclear localization by transforming the *RPS4^{nls}-HA-StrepII* construct into *rps4-2* plants. Three independent transgenic lines (RPS4^{nls}-HS #1-3) were selected on the basis of similar RPS4-fusion-protein accumulation compared to the complementing RPS4-HS line (Figure 2B). The nuclear pool of RPS4^{nls}-HA-StrepII was strongly diminished in all three RPS4^{nls}-HS lines (to 0%–2% of the total RPS4^{nls} pool) (Figure 2B). Therefore RPS4 amino acids K1171 to R1178 comprise a functional NLS required for nuclear import of the receptor. Exclusion of RPS4 protein from nuclei did not result in elevated protein levels in the nonnuclear fraction (Figure 2B), probably reflecting the fact that only a minor proportion of the cellular RPS4 pool accumulates in the nucleus.

To establish whether RPS4 nuclear localization is required for RPS4 resistance, we tested lines RPS4^{nls}-HS #1-3 for complementation of the *rps4-2* mutant phenotype. Expression of RPS4^{nls}-HA-StrepII fusion protein failed to complement the *rps4* mutant when challenged with *Pst*-AvrRps4 (Figure 2C). Thus, depletion of the nuclear RPS4 pool correlates with loss of RPS4 function. We then used noninvasive imaging combined with a transient *Agrobacterium*-tobacco expression system to test the localization and functionality of yellow fluorescent protein (YFP)-tagged RPS4. Overexpression of *RPS4* in *Nicotiana tabacum* triggers an *AvrRps4*-independent cell death response that requires a functional NB domain P loop motif for efficient ATP binding [14, 15]. Cell death induced by *RPS4* in *N. benthamiana* was dependent on *NbEDS1*, *NbSGT1*, and *NbHSP90* orthologs, reflecting the genetic requirements of effector-induced resistance mediated by TIR-NB-LRR proteins [15]. Transient expression of YFP-RPS4 from the *35S* promoter in *N. tabacum* produced strong fluorescence inside nuclei and a weaker fluorescent signal in nonnuclear compartments at 24 hr (Figure 3A) and induced confluent cell death 40–48 hr after infiltration (Figure 3B). The YFP-RPS4^{nls} variant was excluded from *N. tabacum* nuclei and failed to elicit cell death (Figures 3A and 3B), although its total accumulation at 24 hr was similar to YFP-RPS4 (Figure 3C). Because the quadruple lysine-to-alanine exchange in the NLS might affect RPS4 functions other than localization, we fused the SV40 large-T antigen NLS (PKKKRKV, [16]) to the YFP-RPS4^{nls} C terminus. This restored both accumulation of RPS4 in the nucleus and triggering of cell death (Figures 3A and 3B). A K242A mutant of YFP-RPS4 lacking a functional P loop motif did not elicit cell death, as reported previously for HA-tagged RPS4 variants [15], but accumulated in nuclei (Figures 3A–3C). Thus, the ability to bind ATP and potentially form receptor oligomers [2, 17] appears not to be a prerequisite for nuclear import of RPS4. The necessity for RPS4 nuclear localization to induce AvrRps4-independent cell death in tobacco is consistent with a postactivation function of RPS4 requiring its translocation to the nucleus.

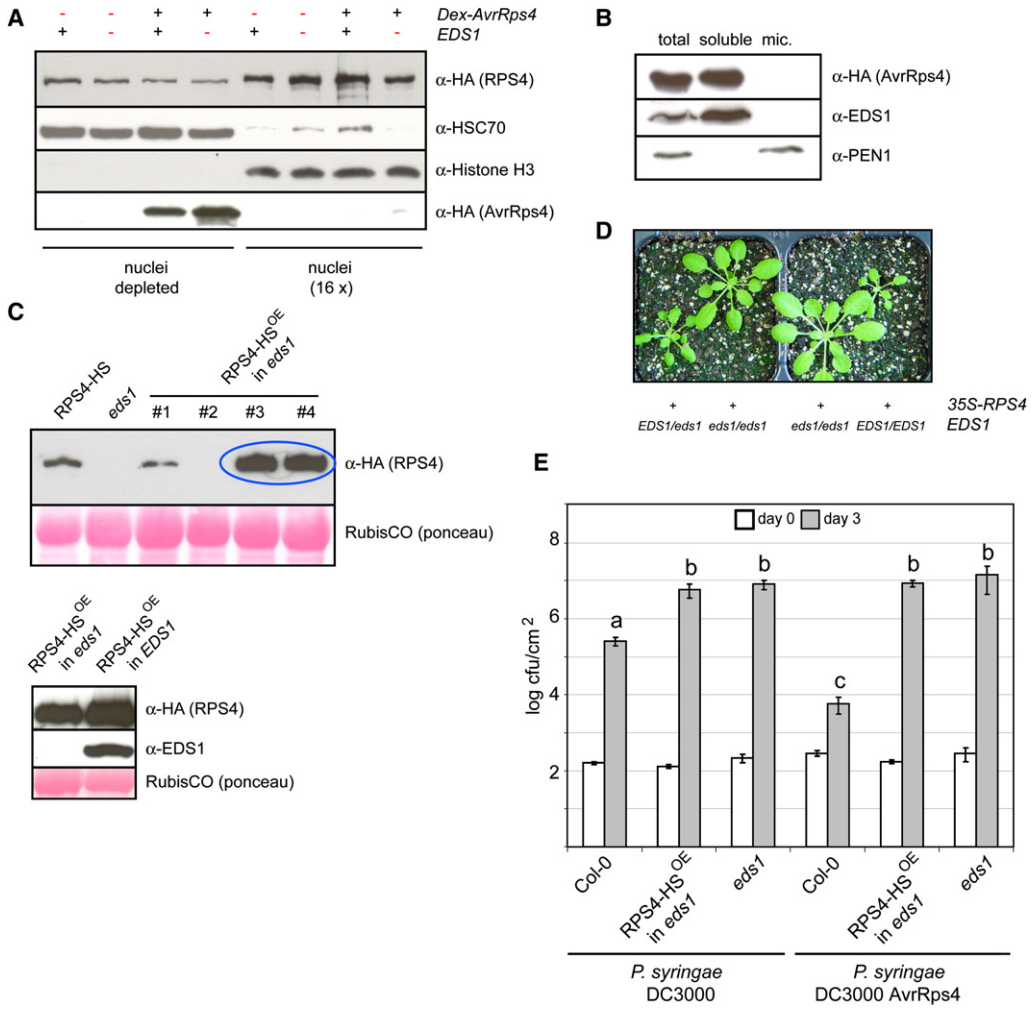

Figure 4. EDS1 Is an Indispensable Component of RPS4-Triggered Resistance

(A) Separation of Dex-induced leaf tissue (24 hr after Dex application) into nuclei and nuclei-depleted fractions. All plants carried the *RPS4-HA-StrepII* transgene and segregated for the *Dex-AvrRps4-HA* construct and the *eds1* mutation as indicated. Fractions were probed on Western blots with the indicated antibodies.

(B) Dex-induced total protein of the transgenic Col-0 *AvrRps4-HA* line [19] was separated into soluble and microsomal fractions by ultracentrifugation. Antisera specific for EDS1 and PEN1 served as soluble and microsomal markers, respectively.

(C) Western blot of total protein extracts from line RPS4-HS, the *eds1* mutant, and four independent transgenic lines expressing *35S:RPS4-HA-StrepII* in *eds1* probed with α-HA antibody (upper panel). The lower panel contains a western blot showing RPS4-HA-SII levels in overexpression line #3 in *eds1* and *EDS1* backgrounds.

(D) Representative individuals from an F_2 population segregating for the *RPS4-HA-StrepII* overexpression construct and the *eds1* mutation as indicated.

(E) *Pst* DC3000 expressing AvrRps4 or carrying the empty vector (as shown) was infiltrated into leaves of Col-0, *eds1* mutant, or the RPS4-HA-StrepII overexpression line #3 (infection conditions as in Figure 2C). Lowercase letters a, b, c, and d indicate significant differences between values of four replicate samples.

EDS1 Is an Integral Signal Transducer of RPS4-Triggered Immunity

We examined whether *EDS1* influences RPS4 protein levels or localization by crossing the RPS4-HS line into Col-0 *eds1* [18]. Total RPS4-HA-StrepII protein accumulation (data not shown) and endomembrane versus nuclear partitioning (Figure 4A) were not altered in Col *eds1* healthy leaf tissues. Therefore, the requirement of TIR-type NB-LRR receptors for *EDS1* appears not to be at the level of receptor assembly or distribution in the cell prior to pathogen sensing.

We then tested whether RPS4 localization changes after activation by its cognate effector, AvrRps4. To ensure a synchronous leaf tissue response, we used a dexamethasone (Dex)-inducible transgenic line expressing HA-tagged AvrRps4 protein. This yields a ~11 kDa C-terminal fragment (AvrRps4-HA^C) that is necessary and sufficient to induce RPS4-mediated defenses [19]. We crossed the Dex-inducible AvrRps4-HA line into transgenic lines expressing RPS4-HA-StrepII in a *rps4-2* or *rps4-2/eds1* background and found that expression of AvrRps4 triggered *EDS1*-dependent cell death (Figure S4). The AvrRps4-HA^C fragment was first detected in western blots 24 hr after Dex application, and so this time point was chosen for the assessment of RPS4 localization. Neither AvrRps4 expression nor the

presence of EDS1 changed distribution of RPS4 between the nuclei-depleted and nuclear fractions (Figure 4A), suggesting that major relocalization of RPS4 does not occur upon AvrRps4 recognition. This result also argues against a role of EDS1 in regulating RPS4 localization once the receptor is activated by AvrRps4. Inducible AvrRps4-HA^{C} was detected in the nonnuclear fraction and remained in the supernatant of a 100,000 × g ultracentrifugation step (Figures 4A and 4B). Thus inducible AvrRps4-HA^{C} does not cofractionate with either the nuclear or endomembrane-associated RPS4 pools, suggesting that recognition of AvrRps4 by RPS4 might be indirect and outside the nucleus. Alternatively, a small amount of AvrRps4 that is below the detection limit of our western blots could be sensed in the endomembrane system or nucleus (directly or indirectly) by RPS4.

Because *eds1* did not influence accumulation or localization of RPS4-HA-StrepII protein (Figure 4A), we hypothesized that *EDS1* functions downstream of the activated RPS4 receptor. Previously, EDS1 was found to amplify defense signals in plant tissues beyond hypersensitive cell death foci triggered by *EDS1*-independent CC-NB-LRR immune receptors [20]. TIR-NB-LRR proteins might therefore recruit EDS1 as a defense potentiator if they are inherently weak immune receptors. Deregulated expression of RPS4 in *Arabidopsis* would then be expected to at least partially override the requirement for *EDS1* to trigger resistance and cell death. To test this, we generated transgenic lines expressing *RPS4-HA-StrepII* under control of the strong CaMV *35S* promoter in a *rps4-2* or *eds1* background. Few transgenic *rps4-2* plants were recovered. All were dwarf, developed necrotic lesions, and eventually died, consistent with RPS4 becoming activated through overexpression [4, 10, 21]. By contrast, *RPS4-HA-StrepII* overexpression lines in *eds1* were viable and indistinguishable from wild-type (data not shown). Among these, several lines were identified that expressed RPS4-HA-StrepII protein at substantially higher levels than the native promoter:RPS4-HA-StrepII line (Figure 4C). We crossed RPS4-HS^{OE} #3 (Figure 4C, upper panel) to wild-type Col-0 and found that high levels of RPS4-HA-StrepII were maintained (Figure 4C, lower panel). In a segregating F_2 population (Figure 4D), dwarfism cosegregated with the presence of an *RPS4* overexpression construct and at least one functional *EDS1* allele in 25 dwarf and 20 normal plants examined. Thus, EDS1 does not alter RPS4 over accumulation but blocks its consequences in growth inhibition. In bacterial-growth assays, RPS4-HA-StrepII overexpression failed to attenuate the extreme susceptibility of *eds1* to virulent *Pst* DC3000 or avirulent *Pst*-AvrRps4 (Figure 4E). Therefore, even activation of overexpressed RPS4-HA-StrepII by its cognate effector does not compensate for the absence of *EDS1*. We concluded that *EDS1* is an indispensible component of TIR-NB-LRR-receptor-mediated resistance and cannot be bypassed by a stronger initial R protein stimulus.

In order to position EDS1 more precisely in the RPS4-triggered defense pathway, we examined transcript profiles of wild-type (Ws-0) and *eds1-1* null mutants in response to *Pst*-AvrRps4 that were performed previously with Affymetrix ATH1 GeneChips [18] (http://www.ebi.ac.uk/arrayexpress/). In contrast to 116/17 induced/repressed genes in Ws-0, only 2/1 induced/repressed genes were identified in *eds1-1* (Table S1). Also, transcriptional upregulation of several strongly pathogen-induced genes that were registered as being *EDS1* dependent in Ws-0 was abolished in *eds1-1* (Table S2). These data support a EDS1 function that links activated RPS4 to changes in gene expression in the nucleus.

Here, we provide evidence that nuclear accumulation of RPS4 is necessary for triggering the immune response and that EDS1 connects activated RPS4 to defense gene transcription in the nucleus. Elicitor-induced oligomerization of tobacco N protein also did not require EDS1, consistent with EDS1 functioning downstream of TIR-NB-LRR-receptor activation [17]. A postactivation signaling activity of EDS1 might occur in the nucleus because subpools of both RPS4 and EDS1 localize here. However, we cannot exclude the possibility that EDS1 in the cytoplasm modifies activated RPS4 such that it enters the nucleus as a signaling-competent molecule. A proportion of the barley CC-NB-LRR protein MLA10 [22] and tobacco N [11] also accumulate in nuclei and require nuclear localization to confer resistance, although they do not possess strong NLS motifs. Thus, a nuclear function for NB-LRR receptors of different structural classes seems to be conserved in species as diverse as barley, tobacco, and *Arabidopsis*. Some plant NB-LRR receptors appear to connect intimately to the transcriptional machinery [22, 23], and the mammalian NOD/CATERPILLER protein CIITA acts as a master transcriptional regulator of *MHC class II* gene expression [24, 25]. It remains to be established whether the nucleus is the key location for other animal and plant receptors to trigger innate immune responses.

Experimental Procedures

Plant Materials, Pathogen Isolates, and Pathology Assays

Arabidopsis wild-type accessions and the *eds1* mutant have been described [5, 18]. The *BON1-3HA*, *GFP-HDEL*, and Dex-inducible *AvrRps4-HA* transgenic lines are published [9, 19, 26]. *Rps4* T DNA insertion mutants were identified with the SIGnAL T DNA Express *Arabidopsis* gene-mapping tool (http://signal.salk.edu). The *rps4-2* mutant (SALK_057697) was obtained from NASC (http://arabidopsis.info), and *rps4-3* (GABI_13F04) was obtained from the GABI-Kat collection (http://www.gabi-kat.de). We verified homozygous insertion lines by sequencing a PCR product obtained with primers specific for the T DNA left border in combination with *RPS4*-specific primers (these and other primer sequences are available on request). Sequencing revealed that the *RPS4* ORF was disrupted after bases G1441 and C1367 in *rps4-2* and *rps4-3*, respectively. Plants were soil grown in controlled environment chambers under a 10 hr light regime (150–200 $\mu E/m^2s$) at 22°C and 65% relative humidity. Vacuum infiltration of 4- to 5-week-old plants with *Pst* at 4×10^5 cfu/ml, bacterial-growth assays, and lactophenol trypan-blue staining were preformed as in [18]. Cultivation of *N. tabacum* SR1 plants was as described [27].

Transgenic *Arabidopsis* Lines Expressing HA-StrepII-Tagged RPS4

pGreenII 0229 vectors carrying the Col-0 *RPS4* genomic sequence under transcriptional control of the CaMV 35S promoter or 511 bp endogenous 5′ regulatory sequence were obtained from S. Dorey (Sainsbury laboratory, Norwich, UK). We cloned PCR products via BamHI into the pGreenII 0229 vectors *p35S:RPS4* and *pRPS4:RPS4* to generate, respectively, *p35S:RPS4-HA-StrepII and pRPS4:RPS4-HA-StrepII*. Site-directed mutagenesis of the predicted *RPS4* NLS sequence was performed with the Stratagene Quick-change site

directed mutagenesis kit according to the manufacturer's conditions. *Arabidopsis* stable transformants were made with the floral-dip method as described previously [18].

Agrobacterium-Mediated Transient Expression of *RPS4* in Tobacco

Gateway (Invitrogen)-compatible entry clones in pENTR4 (Invitrogen) harboring either the Col-0 genomic *RPS4* sequence or the same sequence with the mutated NLS version were generated. The SV40 large T-antigen NLS was fused to the *RPS4-nls* construct by PCR amplification of the C-terminal part with a gene-specific primer and primer RPS4 + NLS (5′-TGGGATCCGACCTTCCGTTT CTTCTTTGGGGCGAAATTCTTAACCGTGT-3′). The genomic *RPS4* K242A P loop mutant variant [15] was constructed with the Stratagene Quick-change site directed mutagenesis kit according to the manufacturer's conditions in pENTR4. To create N-terminal *YFP-RPS4* fusion constructs, we modified the Gateway (Invitrogen)-compatible pJH20-GW overexpression vector (obtained from K. Shirasu, RIKEN, Yokohama, Japan) by inserting the *YFP* coding sequence [28] between the CaMV 35S promoter and the attL1 region. The *RPS4* entry clones were ligated into pJH20-YFP-GW with the Invitrogen LR clonase kit. Transient expression of *RPS4* in *N. tabacum* was performed as described by Zhang et al. [15].

Protein-Expression Analysis

We prepared total protein extracts from *Arabidopsis* or tobacco by grinding leaf material in liquid nitrogen. Ground tissue was thawed in 1 ml extraction buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.5% Triton X-100, and 1× Sigma protease-inhibitor cocktail) per g leaf material. Sample volumes were mixed with an equal volume of 2× SDS-PAGE loading buffer, boiled for 5 min, and stored at −20°C. Protein extracts for soluble versus microsomal fractionations were prepared as above, except that we resuspended ground tissue in extraction buffer B (100 mM Tris-HCl [pH 7.5], 12% sucrose, 1 mM EDTA, 5 mM DTT, and 1× Sigma protease-inhibitor cocktail), filtered it through two layers of Miracloth (Calbiochem), and centrifuged it for 15 min at 2000 × g (4°C) to remove nuclei and cell debris. The supernatant was further fractionated at 100,000 × g (1 hr; 4°C). For RPS4-HA-StrepII solubility assays, 2000 × g supernatants were incubated in extraction buffer B that was supplemented with NaCl, urea, or Triton X-100 at the given concentrations, rotated for 30 min at 4°C, and centrifuged at 100,000 × g as above. Microsomal pellets were resuspended in extraction buffer B containing 1% Triton X-100. Sample volumes corresponding to equal amounts of starting material were mixed with 2× SDS-PAGE loading buffer and stored at −20°C. Nuclear fractionation of *Arabidopsis* tissue was performed as in Feys et al. [5]. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose or PVDF membranes, and protein transfer was monitored by Ponceau S staining (Sigma-Aldrich). Commercial antibodies used were α-HA 3F10 (Roche), α-histone H3 (Abcam), α-HSC70 plant cytosolic (Stressgen), α-GFP mouse monoclonal (Roche), and α-PEPC (Rockland). The α-EDS1 antiserum has been described [5]. α-BIP and α-PEN1 antisera were kindly provided by C. Koncz and P. Schulze-Lefert, respectively (MPIZ, Cologne). Rabbit polyclonal α-RPS4 antiserum was raised against the L*er* RPS4 NB domain expressed in *E. coli*.

Aqueous Two-Phase separation

Purification of *Arabidopsis* plasma membranes was performed as described by Larsson et al. [8] with all volumes scaled down to 20% and a polymer concentration of 6.2% (w/v).

Semiquantitative RT-PCR Analysis

Total RNA was extracted with Tri Reagent (Sigma) according to the manufacturer's protocol. RT reactions were performed with 1 μg of total RNA and 0.5 μg of oligo(dT)$_{18}$ primer at 42°C with reverse transcriptase (Invitrogen) and Protector RNAase inhibitor (Roche) in a 25 μl reaction volume. Aliquots of 1 μl RT-reaction product were subsequently used for semiquantitative RT-PCR analysis with primers *i4*-F (5′-ACGGCTGTAGTTCGCTGAAG-3′) and *i4*-R (5′-ACAAGCGG CTGACTTGATCT-3′) for *RPS4* and primers *actin*-F (5′- TGCGAC AATGGA ACTGGAATG −3′) and *actin*-R (5′- GCTTTTTAAGCCTTT GATCTTGAGA −3′) for *ACTIN*.

Confocal Imaging

Leaf discs of *YFP-RPS4* expressing tobacco leaves were excised, mounted in 60% glycerol onto object slides, and viewed with a confocal laser-scanning microscope (Leica TCS 4D).

Supplemental Data

Four figures and two tables are available at http://www.current-biology.com/cgi/content/full/17/23/2023/DC1/.

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References

1. Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: Shaping the evolution of the plant immune response. Cell *124*, 803–814.
2. Takken, F.L., Albrecht, M., and Tameling, W.I. (2006). Resistance proteins: Molecular switches of plant defence. Curr. Opin. Plant Biol. *9*, 383–390.
3. Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G., and Katagiri, F. (2003). Quantitative nature of *Arabidopsis* responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell *15*, 317–330.
4. Gassmann, W., Hinsch, M.E., and Staskawicz, B.J. (1999). The *Arabidopsis RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. Plant J. *20*, 265–277.
5. Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005). *Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. Plant Cell *17*, 2601–2613.
6. Wiermer, M., Feys, B.J., and Parker, J.E. (2005). Plant immunity: The EDS1 regulatory node. Curr. Opin. Plant Biol. *8*, 383–389.
7. Tornero, P., Chao, R.A., Luthin, W.N., Goff, S.A., and Dangl, J.L. (2002). Large-scale structure-function analysis of the *Arabidopsis* RPM1 disease resistance protein. Plant Cell *14*, 435–450.
8. Larsson, C., Widell, S., and Kjekkbom, P. (1987). Preparation of high-purity plasma membranes. Methods Enzymol. *148*, 558–568.
9. Hua, J., Grisafi, P., Cheng, S.H., and Fink, G.R. (2001). Plant growth homeostasis is controlled by the *Arabidopsis BON1* and *BAP1* genes. Genes Dev. *15*, 2263–2272.
10. Weaver, M.L., Swiderski, M.R., Li, Y., and Jones, J.D. (2006). The *Arabidopsis thaliana* TIR-NB-LRR R-protein, RPP1A; protein localization and constitutive activation of defence by truncated alleles in tobacco and *Arabidopsis*. Plant J. *47*, 829–840.
11. Burch-Smith, T.M., Schiff, M., Caplan, J.L., Tsao, J., Kirk, C., and Dinesh-Kumar, S.P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. PLoS Biol. *5*, e68.
12. Schornack, S., Peter, K., Bonas, U., and Lahaye, T. (2005). Expression levels of *avrBs3*-like genes affect recognition specificity in tomato *Bs4*- but not in pepper *Bs3*-mediated perception. Mol. Plant Microbe Interact. *18*, 1215–1225.
13. Horton, P., Park, K.J., Obayashi, T., and Nakai, K. (2006). Protein subcellular localization prediction with WoLF psort. Proceedings of the 4th Annual Asia Pacific Bioinformatics Conference APBC06, Taipei, Taiwan, 39–48.
14. Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A., and Cornelissen, B.J. (2002). The tomato *R* gene products I-2 and MI-1 are functional ATP binding proteins with ATPase activity. Plant Cell *14*, 2929–2939.

15. Zhang, Y., Dorey, S., Swiderski, M., and Jones, J.D. (2004). Expression of *RPS4* in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. Plant J. *40*, 213–224.
16. Kalderon, D., Richardson, W.D., Markham, A.F., and Smith, A.E. (1984). Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature *311*, 33–38.
17. Mestre, P., and Baulcombe, D.C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. Plant Cell *18*, 491–501.
18. Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J., and Parker, J.E. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase *FMO1* and the Nudix hydrolase *NUDT7*. Plant Cell *18*, 1038–1051.
19. Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L. (2003). *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell *112*, 379–389.
20. Rusterucci, C., Aviv, D.H., Holt, B.F., 3rd, Dangl, J.L., and Parker, J.E. (2001). The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in *Arabidopsis*. Plant Cell *13*, 2211–2224.
21. Oldroyd, G.E.D., and Staskawicz, B.J. (1998). Genetically engineered broad-spectrum disease resistance in tomato. Proc. Natl. Acad. Sci. USA *95*, 10300–10305.
22. Shen, Q.H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I.E., and Schulze-Lefert, P. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science *315*, 1098–1103.
23. Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. USA *100*, 8024–8029.
24. Harton, J.A., O'Connor, W., Jr., Conti, B.J., Linhoff, M.W., and Ting, J.P. (2002). Leucine-rich repeats of the class II transactivator control its rate of nuclear accumulation. Hum. Immunol. *63*, 588–601.
25. Ting, J.P., Kastner, D.L., and Hoffman, H.M. (2006). CATERPILLERs, pyrin and hereditary immunological disorders. Nat. Rev. Immunol. *6*, 183–195.
26. Matsushima, R., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2003). A novel ER-derived compartment, the ER body, selectively accumulates a beta-glucosidase with an ER-retention signal in *Arabidopsis*. Plant J. *33*, 493–502.
27. Romeis, T., Ludwig, A.A., Martin, R., and Jones, J.D. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. *20*, 5556–5567.
28. Shah, K., Gadella, T.W., Jr., van Erp, H., Hecht, V., and de Vries, S.C. (2001). Subcellular localization and oligomerization of the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 protein. J. Mol. Biol. *309*, 641–655.