Novel effector recognition capacity engineered into a paired NLR complex

Shanshan Wang^{1#}, Weijie Huang^{2#}, Zane Duxbury^{1,3}, Saskia A. Hogenhout², Jonathan D. G. Jones^{1*}

Affiliations:

¹ The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, Norwich NR4 7UH, United Kingdom

² Department of Crop Genetics, John Innes Center, NR4 7UH Norwich, United Kingdom

³ Present address: Jealott's Hill International Research Centre, Syngenta, Bracknell RG42 6EY, United Kingdom

#S.W. and W.H. contributed equally to this work

* Correspondence to: jonathan.jones@tsl.ac.uk

Abstract

The Arabidopsis *RRS1-R Resistance* gene confers recognition of the bacterial acetyltransferase PopP2 and another bacterial effector, AvrRps4. The *RRS1-S* allele recognizes AvrRps4 but not PopP2. *RRS1-R/RRS1-S* heterozygotes cannot recognize PopP2. *RRS1-R* and *RRS1-S* also suppress the constitutive RPS4-dependent autoactivity of *RRS1-R^{slh1}*. Phytoplasmas cause important plant diseases, and their effectors can cause degradation of specific host proteins. We tested whether attaching a pathogen effector-dependent degron to *RRS1-R*, enabling its degradation by phytoplasma effector SAP05, could derepress *RRS1-R^{slh1}* autoactivity, resulting in SAP05-dependent resistance. In transient assays in tobacco, *RRS1-R*-derived constructs can confer a hypersensitive response (HR) to SAP05. However, phytoplasma infection assays in transgenic Arabidopsis resulted in delayed disease symptoms but not full resistance. We provide a proof-of-concept strategy utilizing the recessiveness of a plant immune receptor gene to engineer recognition of a pathogen effector that promotes degradation of a specific host protein.

Key words: synthetic NLR, *RRS1/RPS4*, phytoplasma, SAP05, plant immunity

Introduction

Plant immunity to pathogens involves both cell surface and intracellular receptors¹. The cell surface receptors perceive (mostly) conserved pathogen-associated molecular patterns (PAMPs), and intracellular receptors recognise pathogen virulence factor ("effector") proteins delivered into the cell¹. In both plants and animals, the intracellular nucleotide-binding leucine-rich repeat receptor (NLR) family of proteins contributes to innate immunity². Plant NLRs are activated upon perception of effector proteins. Activated NLRs lead to various defense responses towards pathogens, including a hypersensitive cell death response (HR) that restricts pathogens to the infection site, and the activation of transcription of defense-related genes. Since the isolation of the NLR-encoding resistance genes *N* from *Nicotiana tabacum*³, and *RPS2* from *Arabidopsis thaliana*⁴, hundreds of plant NLR genes have been cloned⁵. However, unlike most cell surface receptors, NLRs recognise specific cognate effectors from recognized pathogen races. Expanding the recognition spectrum of NLRs might provide novel resistance resources for future crop disease prevention.

There have been important recent advances in our understanding of how NLRs recognise their cognate effectors and activate immunity. Most plant NLRs carry an N-terminal Toll/interleukin-1 receptor/resistance protein (TIR) domain, a coiled coil (CC) domain or an RPW8 domain. They share a central nucleotide-binding site (NBS), which switches the NLR from inactive to active state (often correlated with ATP binding), a C-terminal leucine-rich repeat (LRR) domain and other C-terminal domains for effector recognition and self-regulation of the NLR structure. Some NLRs carry an integrated decoy domain that mimics authentic virulence targets of pathogen effectors⁶⁻⁹.

The structures of activated forms of NLRC4, a mammalian NLR, and plant NLRs ZAR1, Roq1 and RPP1 demonstrate that upon effector recognition, NLR proteins undergo conformational changes and form oligomers which impose induced proximity on their N-terminal signalling domains, resulting in activation of downstream immune signalling⁹⁻¹³. The conserved architectures and molecular mechanism of NLRs suggest the possibility of moving NLRs between species to confer new resistances¹⁴.

Wild species constitute a disease resistance (R) gene resource. However, time and effort are required to clone new *R* genes, and to validate the function and dissect the molecular mechanism of each NLR. Therefore, engineering plant NLRs is an attractive alternative potential route to gain novel resistance. Gain of function mutagenesis screens have been performed to select alleles of Rx, R3a, I2 or L6 conferring response to new effectors¹⁵⁻¹⁸. Arabidopsis RPS5 monitors the guard cell PBS1 for cleavage by AvrPphB from *Pseudomonas syringae*¹⁹, and modification of the PBS1 proteolytic target site to other proteolytic target sites enables immune response to AvrRpt2 from *P. syringae*, tobacco etch virus (TEV) and turnip mosaic virus (TuMV)²⁰. Pik and Pia, paired NLRs from rice, with a Heavy Metal Associated (HMA) domain integrated in the sensor NLR Pik, function to recognise effector from *Magnaporthe oryzae*²¹. Modification of the HMA domain expanded the range of effector recognition²²⁻²⁵.

In Arabidopsis, RRS1-R (*Resistance to Ralstonia solanacearum* 1) and RPS4 (*Resistance to Pseudomonas syringae* 4) are well-studied paired TIR-NLRs, functioning together to recognize bacterial effector AvrRps4 from *P. syringae*, PopP2 from *R. solanacearum* and an unknown effector from *Colletotrichum higginsianum*²⁶⁻²⁸. The effectors are recognized via direct interaction with and/or modification of the integrated C-terminal WRKY domain of RRS1, followed by defense activation through RPS4²⁹⁻³². The Arabidopsis RRS1-R^{slh1} (sensitive to low humidity 1) mutant with a single leucine insertion in WRKY domain causes an RPS4-dependent effector-independent autoimmune phenotype^{33,34}. Most NLR genes are dominant, but like RRS1-R, RRS1-R^{slh1} is also recessive, which means the autoimmune phenotype can be suppressed by inactive forms of RRS1 such as RRS1-R from Arabidopsis Ws-2 or RRS1-S from Col-0. The RRS1-R^{slh1}-induced-RPS4-dependent autoactivity (hereafter RRS1-R^{slh1} autoactivity) overlaps strongly with AvrRps4- and PopP2- induced RRS1/RPS4 mediated response at transcriptional level³⁴, and RRS1-R^{slh1} autoactivity could enable engineering effector-triggered immunity.

Here we designed a system consisting of an autoactive RRS1-R^{slh1}, an RPS4 and an inactive RRS1 with an extra domain which can be recognised and degraded by the cognate effector. Upon effector action, RRS1-R^{slh1} autoimmunity is derepressed, and defense responses activated. To create this, we selected

several effectors and their corresponding targets. SAP05, an effector from insect-transmitted phytopathogenic bacteria, degrades GATA zinc finger domain transcription factor proteins³⁵. We fused a GATA SAP05-dependent degron domain to RRS1-R. Co-expression of this fusion protein represses autoimmunity of RRS1-R^{slh1}; this is derepressed by coexpression with SAP05, restoring the RRS1-R^{slh1}-induced-RPS4-dependent hypersensitive response (HR) in transient assays. Transgenic Arabidopsis with the engineered resistance proteins show delayed disease symptoms after phytoplasma infection though not full resistance. This synthetic NLR combination results in new effector recognition capacity, and this approach could be applied to recognise a broad range of effectors from phytopathogens and serve as new source of plant resistance.

Results

Constructing an RRS1-derived allele that represses the autoactivity of RRS1-R^{slh1}

We set out to engineer a system that contains three components; an autoactive RRS1-R^{slh1}, RPS4, and an RRS1-derived variant carrying an effector target domain, here designated as RRS1-X (Fig. 1a). Effector action promotes degradation of RRS1-X, thus derepressing the RRS1-R^{slh1} autoactivity. RRS1 and RPS4 are nuclear localized NLRs, so nuclear-localized host targets and effectors were prioritized for evaluation. Transcription factors (TFs) are often targeted by effectors, which can lead to their degradation (Table. S1). For example, the phytoplasma effector SAP11 interacts with and destabilizes multiple TEOSINTE BRANCHED 1-CYCLOIDEA-PROLIFERATING CELL FACTOR (TCP) transcription factors³⁶⁻³⁸, SAP54 directly binds and promotes degradation of MADS domain transcription factor (MTF) family proteins, including SEP3, AP1 or SOC1³⁹, and SAP05 directly binds the zinc finger domains of SQUAMOSA promoter-binding proteins (SBP) and GATA domain transcription factors and mediates their degradation³⁵. *Pseudomonas syringae* Type III Effector HopBB1 promotes the degradation of TCP14 by direct binding⁴⁰, and HopX1 eliminates JAZ proteins by cleaving the ZIM domain⁴¹.

The C- terminus of RRS1 plays a crucial role in maintaining the inactive state of the RRS1/RPS4 complex as well as in responses to effectors^{31,32}. The RRS1-R allele has an additional 83 amino acids at the C terminus compared to RRS1-S. Both RRS1-R and RRS1-S repress the autoactivity of RRS1-*R^{slh1}*. Therefore, RRS1-R or RRS1-S were fused with target proteins/domains. Since NLR protein derivatives can easily lose function or become autoactive, a variety of linkers including Glycine Serine [GS] tandem repeat sequences, Asn-Ala-Ala-Ile-Arg-Ser [NAAIRS] tandem repeat sequences^{42,43} or a combination of both were applied to link RRS1 to the target protein/domain.

We transiently co-expressed RRS1 fusion proteins with RPS4 using agrobacterial infiltration into *Nicotiana tabacum* leaves to test for RRS1 fusion protein autoactivity. RRS1-R-[GS]₁₀-TCP14, RRS1-R-[GS]₁₀-JAZ1 and RRS1-R-[GS]₁₀-GFP show RPS4-dependent autoactivity (Fig. S1a). We hypothesized this may be due to the size of the fused protein JAZ1 (253 amino acids), TCP14 (489 amino acids) and GFP (238 amino acids). The minimum size of target protein domain which responds to effector was then investigated. A keratin-like domain (KD) from MADS box transcription factors is sufficient to interact with phytoplasma effector SAP54³⁹ and was fused to RRS1. Fusion proteins of RRS1-R carrying the KD with 3 different linkers show strong autoactivity when transiently co-expressed with RPS4 in *N. tabacum* (Fig. S1b) and the same strong RPS4-dependent autoactivity is observed with RRS1-S allele and KD fusion protein (Fig. S1c). We also fused the SBP domain from squamosa promoter-like 11 (SPL11) and GATA domain from GATA18, which are the targets of phytoplasma effector SAP05, to RRS1-R. All the tested fusion proteins show RPS4-dependent autoactivity, though RRS1-R-[GS]₁₀-GATA-HF (His-FLAG) showed weak autoactivity (Fig. S1d). These results suggest that RRS1 structure is tightly self-regulated by intramolecular domain interactions and extra domains may disrupt the inactive state of RRS1/RPS4 complex.

RRS1-R^{SH/AA} eliminates the autoactivity of the fusion protein

We tested an alternative method to eliminate the autoactivity of the RRS1 fusion protein. The structure of RRS1 and RPS4 TIR domains reveals that the heterodimerization of RPS4 TIR and RRS1 TIR is dependent on the integrity of the α A- and α E-helices (the AE interface), and mutations disrupting the interface prevent the signalling induction of RRS1/RPS4⁴⁴. Because RRS1-R^{SH/AA}, with H25A S26A

mutations in the AE interface, abolishes RRS1/RPS4 function, but can repress RRS1-R^{slh1} autoactivity (Fig. 1a)^{34,44}, we tested if these mutations eliminate the autoactivity of the fusion protein. Unlike the autoactive RRS1-R-[GS]₄-[NAAIR]₂-[GS]₄-KD-HF, RRS1-R^{SH/AA}-[GS]₄-[NAAIR]₂-[GS]₄-KD does not show autoactivity with RPS4 (Fig. S2a). The TCP domain of transcription factor TCP2 (TCPd) or TCP18 (TCP18d), which is sufficient to associate with SAP11³⁶, and MED19a which is destabilized by *Hyaloperonospora arabidopsidis* effector HaRXL44, were fused to RRS1-R^{SH/AA} respectively^{36,45}. A 95 amino-acid unstructured region (Unstr) from *S. cerevisiae* cytochrome *b*₂ was appended to the C terminus of TCPd to facilitate a rapid proteasome-dependent degradation⁴⁶. RRS1-R^{SH/AA}-[GS]₁₀-TCP2d-FLAGUnstr, RRS1-R^{SH/AA}-[GS]₁₀-TCP18d-FLAG-Unstr, RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF and RRS1-R^{SH/AA}-[GS]₁₀-MED19a were not autoactive when co-expressed with RPS4 in *N. tabacum* (Fig. S2a, Fig. 1b and 1d). Co-expression of RRS1-R^{slh1} and RPS4, RRS1-R^{SH/AA}-[GS]₁₀-GATA fully suppress RRS1-R^{slh1} autoactivity (Fig. 1c and 1e), whilst with the rest, only partial suppression was observed (Fig. S2b and S3a). Protein expression level were verified by western blot (Fig. 1g and S2c and S3b). Intriguingly, RRS1-R^{SH/AA}-[NAAIR]₄-GATA-HF cannot suppress RRS1-R^{slh1} autoimmunity (Fig. S3a), suggesting that the linker region is important to maintain the inactive state of the complex.

Constructing a RRS1- R^{SH/AA} allele with a GATA domain that promotes SAP05-dependent degradation

To determine whether RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF responds to effector SAP05, we performed transient expression assays in *N. tabacum* and found that expressing RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF, RRS1-R^{slh1}, RPS4 with SAP05 but not with control GUS-HF, triggered HR (Fig. 2a and 2b). RRS1-R^{SH/AA}-HF did not respond to SAP05 (Fig. S4). Consistent with the recent report that SAP05 binds and subsequently degrades GATA domain-containing proteins³⁵, RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF, but not RRS1-R^{SH/AA}-HF, co-immunoprecipitated with SAP05 (Fig. 2c). RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF, but not RRS1-R^{SH/AA}-HF, was degraded when co-expressed in *N. benthamiana* with SAP05 in the presence of RPS4-HA and RRS1-R^{slh1}-V5 (Fig. 2d). Transient assays in *N. benthamiana* and *N. tabacum* confirmed that in the presence of RPS4 and RRS1-R^{slh1}, SAP05 mediates RRS1-R^{SH/AA}-[GS]₁₀-GATA degradation and therefore can derepress RRS1-R^{slh1} autoactivity.

Transgenic Arabidopsis with RRS1-R^{SH/AA}-[GS]₁₀-GATA show delayed disease symptoms after phytoplasma infection

Using Golden Gate cloning⁴⁷, binary vectors carrying RRS1-R^{shl}-V5 with RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF (SH/AA-GATA) or RRS1-R^{SH/AA}-HF (SH/AA) were generated for Arabidopsis transformation (Fig. 3a). We use the pAtSSR16 (AT4G34620) promoter to drive the RRS1 alleles and FastRed as selection marker⁴⁸. The constructs were transformed into *Col-0/rrs1-3/rrs1b-1* background. Growth of the transgenic plants was indistinguishable from Col-0 or *Col-0/rrs1-3/rrs1b-1* (Fig. S5a). Two independent transgenic Arabidopsis lines were used for phytoplasma infection assays, of which 6 individual plants were sampled to confirm the protein expression by Western blot (Fig. S5b).

Phytoplasmas are vectored by sap-feeding insects, including leafhopper, planthoppers and psyllids of the order Hemiptera⁴⁹. We infected plants with AY-WB (Aster Yellows Witches'-Broom) phytoplasma carrying the SAP05 effector³⁵ via transmission with aster leafhoppers (*Macrostelus quadrilineatus*) (Fig. 3b). The symptoms of AY-WB-infected Arabidopsis plants include small and deformed leaves, long petioles, pale green leaf colours, and narrow leaf blades^{50,51}. After 3-5 weeks, some of the infected plants displayed severe symptoms whilst others displayed mild symptoms (Fig. 3c and S6). Seven to 10 weeks post infection (wpi), all the infected plants eventually died (Fig. S7).

We observed a delayed AY-WB phytoplasma disease symptom progression on infected SH/AA-GATA transgenic plants, compared to SH/AA, *Col-0/rrs1-3/rrs1b-1* or Col-0, 3-5 weeks after infection. The SH/AA-GATA-9# line shows a significant reduction in the number of plants with severe symptoms, while the reduction in line SH/AA-GATA-10# was not statistically significant (Fig. 3d). However, all infected plants including Col-0, *Col-0/rrs1-3/rrs1b-1*, SH/AA-2#, -7#, SH/AA-GATA-9#, and -10# died 7-10 weeks after phytoplasma infection (Fig. S7b), indicating that the transgenic Arabidopsis SH/AA-GATA are not fully resistant to AY-WB phytoplasma.

In this three-component system, the fusion protein RRS1-R^{SH/AA}-GATA suppressed the RPS4-dependent RRS1-R^{shl} mediated autoactivity in the absence of effector. Upon effector recognition, the GATA domain fusion protein is degraded, restoring RRS1-R^{shl} mediated autoactivity (Fig. 4). In the transient assay in *N. tabacum* and *N. benthamiana*, the HR occurs upon effector delivery (Fig. 2a). In

transgenic Arabidopsis, this system delays the disease symptom progression upon phytoplasma infection (Fig. 3c and 3d), but the transgenic plants do not show full resistance.

Discussion

Breeding crops to reduce losses to plant pathogens and pests involves judicious use of *Resistance* (*R*) genes, which usually encode NLR proteins that act as intracellular immune receptors which recognise effectors from pathogens and activate defense. Numerous NLR-encoding genes have been cloned, and there are widespread efforts to engineer known NLRs to expand their recognition capacity. We report here an approach to expanding the recognition capacity of the Arabidopsis paired NLRs RRS1 and RPS4. Transgenic tomato carrying RRS1-RPS4 show elevated resistance to *R. solanacearum* and *Pseudomonas syringae* pv. *tomato* DC3000 carrying the effector AvrRps4, and transgenic cucumber plants carrying RRS1/RPS4 are highly resistant to *Colletotrichum orbiculare*⁵². These data indicate that engineered RRS1/RPS4 can function in other species.

Integrated domains as new source of resistance

The RRS1-R/RPS4 paired NLR recognises AvrRps4 and PopP2 via the integrated WRKY domain at the C terminus of RRS1-R. The discovery of non-canonical integrated domains in NLRs suggests a novel path for engineering NLR recognition capacity⁶⁻⁸. Rice NLR Pik-1 alleles which contain integrated HMA domains were designed to gain binding affinity and recognition capacity to the corresponding allelic effectors based on structural information or phylogenetic analysis. The modified Pik alleles activated immune responses in *N. benthamiana*²²⁻²⁵. Another HMA-containing rice NLR, RGA5 (which functions with RGA4), carrying an engineered HMA domain was able to perceive a new ligand in *N. benthamiana*⁵³. In our system, RRS1-R^{SH/AA}-GATA recognizes an effector that has not been reported to be recognised by any NLR. Ideally, synthetic plant NLRs with any effector binding domain may result in new resistance genes. However, judging the best position to insert an ID is challenging⁵⁴. Full length NLR structural studies will illuminate attempts to achieve such engineering. Here, for RRS1-R^{SH/AA}-GATA, with the linker [GS]₁₀ but not [NAAIR]₄, can suppress RRS1-R^{slh1} autoactivity (Fig. S3), though this advance was achieved by trial-and-error rather than design.

A novel approach for NLR engineering

Synthetic NLRs with modified HMA domains have been engineered with expanded recognition of allelic variants of effectors²²⁻²⁵. However, further enhancement of the effector spectrum for NLR engineering would be desirable. With more knowledge of effectors and host targets, new strategies are emerging for synthetic NLRs that can respond to divergent effectors. In the Arabidopsis PBS1/RPS5 decoy system, PBS1 contains a protease cleavage site that serves as decoy and NLR RPS5 is activated by proteolytic cleavage of PBS1. This system mediates detection of the effector protein AvrPphB from *Pseudomonas syringae*¹⁹. By swapping the proteolytic target site of PBS1 to protease recognition sites of AvrRpt2 from *P. syringae* or NIa protease from TEV or TuMV, the engineered PBS1 with RPS5 confers resistance to *P. syringae* carrying AvrRpt2, TEV or TuMV²⁰. In our system, we derepress RRS1-*R^{slh1}* autoimmunity by attaching an effector-dependent degron to inactive, repressing RRS1-*R^{slh1}*. We generated the chimeric repressor RRS1-*R^{SH/AA}*-GATA to be degraded in response to a phytoplasma effector SAP05, thus broadening the NLR recognition specificity. Incorporating SH/AA mutation in the signalling TIR domain was required to prevent the autoactivity of the fusion protein. With the expanding knowledge of NLRs and effectors, additional alternative strategies to design synthetic NLRs will enable further engineering to expand NLR recognition specificity.

HR is not always associated with resistance

HR is a form of localized cell death that occurs at the pathogen infection sites, and is often correlated recognition of a pathogen effector by a plant R protein⁵⁵. Recent data show how surface receptor- and intracellular receptor-mediated immunity mechanisms mutually potentiate, culminating in an HR^{56,57}. In plant-virus pathosystems, resistance can occur in the absence of cell death. Potato *Rx* mediates extreme resistance to various Potato Virus X (PVX) strains in *Nicotiana* spp and potato, without visible cell death⁵⁸. Transgenic *N. benthamiana* plants carrying the tomato *Tm-2²* gene show extreme resistance to Tobacco Mosaic Virus (TMV) in the absence of cell death⁵⁹. In the plant-oomycete pathosystem, potato *Rpi-chc1.2* confers HR on coexpression with multiple *Phytophthora infestans* effector PexRD31 paralogs, but doesn't always confer late blight resistance⁶⁰. The lack of full correlation of cell death and resistance is also observed in Arabidopsis and bacterial pathogens. The Arabidopsis *nrg1* mutant lacks

HR induced by RRS1/RPS4 and RRS1B/RPS4B but still maintains the resistance to *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying effector AvrRps4 or AvrRpt2 (recognised by CC-NLR RPS2)⁶¹. PAD4 is dispensable for RRS1/RPS4 mediated cell death but required for the resistance to DC3000 with AvrRps4⁶². RGA5 with a modified HMA domain can confer HR to a new ligand, AVR-PikD in *N. benthamiana* but does not confer resistance in the homologous rice system⁵³.

Despite effector-dependent HR in transient assays in *Nicotiana* sp., transgenic Arabidopsis carrying RPS4, RRS1-R^{slh1} and RRS1-R^{SH/AA}-GATA were not resistant to Phytoplasma infection, though they did show delayed symptoms. This further exemplifies how HR does not always correlate to resistance. Phytoplasmas locate to the phloem and it remains an open question whether NLRs induce HR in all cell types. Also, whereas SAP05 is likely to unload from the phloem and migrate to other cells, it is not yet clear which cells the effector migrates to and whether these cells have the signaling pathway that translates NLR activation into an HR. Finally, SAP05 degrades plant transcription factors and changes developmental process³⁵ and may interfere with defense induction upstream of NLR signaling. Conceivably, there is insufficient SAP05 delivery from phytoplasma infection to enable enough RRS1-R^{SH/AA}-GATA degradation to fully derepress immunity. Caution is required in selecting the appropriate effectors for NLR engineering. It is likely that a balance needs to be struck in our system between expressing sufficient RRS1-R^{SH/AA}-GATA to avoid any constitutive defense activation by RRS1-R^{slh1}, while avoiding expressing so much RRS1-R^{slh1} that a pathogen effector fails to remove enough to derepress RRS1-R^{slh1}.

TNL-mediated cell death and resistance requires a non-NLR protein, Enhanced Disease Susceptibility1 (EDS1) in Arabidopsis. EDS1 forms EDS1-SAG101 or EDS1-PAD4 complexes to determine the cell death and pathogen growth restriction respectively^{63,64}, and EDS1-SAG101 engages with the RPW8-NLR NRG1 upon effector recognition⁶⁵. Since the engineered NLR is RPS4-dependent, it is likely to signal via these modules. NLR protein levels also determine the strength of resistance⁵⁹; R genes are usually semi-dominant, and transgenic lines with sufficient NLR expression are likely required for robust resistance. Last but not least, engineering the resistance to a phloem-limited insect-transmitted plant pathogen might require more knowledge of defense in phloem.

In summary, this study provides a new strategy utilizing the concept of integrated domain and the recessiveness of RRS1 to expand NLR-dependent effector recognition capacity.

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459 **Contribution**

460 J.D.G.J. and S.A.H. conceptualized the study and obtained funding. S.W. and J.D.G.J. designed the
461 experiment. S.W., W.H. and Z.D. performed the experiments and data analysis. S.W. and J.D.G.J. wrote
462 the manuscript. All the authors edited the manuscript.

463 **Competing interests**

464 The authors declare no competing interests.

465 **Supplementary Materials:**

466 Materials and Methods

467 Figs. S1 to S7

468 Table S1 to S4

Figure 1

Figure 1. RRS1-R^{SH/AA}-[GS]₁₀-GATA suppresses RRS1-R^{slh1} autoactivity. **(a)** Schematic diagram of RRS1/RPS4 complexes. RPS4-dependent autoactivity of RRS1 (RRS1-R^{slh1}) can be suppressed by inactive RRS1 allele (RRS1-R/RRS1-S/RRS1-R^{SH/AA}). **(b)** HR assay in *N. tabacum* shows RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF is not autoactive. **(c)** HR assay in *N. tabacum* shows RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF suppresses RPS4-dependent RRS1-R^{slh1} autoactivity. **(b and c)** *N. tabacum* leaves were

coinfiltrated with *A. tumefaciens* strains carrying the indicated constructs. Photos were taken 3-4 days post infiltration (dpi). **(d and e)** HR index of visually assessed tobacco infiltrations for **(b)** and **(c)** respectively. Each sample represents co-infiltrations of agrobacteria with different construct (indicated on the x axis) with a sample size of n= 12, 12 and 12 for **(d)** and n=15, 15 and 15 for **(e)**. Errorbars show SD. Difference of HR levels was assessed by One way ANOVA (Analysis of variance) followed by a Turkey HSD test. Different letters indicate significant difference at level $p < 0.05$. **(f)** Representative image of HR index used to quantify cell death in tobacco leaf. **(g)** Protein expression analysis of the constructs. *N. benthamiana* leaves were coinfiltrated with *A. tumefaciens* strains carrying the indicated constructs and harvested after 48 hours post infiltration. Total proteins were immunoblotted with anti-FLAG antibodies for RRS1-R^{SH/AA}-HF (His-FLAG), RRS-R^{slh1}-HF or RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF.

Figure 2

Figure 2. RRS1-R^{SH/AA}-GATA responds to SAP05 and derepresses the RPS4-dependent RRS1-R^{slh1} autoactivity. **(a)** RRS1-R^{SH/AA}-[GS]₁₀-GATA restores RRS1-R^{slh1}-mediated RPS4-dependent HR when coinfiltrated with SAP05. *N. tabacum* leaves were coinfiltrated with *A. tumefaciens* strains carrying the indicated constructs. Photos were taken at 4 dpi. **(b)** HR index of visually assessed tobacco infiltrations of **(a)**. Each sample represents co-infiltrations of agrobacteria with different construct (indicated on the x axis) with a sample size of n= 17, 17 and 13. Errorbars show SD. Difference of HR levels was assessed by One way ANOVA (Analysis of variance) followed by a Turkey HSD test. Different letters indicate significant difference at level $p < 0.05$. **(c)** RRS1-R^{SH/AA}-[GS]₁₀-GATA interact with SAP05 in planta. *N. benthamiana* leaves were transiently coinfiltrated with *A. tumefaciens* strains carrying SAP05, RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF or RRS1-R^{SH/AA}-HF (as indicated by a + or -symbol). Two days after infiltration, the samples were harvested and proteins with FLAG epitope were immunoprecipitated and subjected to SDS-PAGE. Proteins were immunoblotted by anti-FLAG or anti-SAP05 antibody. Rubisco stained by Ponceau (Pon S) served as loading control. This experiment was performed twice with the same result. **(d)** RRS1-R^{SH/AA}-[GS]₁₀-GATA is degraded by SAP05 in planta. *N. benthamiana* leaves were transiently coinfiltrated with *A. tumefaciens* strains carrying RPS4-HA, RRS1-R^{slh1}-V5, SAP05, GUS-HF, RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF or RRS1-R^{SH/AA}-HF (as indicated by a + or -symbol).

Rubisco stained by Ponceau (Pon S) served as loading control. Samples were harvested at 3 dpi and the total protein were subjected to SDS-PAGE and immunoblotted by anti-FLAG or anti-SAP05 antibody. Two replicates were shown here. The experiment was repeated for three times with similar result.

Figure 3

Figure 3. Transgenic Arabidopsis with $RRS1-R^{SH/AA}$ -[GS]₁₀-GATA and $RRS1-R^{slh1}$ has delayed disease symptoms after phytoplasma infection. **(a)** Schematic of constructs that were transformed into Arabidopsis. Arrow indicates the promoter, grey box indicates the gene, other coloured box indicates the linker region, epitope or GATA domain and terminators are in the last box. **(b)** Cartoon to show the

phytoplasma infection. Leafhoppers carrying the phytoplasma were caged on one leaf for 3-4 days. **(c)** Disease development after phytoplasma infection. Infected Arabidopsis developed severe or mild symptoms 3-5 weeks post infection (wpi). **(d)** Statistical analysis of Arabidopsis 3-5 wpi with severe symptom. Infected Arabidopsis symptoms were characterised as severe or mild. Three independent infection experiments were performed, each line was tested for at least two times with the total number of plant $n = 14, 23, 24, 14, 23$ or 13 . Student's t test was used to analyse the difference level between groups. P value was indicated above the groups and error bar showed the 95% confidence interval). The analysis results are in Dataset_01.

Figure 4

Figure 4. Schematic representation of engineering RRS1 with degron domain to recognize novel effector. **(a)** RRS1-R^{slh1} causes RPS4-dependent autoactivity. **(b)** Phytoplasma effector SAP05 targets GATA transcription factor and promotes proteasome-dependent degradation. **(c)** RRS1-R wildtype can repress RRS1-R^{slh1} autoactivity. **(d)** RRS1-R^{slh1} autoactivity can also be repressed by RRS1-R^{SH/AA}-GATA. **(e)** The degradation of RRS1-R^{SH/AA}-GATA by SAP05 derepresses RRS1-R^{slh1}-induced-RPS4-dependent autoactivity.

Supplementary Figure

Figure S1

Fig. S1 HR assays to test the RPS4-dependent autoactivity of RRS1-X constructs. *N. tabacum* leaves were co-infiltrated with *A. tumefaciens* strains (each at OD₆₀₀ = 0.5) carrying RPS4 and the indicating RRS1-X construct. (a) RPS4 and RRS1-R-[GS]₁₀-GFP, RRS1-R-[GS]₁₀-TCP14, RRS1-R-[GS]₁₀-JAZ1, showed RPS4-dependent autoactivity. (b) RRS1-R and KD fusion protein with three different linkers ([GS]₁₀, [NAAIRS]₄ and [GS]₁₀-[NAAIRS]₄-[GS]₁₀) all displayed RPS4-dependent autoactivity. (c) RRS1-S and KD fusion protein with 4 different linkers ([GS]₁₀, [NAAIRS]₄, [GS]₁₀-[NAAIRS]₄-[GS]₁₀ and HF) all displayed RPS4-dependent autoactivity. (d) RRS1-R-[NAAIRS]₄-SBP-HF and RRS1-R-[NAAIRS]₄-GATA-HF displayed strong RPS4-dependent autoactivity whilst RRS1-R-[GS]₁₀-GATA-HF displayed weak RPS4-dependent autoactivity. HR was visually assessed and photographed at 3 days post-infiltration (3 dpi). When co-infiltrated with RPS4, RRS1-R^{slh1}-HF (HR) and RRS1-R-HF (no HR) were used as control. The numbers in parentheses are the number of leaves displaying HR out of the total number of leaves infiltrated. Numbers in red with '*' represents weak HR.

Figure S2

Fig. S2 RRS1-R^{SH/AA}-X constructs do not show RPS4-dependent autoactivity but cannot fully suppress RRS1-R^{sh1} mediated-autoactivity. **(a)** *N. tabacum* leaves were co-infiltrated with *A. tumefaciens* strains (each at OD₆₀₀ = 0.5) carrying RPS4 and RRS1-R^{SH/AA}-[GS]₄-[NAAIR]₂-[GS]₄-KD-HF, RRS1-R^{SH/AA}-[GS]₁₀-MED19a-HF, RRS1-R^{SH/AA}-[GS]₁₀-TCP2d-HF or RRS1-R^{SH/AA}-[GS]₁₀-TCP18d-HF did not show HR. **(b)** All the RRS1-R^{SH/AA}-X constructs tested in (A) cannot fully repress RRS1-R^{sh1}-mediated autoactivity. The numbers in parentheses are the number of leaves displaying HR out of the total number of leaves infiltrated. Numbers in red with '*' represents partial cell death. **(c)** Western blot showed the protein expression level. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strains (OD₆₀₀ = 0.5) carrying RRS1-R^{SH/AA}-[GS]₄-[NAAIR]₂-[GS]₄-KD-HF, RRS1-R^{SH/AA}-[GS]₁₀-MED19a-HF, RRS1-R^{SH/AA}-[GS]₁₀-TCP2d-FlagUnstr or RRS1-R^{SH/AA}-[GS]₁₀-TCP18d-FlagUnstr. Samples were harvested 48 hours post infiltration (hpi) and total protein were extracted and subjected to SDS-PAGE, then immunoblotted for FLAG. The construct highlighted in blue has no FLAG epitope. Ponceau S staining (Pon S) was used as loading control.

Figure S3

Fig. S3 The linker between RRS1-*R^{SH/AA}* and fusion domain is important to suppress RRS1-*R^{slh1}*-induced-autoactivity. **(a)** *N. tabacum* leaves were co-infiltrated with *A. tumefaciens* strains (each at OD₆₀₀ = 0.5) carrying RPS4, RRS1-*R^{slh1}* and RRS1-*R^{SH/AA}*-[NAAIR]₄-GATA-HF, RRS1-*R^{SH/AA}*-[GS]₁₀-GATA-HF, RRS1-*R^{SH/AA}*-[NAAIR]₄-SBP-HF or RRS1-*R^{SH/AA}*-[GS]₁₀-SBP-HF. Only RRS1-*R^{SH/AA}*-[GS]₁₀-GATA-HF can fully suppress RRS1-*R^{slh1}* mediated-autoactivity. **(b)** Western blot is to show the protein expression of RRS1-*R^{SH/AA}*-[NAAIR]₄-GATA-HF and RRS1-*R^{SH/AA}*-[GS]₁₀-GATA-HF. The numbers in parentheses are the number of leaves displaying HR out of the total number of leaves infiltrated.

Figure S4

Fig. S4 RRS1-R^{SH/AA} does not respond to SAP05. *N. tabacum* leaves were co-infiltrated with *A. tumefaciens* strains (each at OD₆₀₀ = 0.5, strain with SAP05 OD₆₀₀ = 1) carrying RPS4, RRS1-R^{slh1}, RRS1-R^{SH/AA}-HF and SAP05 or GUS-HF. RRS1-R^{SH/AA}-HF did not show cell death when co-expressed with SAP05. The numbers in parentheses are the number of leaves displaying HR out of the total number of leaves infiltrated.

Figure S5

Fig. S5 Phenotype and protein expression level of transgenic Arabidopsis. **(a)** Phenotype of the indicated 3-4 weeks-old *Arabidopsis* plants. Transgenic Arabidopsis lines (T2 plants) transformed with RRS1-R^{slh1}-V5 and RRS1-R^{SH/AA}-HF or RRS1-R^{SH/AA}-[GS]₁₀-GATA-HF were labeled as SH/AA or SH/AA-GATA respectively. **(b)** Western blot results to show the protein expression level of each transgenic line. Arabidopsis leaves were harvested from 3-4 weeks-old plants individually. Total protein was extracted and subjected to SDS-PAGE and immunoblotted for FLAG or V5. Pon S was used as loading control.

Figure S6

Fig. S6 Disease symptom of Arabidopsis 4 weeks post phytoplasma infection.

Figure S7

Fig. S7 Disease symptom of Arabidopsis in the late stage after phytoplasma infection. **(a)** Timeline of infection assay and typical symptom of Arabidopsis plant 7-9 weeks post phytoplasma infection. **(b)** Arabidopsis were dying 7 weeks post phytoplasma infection.