Supplemental Material and Methods

Multiple sequence alignment

Protein amino acids sequences were retrieved from The Arabidopsis Information Resource (TAIR) and NCBI GenBank databases under the following accession numbers: *Ab*TN2, AT1G17615; *Ab*ZAR1, AT3G50950; *Ab*RPS4, AT5G45250; *Ab*RPS2, AT3G03600; *Ab*RPS5, AT1G12220; *Ab*ADR1, AT1G33560; *Ab*RPM1, AT3G07040; *Ab*NRG1a, AT5G66900; *Nb*NRC2, ALQ52761; *Nb*NRC3, QER78240; *Nb*NRC4, QER78241; *Nb*Roq1, ATD14363; *Sl*I-2, AAD27815;*St*Rx, CAB50786.

The sequence alignments were generate using online EMBL-EBI Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The resulting alignment file was loaded in the Web service ESPript (http://espript.ibcp.fr/ESPript/ESPript/) (Robert and Gouet, 2014) to produce the alignment figure.

Plasmid construction

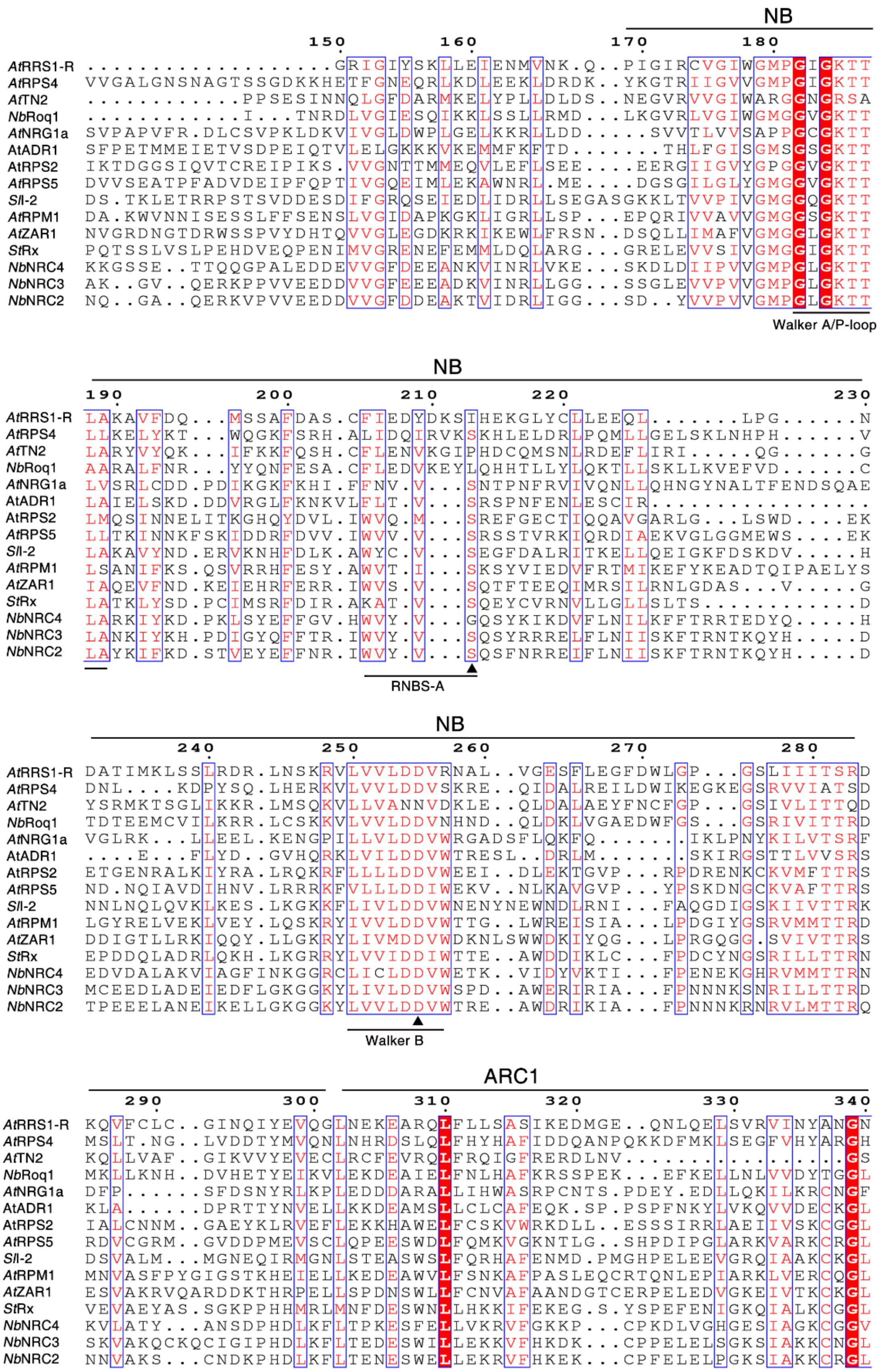
The C-terminally HF-tagged RPS4 variant constructs were generated using the Golden Gate assembly cloning procedure described previously (Ma et al., 2018). To generate RPS4 mutant, the fragments were amplified from wild-type RPS4 template using Goldern Gate cloning with primers containing BbsI site and the introduced mutations. All final constructs used in this study were validated by DNA sequencing. Primers sequences used for plasmid construction are shown in Table S1.

Agrobacterium-Mediated Tobacco Transient Assays (HR)

Agrobacterium-mediated transient expression were performed according to the method described previously in Guo et al. (2020). Briefly, *Agrobacterium* strain GV3101 harbouring indicated binary constructs were streaked on selective L-medium agar plates and grown for 48 h at 28°C. Bacteria were scraped from agar plates and then re-suspended in infiltration medium (10mM $MgCl_2$, 10mM MES, pH 5.6). For co-expression, each bacterial suspension carrying individual constructs was adjusted to OD_{600}=0.5 in the final mix for infiltration. The abaxial leaf sections of 5-week-old *Nicotiana. tabacum* fully-expanded leaves were infiltrated with 1 ml needleless syringe. HR phenotypes were photographed at 5 dpi.

Immunoblot analysis

Immunoblot analysis was performed according to the method described previously in Guo et al. (2020). Briefly, *Nicotiana benthamiana* leaves after transiently expressing the indicated constructs were ground in liquid nitrogen and then mixed with an equal volume of ice-cold GTEN buffer (10% glycerol, 150 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) supplemented with 10 mM DTT, 0.2% (vol/vol) Nodinet-40, Anti-protease tablet (Complete EDTA-free Roche) and 2% PVPP (Polyvinylpolypyrrolidone). The lysates were then centrifuged at 5000 g for 30 min at 4°C, and the supernatant was filtered through 0.45 mm filters. The filtered protein extract mixed with 3xSDS loading buffer (30% glycerol, 3.3% SDS, 94 mM Tris-HCl pH 6.8, 0.05% (vol/vol) bromophenol blue) supplemented with 10 mM DTT were separated by SDS-PAGE. Proteins were transferred to Immobilon-P PVDF membranes (Merck Millipore) and incubated with Horseradish Peroxidate (HRP) conjugated antibodies (Anti-FLAG M2, 1:10000 dilution). Chemiluminescence detection was carried out by incubating the membrane with developing reagents (SuperSignal West Pico & West Femto) and followed by using ImageQuant LAS 4000 (Life Sciences). For quantification, the relative intensities of immunoblotting band were processed and quantified with ImageJ software (National Institutes of Health).


NB
150 160 170 180
AtRRS1-R ....................GRIGIYSKLLEIENMVNK.Q..PIGIRCVGIWGMPGIGKTT
AtRPS4 VVGALGNSNAGTSSGDKKHETFGNEQRLKDLEEKLDRDK..YKGTRIIGVVGMPGIGKTT
AtTN2 ............PPSESINNQLGFDARMKELYPLLDLDS..NEGVRVVGIWARGGNGRSA
NbRoq1 ............I...TNRDLVGIESQIKKLSSLLRMD...LKGVRLVGIWGMGGVGKTT
AtNRG1a SVPAPVFR.DLCSVPKLDKVIVGLDWPLGELKKRLLDD.....SVVTLVVSAPPGCGKTT
AtADR1 SFPETMMEIETVSDPEIQTVLELGKKKVKEMMFKFTD.......THLFGISGMSGSGKTT
AtRPS2 IKTDGGSIQVTCREIPIKS.VVGNTTMMEQVLEFLSEE....EERGIIGVYGPGGVGKTT
AtRPS5 DVVSEATPFADVDEIPFQPTIVGQEIMLEKAWNRL.ME....DGSGILGLYGMGGVGKTT
SlI-2 DS.TKLETRRPSTSVDDESDIFGRQSEIEDLIDRLLSEGASGKKLTVVPIVGMGGQGKTT
AtRPM1 DA.KWVNNISESSLFFSENSLVGIDAPKGKLIGRLLSP...EPQRIVVAVVGMGGSGKTT
AtZAR1 NVGRDNGTDRWSSPVYDHTQVVGLEGDKRKIKEWLFRSN..DSQLLIMAFVGMGGLGKTT
StRx PQTSSLVSLPEHDVEQPENIMVGRENEFEMMLDQLARG...GRELEVVSIVGMGGIGKTT
NbNRC4 KKGSSE..TTQQGPALEDDEVVGFDEEANKVINRLVKE...SKDLDIIPVVGMPGLGKTT
NbNRC3 AK..GV..QERKPPVVEEDDVVGFEEEADKVINRLLGG...SSGLEVVPVVGMPGLGKTT
NbNRC2 NQ..GA..QERKVPVVEEDDVVGFDDEAKTVIDRLIGG...SD..YVVPVVGMPGLGKTT
Walker A/P-loop
NB
190 200 210 220 230
AtRRS1-R LAKAVFDQ...MSSAFDAS.CFIEDYDKSIHEKGLYCLLEEQL........LPG......N
AtRPS4 LLKELYKT...WQGKFSRH.ALIDQIRVKSKHLELDRLPQMLLGELSKLNHPH......V
AtTN2 LARYVYQK...IFKKFQSH.CFLENVKGIPHDCQMSNLRDEFLIRI.....QG......G
NbRoq1 AARALFNR...YYQNFESA.CFLEDVKEYLQHHTLLYLQKTLLSKLLKVEFVD......C
AtNRG1a LVSRLCDD.PDIKGKFKHI.FFNV.V...SNTPNFRVIVQNLLQHNGYNALTFENDSQAE
AtADR1 LAIELSKD.DDVRGLFKNKVLFLT.V...SRSPNFENLESCIR.................
AtRPS2 LMQSINNELITKGHQYDVL.IWVQ.M...SREFGECTIQQAVGARLG...LSWD....EK
AtRPS5 LLTKINNKFSKIDDRFDVV.IWVV.V...SRSSTVRKIQRDIAEKVGLGGMEWS....EK
SlI-2 LAKAVYND.ERVKNHFDLK.AWYC.V...SEGFDALRITKELLQEIGKFDSKDV.....H
AtRPM1 LSANIFKS.QSVRRHFESY.AWVT.I...SKSYVIEDVFRTMIKEFYKEADTQIPAELYS
AtZAR1 IAQEVFND.KEIEHRFERR.IWVS.V...SQTFTEEQIMRSILRNLGDAS...V.....G
StRx LATKLYSD.PCIMSRFDIR.AKAT.V...SQEYCVRNVLLGLLSLTS............D
NbNRC4 LARKIYKD.PKLSYEFFGV.HWVY.V...GQSYKIKDVFLNILKFFTRRTEDYQ.....H
NbNRC3 LANKIYKH.PDIGYQFFTR.IWVY.V...SQSYRRRELFLNIISKFTRNTKQYH.....D
NbNRC2 LAYKIFKD.STVEYEFFNR.IWVY.V...SQSFNRREIFLNIISKFTRNTKQYH.....D
RNBS-A
NB
240 250 260 270 280
AtRRS1-R DATIMKLSSLRDR.LNSKRVLVVLDDVRNAL..VGESFLEGFDWLGP...GSLIIITSRD
AtRPS4 DNL....KDPYSQ.LHERKVLVVLDDVSKRE..QIDALREILDWIKEGKEGSRVVIATSD
AtTN2 YSRMKTSGLIKKR.LMSQKVLLVANNVDKLE..QLDALAEYFNCFGP...GSIVLITTQD
NbRoq1 TDTEEMCVILKRR.LCSKKVLVVLDDVNHND..QLDKLVGAEDWFGS...GSRIVITTRD
AtNRG1a VGLRK...LLEEL.KENGPILLVLDDVWRGADSFLQKFQ.......IKLPNYKILVTSRF
AtADR1 ....E...FLYD..GVHQRKLVILDDVWTRESL..DRLM.......SKIRGSTTLVVSRS
AtRPS2 ETGENRALKIYRA.LRQKRFLLLLDDVWEEI..DLEKTGVP...RPDRENKCKVMFTTRS
AtRPS5 ND.NQIAVDIHNV.LRRRKFVLLLDDIWEKV..NLKAVGVP...YPSKDNGCKVAFTTRS
SlI-2 NNLNQLQVKLKES.LKGKKFLIVLDDVWNENYNEWNDLRNI...FAQGDIGSKIIVTTRK
AtRPM1 LGYRELVEKLVEY.LQSKRYIVVLDDVWTTG..LWREISIA...LPDGIYGSRVMMTTRD
AtZAR1 DDIGTLLRKIQQY.LLGKRYLIVMDDVWDKNLSWWDKIYQG...LPRGQGG.SVIVTTRS
StRx EPDDQLADRLQKH.LKGRRYLVVIDDIWTTE..AWDDIKLC...FPDCYNGSRILLTTRN
NbNRC4 EDVDALAKVIAGFINKGGRCLICLDDVWETK..VIDYVKTI...FPENEKGHRVMMTTRN
NbNRC3 MCEEDLADEIEDFLGKGGKYLIVLDDVWSPD..AWERIRIA...FPNNNKSNRILLTTRD
NbNRC2 TPEEELANEIKELLGKGGKYLVVLDDVWTRE..AWDRIKIA...FPNNNKRNRVLMTTRQ
Walker B
ARC1
290 300 310 320 330 340
AtRRS1-R KQVFCLC..GINQIYEVQGLNEKEARQLFLLSASIKEDMGE..QNLQELSVRVINYANGN
AtRPS4 MSLT.NG..LVDDTYMVQNLNHRDSLQLFHYHAFIDDQANPQKKDFMKLSEGFVHYARGH
AtTN2 KQLLVAF..GIKVVYEVECLRCFEVRQLFRQIGFRERDLNV...................GS
NbRoq1 MKLLKNH..DVHETYEIKVLEKDEAIELFNLHAFKRSSPEK...EFKELLNLVVDYTGGL
AtNRG1a DFP......SFDSNYRLKPLEDDDARALLIHWASRPCNTS.PDEY.EDLLQKILKRCNGF
AtADR1 KLA......DPRTTYNVELLKKDEAMSLLCLCAFEQK.SP.PSPFNKYLVKQVVDECKGL
AtRPS2 IALCNNM..GAEYKLRVEFLEKKHAWELFCSKVWRKDLL..ESSSIRRLAEIIVSKCGGL
AtRPS5 RDVCGRM..GVDDPMEVSCLQPEESWDLFQMKVGKNTLG..SHPDIPGLARKVARKCRGL
SlI-2 DSVALM...MGNEQIRMGNLSTEASWSLFQRHAFENMD.PMGHPELEEVGRQIAAKCKGL
AtRPM1 MNVASFPYGIGSTKHEIELLKEDEAWVLFSNKAFPASLEQCRTQNLEPIARKLVERCQGL
AtZAR1 ESVAKRVQARDDKTHRPELLSPDNSWLLFCNVAFAANDGTCERPELEDVGKEIVTKCKGL
StRx VEVAEYAS.SGKPPHHMRLMNFDESWNLLHKKIFEKEG.S.YSPEFENIGKQIALKCGGL
NbNRC4 KVLATY...ANSDPHDLKFLTPKESFELLVKRVFGKKP...CPKDLVGHGESIAGKCGGV
NbNRC3 SKVAKQCKQCIGIPHDLKFLTEDESWILLEKKVFHKDK...CPPELELSGKSIAKKCNGL
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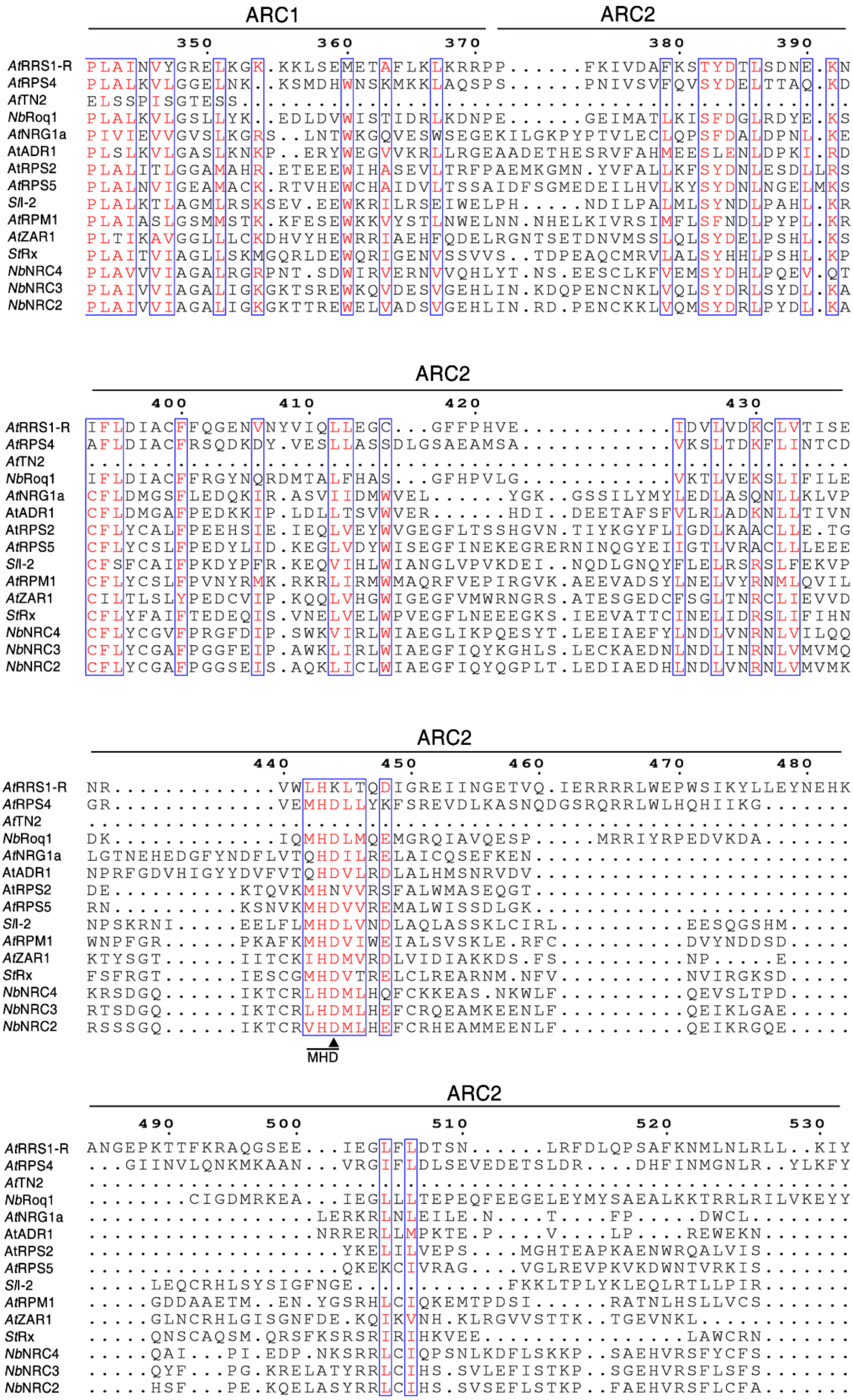
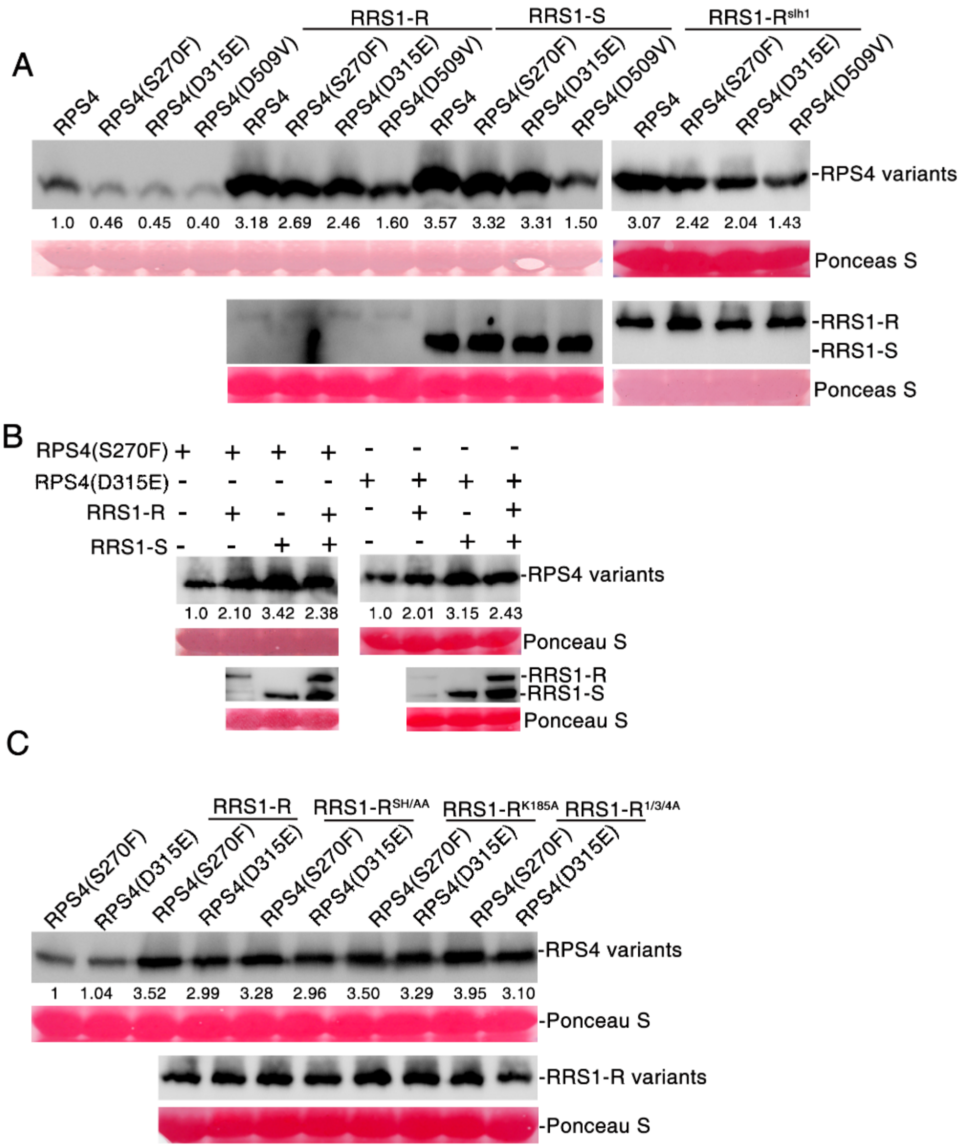

ARC1
ARC2
AtRRS1-R
AtRPS4
AtTN2
NbRoq1
AtNRG1a
AtADR1
AtRPS2
AtRPS5
Sl-2
AtRPM1
AtZAR1
StRx
NbNRC4
NbNRC3
NbNRC2
MHD

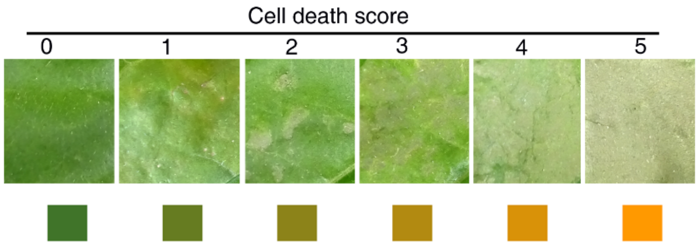
Supplemental Figure S1. Multiple sequence alignment of NB-ARC domains of multiple NLRs.

The amino acid sequence of RPS4 was aligned with representative NLRs from different classes. The subdomain and motifs are annotated as black lines above and below the aligned sequences, respectively. The three conserved amino acid residues for mutation experiments are marked with black triangles. (For details on the alignment, see “Supplemental Materials and Methods.”)



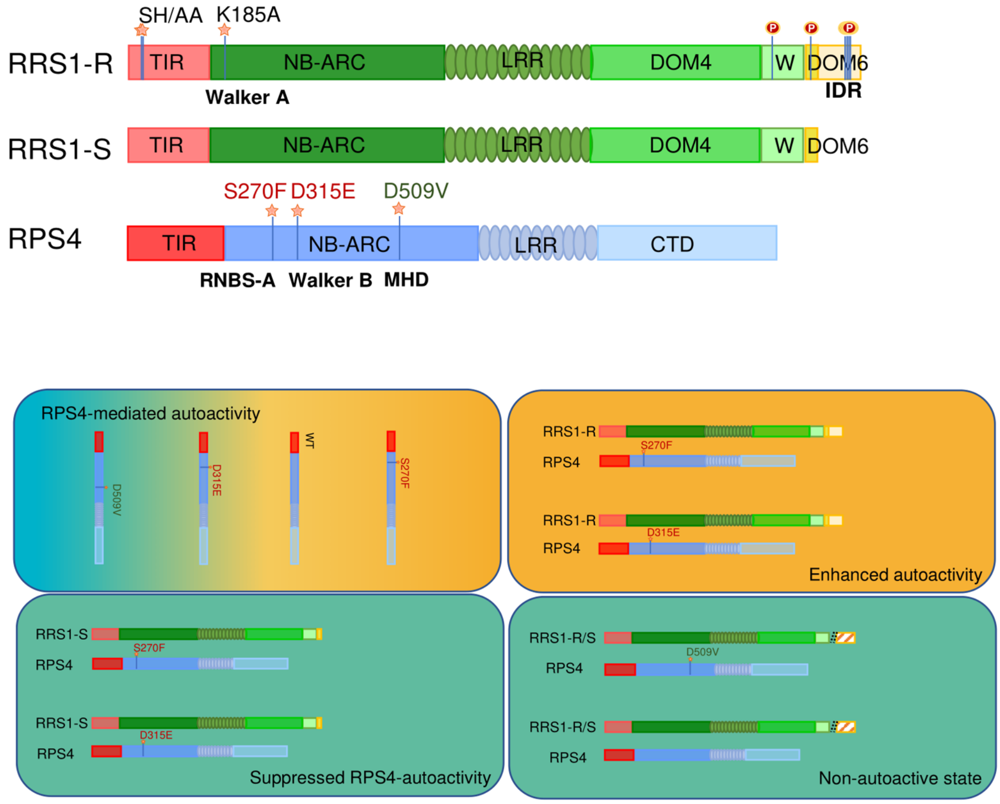
Supplemental Figure S2. Expression levels of wild-type and mutant RPS4.

(A-C) *Nicotiana benthamiana* leaves were agroinfiltrated with indicated constructs to express RPS4 and RRS1-R/S variants. RPS4 variants and RRS1-R/S variants are tagged with HF and GFP and detected by immunoblot assay using anti-FLAG and anti-GFP antibody, respectively. Ponceau S staining of Rubisco served as a loading control. RPS4 variants protein bands shown in western blots were quantified by ImageJ.



Supplemental Figure S3. Representative images of cell death score.

Representative images of the 0–5 scale used to determine the extent of cell death at 5 dpi. 0, no cell death; 5, full cell death.



Supplemental Figure S4. A model illustrating the potentiation of RPS4 autoactive alleles by RRS1-R but not RRS1-S.

The relative activation potential of each RPS4 allele is indicated from cyan (non-autoactive) to yellow (autoactive). The depth of the color represents the (in)activation strength.

Supplementary Table 1. Primers used for plasmid construction in this study.

Purpose	Gene/ Fragments	Vector	Name	Sequence (5'-3')
Generation of mutatants	RPS4(S270F)	pICSL 01005	S270F-F	AATGAAGACATTTTAAGCACTTGGAGTTGGATCGCT
	RPS4(S270F)		S270F-R	AATGAAGACATTAAACTTTACACGGATTTGATCGAT
	RPS4(D315E)		D315E-F	AATGAAGACATGAAGTTAGTAAAAGGGAACAAATAG
	RPS4(D315E)		D315E-R	AATGAAGACATCTTCATCAAGTACAACAAGCACTTT
	RPS4(D509V)		D509V-F	AATGAAGACATGTCCTATTATATAAATTTTCAAGGG
	RPS4(D509V)		D509V-R	AATGAAGACATGGACATGCATCTCCACTCGGCCATC