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Analyses of natural variation indicates that the absence of RPS4/RRS1 and amino acid change in RPS4 cause loss of their functions and resistance to pathogens

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ABSTRACT

A pair of *Arabidopsis thaliana* resistance proteins, RPS4 and RRS1, recognizes the cognate Avr effector from the bacterial pathogens *Pseudomonas syringae* pv. tomato expressing *avrRps4* (*Pst-avrRps4*), *Ralstonia solanacearum*, and the fungal pathogen *Colletotrichum higginsianum* and leads to defense signaling activation against the pathogens. In the present study, we analyzed 14 *A. thaliana* accessions for natural variation in *Pst-avrRps4* and *C. higginsianum* susceptibility, and found new compatible and incompatible *Arabidopsis*–pathogen interactions. We first found that *A. thaliana* accession Cvi-0 is susceptible to *Pst-avrRps4*. Interestingly, the genome sequence assembly indicated that Cvi-0 lost both *RPS4* and *RRS1*, but not *RPS4B* and *RRS1B*, compared to the reference genome sequence from *A. thaliana* accession Col-0. On the other hand, the natural variation analysis of *RPS4* alleles from various *Arabidopsis* accessions revealed that one amino-acid change, Y950H, is responsible for the loss of resistance to *Pst-avrRps4* and *C. higginsianum* in RLD-0. Our data indicate that the amino acid change, Y950H, in RPS4 resulted in the loss of both RPS4 and RRS1 functions and resistance to pathogens.

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Introduction

Plant disease resistance, known as gene-for-gene relationship, requires a resistance (*R*) gene in the host plant and a cognate avirulence (*Avr*) gene in the insect, pest, or pathogen.[1] The *R*-gene product detects the corresponding *Avr* gene product and initiates signal transduction to confer resistance.

Many *R*-genes encode NB-LRR proteins, also known as NLRs, which consist of a central NB-ARC domain (a nucleotide-binding adaptor shared with Apaf-1, plant resistance proteins, and CED-4) and a C-terminal leucine-rich repeat (LRR) domain. NLRs possess either a Toll/interleukin 1 receptor (TIR) domain or a coiled-coil (CC) domain in their N-terminal structures.[2]

In most cases, a single plant NLR recognizes the cognate Avr effector and results in the activation of defense signaling against the pathogen.[3,4] Moreover, some pairs of NLRs are reported to be required for both recognizing the cognate Avr effectors and conferring resistance to pathogens.[5] Our recent studies showed that a pair of *Arabidopsis thaliana* NLRs, RPS4 (Resistance to *Pseudomonas syringae* 4) and RRS1 (Resistance to *Ralstonia solanacearum* 1), mediate recognition of multiple pathogens, such as the fungal pathogen *Colletotrichum higginsianum* (anthracnose) and bacterial pathogens *Pseudomonas syringae* pv. tomato expressing *avrRps4* (*Pst-avrRps4*; bacterial speck) and *Ralstonia solanacearum* (bacterial wilt).[6] In addition, Debieu *et al.*[7] recently reported that RPS4/RRS1 pair was required to confer resistance to *Xanthomonas campestris* pv. *campestris* (black rot). Thus, the paired NLRs are required for recognition of at least three bacterial Avr effectors, AvrRps4 from *Pst-avrRps4*, PopP2 from *R. solanacearum*, and a putative bacterial Avr effector from *X. campestris* pv. *campestris*, and a putative fungal Avr effector from *C. higginsianum*.[6,8-10] Our previous studies also showed that introduction of the NLR gene pair, *RPS4* and *RRS1*, into several crops provided disease resistance to different classes of pathogens.[11,12]

The genes, *RPS4* and *RRS1*, constituting a pair are localized near each other and are encoded in opposite directions. Although both RPS4 and RRS1 are TIR-type NLRs, RRS1 contains a leucine zipper (LZ) motif and a WRKY domain at the C-terminus. The paired NLRs interact with each other physically to form a hetero-complex.[6,13-14]

RPS4 and RRS1 play different roles in effector-triggered immunity in plants. According to the "integrated decoy" model, acetylation of the C-terminal WRKY domain of RRS1 protein triggers activation of NLR complex and thus, initiates the immune response.[15-16] On the other hand, the precise mechanism of how RPS4 interacts with acetylated RRS1 and how RPS4/RRS1 complex triggers defense activation is unclear.

A previous analysis of natural variation in *RPS4* alleles from Col-0, Ws-2, Ler, and RLD-0 accessions revealed that two amino-acid changes, N195D and Y950H, might be responsible for the loss of resistance to *Pst-avrRps4* in RLD.[8,17] The objectives of this study were to investigate whether: 1) the above-mentioned amino acid changes also cause susceptibility to *C. higginsianum* in RLD-0 and Ws-2 background, 2) these amino acid polymorphisms account for the non-functionality of RPS4-RLD, and 3) specific amino acid polymorphisms in RPS4 play a role in the RPS4/RRS1 complex.

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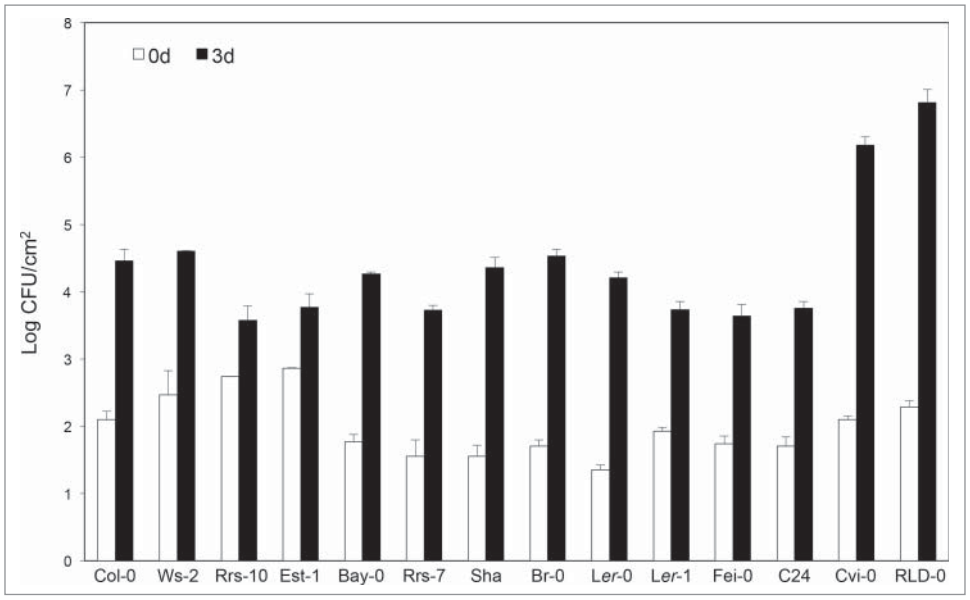

Figure 1. Growth of *Pst-avrRps4* in *Arabidopsis thaliana* accessions after inoculation with *Pst-avrRps4*. Leaves of 5-week-old plants were infiltrated with bacterial suspensions (5×10^4 cfu ml^{-1}). The leaves were harvested at 0 (white columns) and 3 days (black columns) after inoculation. Bacterial growth (cfu cm^{-2}) was assessed using five leaf disks by cell counting. Bars indicate SE. This experiment was repeated three times with similar results.

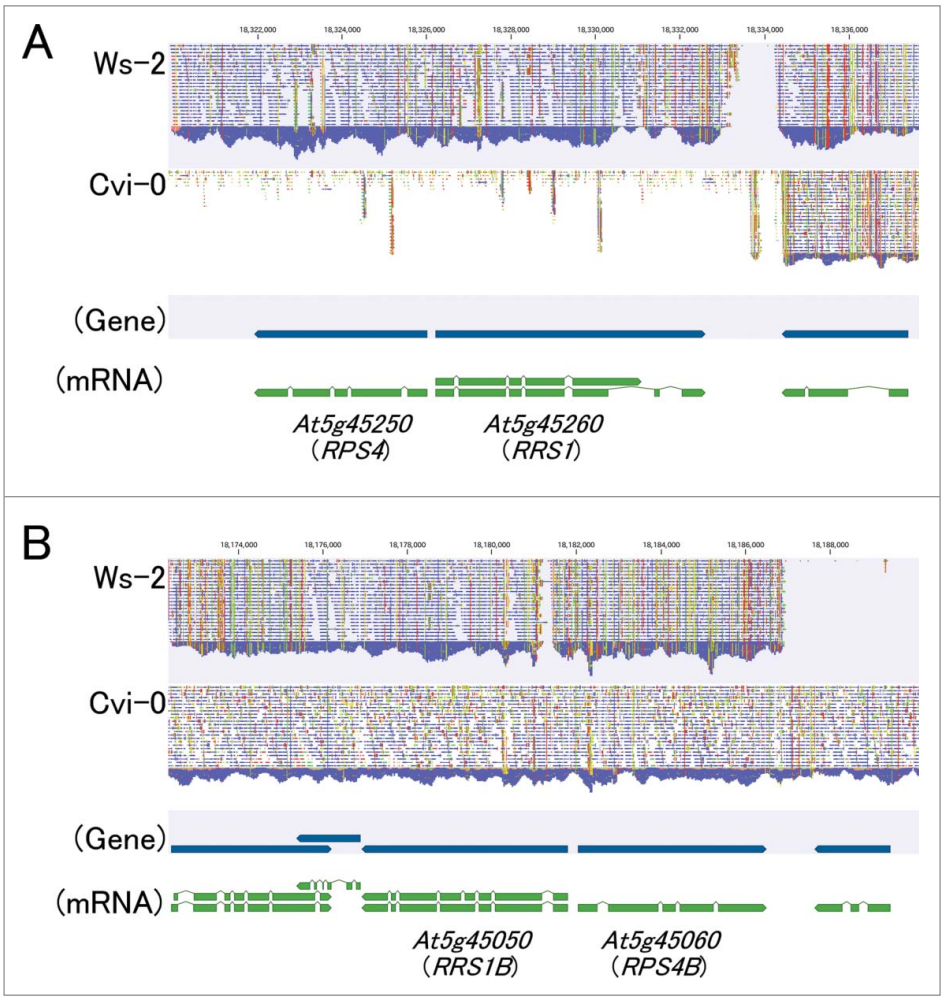

Figure 2. A comparison of the nucleotide sequences of the *RPS4/RRS1* and *RPS4B/RRS1B* alleles between Cvi-0 and Col-0. *RPS4/RRS1* (A) and *RPS4B/RRS1B* (B) from genome sequence data sets for *Arabidopsis thaliana* accession Ws-2 (SRR492407) and Cvi-0 (SRR492239) were compared with the corresponding region of Col-0 reference sequence.

Results

Natural variation in the susceptibility to P. syringae *pv. tomato strain DC3000 expressing* avrRps4 *among* A. thaliana *accessions*

To investigate natural variations in *Pst-avrRps4* susceptibility, we tested 14 *A. thaliana* accessions (Col-0, Ws-2, L*er*-0, L*er*-1, Rrs-7, Rrs-10, Est-1, Bay-0, Sha, Br-0, Fei-0, C24, Cvi-0, and RLD-0). We evaluated the disease reactions of *A. thaliana* accessions to *Pst-avrRps4* based on certain aspects, such as the assays of bacterial growth by colony formation in plate culture of samples from infected leaves. Most of the interactions observed with these accessions were incompatible (Fig. 1). However, a compatible phenotype was found following the inoculation of accessions RLD-0 and Cvi-0 (Fig. 1). *A. thaliana* plants that appeared to be susceptible, developed chlorotic lesions at the inoculation sites, 3–4 day post inoculation (dpi), which expanded further. It is the first report that Cvi-0 is compatible to *Pst-avrRps4*.

Comparison of the nucleotide sequences of RPS4 *and* RRS1 *alleles between Cvi-0 and Col-0*

The evolutionary conservation of *RPS4*/*RRS1* gene pair localized near each other in a head to head arrangement indicates their cooperative function in disease resistance. To understand whether the putative *RPS4-Cvi* and *RRS1-Cvi* are responsible for susceptibility to *C. higginsianum* and *Pst-avrRps4*, we analyzed DNA sequences of the *RPS4-Cvi* and *RRS1-Cvi* alleles. Surprisingly, these genes from *Arabidopsis* 1001 genome project[18] could not be assembled against the corresponding region of Col-0 reference sequence. Although Clark *et al.*[19] reported single-nucleotide polymorphisms (SNPs) in 20 wild accessions of *A. thaliana* using high-density oligonucleotide arrays containing putative *RPS4-Cvi* and *RRS1-Cvi* alleles, the data from *Arabidopsis* 1001 genome project indicate that the putative *RPS4-Cvi* and *RRS1-Cvi* genes are less homologous to the corresponding regions of other *Arabidopsis RPS4*/*RRS1* genes (Fig. 2A). On the other hand, Saucet *et al.*[20] reported that *RPS4B* (*At5g45060*)/*RRS1B* (*At5g45050*) is paralogous and functionally similar to *RPS4*/*RRS1*. RPS4B and RRS1B recognize AvrRps4 but not PopP2. Therefore, we analyzed DNA sequences of *RPS4B-Cvi* and *RRS1B-Cvi* alleles. These genes from *Arabidopsis* 1001 genome project assembled against the corresponding region of Col-0 reference sequence (Fig. 2B).

Responses of A. thaliana *accession RLD-0 to inoculation with* C. higginsianum

In this study, *A. thaliana* accession RLD-0 and Cvi-0 appeared to be compatible to *C. higginsianum*. Light microscopy revealed that infection hyphae developed in the invaded epidermal cells of the susceptible accession Cvi-0 and RLD-0 but not in Ws-2 (Fig. 3).

Susceptibility to C. higginsianum *and* Pst avrRps4: *Which is responsible—RPS4-RLD or RRS1-RLD ?*

To confirm whether *RPS4-RLD* or *RRS1-RLD* is responsible for susceptibility to *C. higginsianum* and *Pst-avrRps4*, we introduced

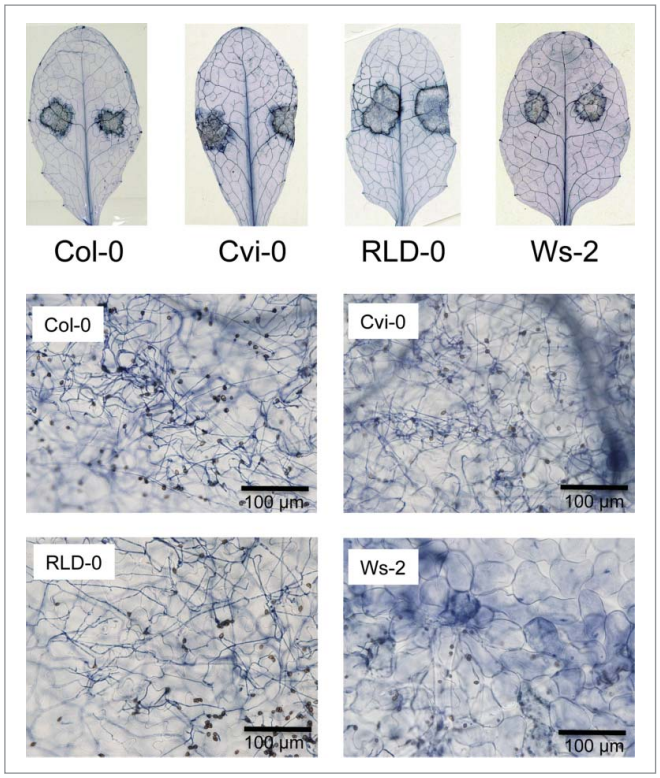

Figure 3. Infection phenotypes of leaves inoculated with *C. higginsianum*. Mature leaves of 28-day-old plants were inoculated by placing 5 μl of spore suspension of *C. higginsianum* (5×10^5 spores ml^{-1}) on each side of the leaf. The leaves were harvested at 6 dpi and stained with trypan blue. Each picture shows a representative of three independent experiments.

either 6.3-kbp genomic *RRS1-Ws* fragments, including approximately 1.7-kbp upstream and 176-bp downstream regions (Ws-2 background),[6] or the 6-kbp genomic *RPS4-Ws* fragments, including approximately 2-kbp upstream and 109-bp downstream regions (Ws-2 background)[6] into the susceptible accession RLD-0. *RPS4-Ws* transgenic RLD-0 plants conferred resistance to *C. higginsianum* and *Pst-avrRps4*, but *RRS1-Ws* transgenic RLD-0 plants were susceptible to the pathogen (Fig. 4). We concluded that *RPS4-RLD* is responsible for resistance to *C. higginsianum* and *Pst-avrRps4*.

Complementation of mutated RPS4 (N195D or Y950H) to rps4-21 *mutants*

Amino acid sequence variations in RPS4 proteins among 20 *Arabidopsis* accessions are shown in Fig. 5. Only one amino-acid substitution, Y950H, observed in RPS4-RLD is not shared with RPS4 from any of the other accessions. In addition, Gassmann *et al.* reported that N195D and/or Y950H were responsible for the loss of resistance to *Pst-avrRps4* in RLD-0[8,17]. Therefore, the mutated *RPS4-Ws* clones (Ws-2 background) with the amino acid changes, N195D and Y950H, were designated as $RPS4\text{-}Ws^{N195D}$ and $RPS4\text{-}Ws^{Y950H}$, respectively. Subsequently, these clones were complemented into *rps4-21* mutants (Ws-2 background).[6]

To determine whether N195 and Y950 are required for resistance to *C. higginsianum*, fungal infection levels were monitored in

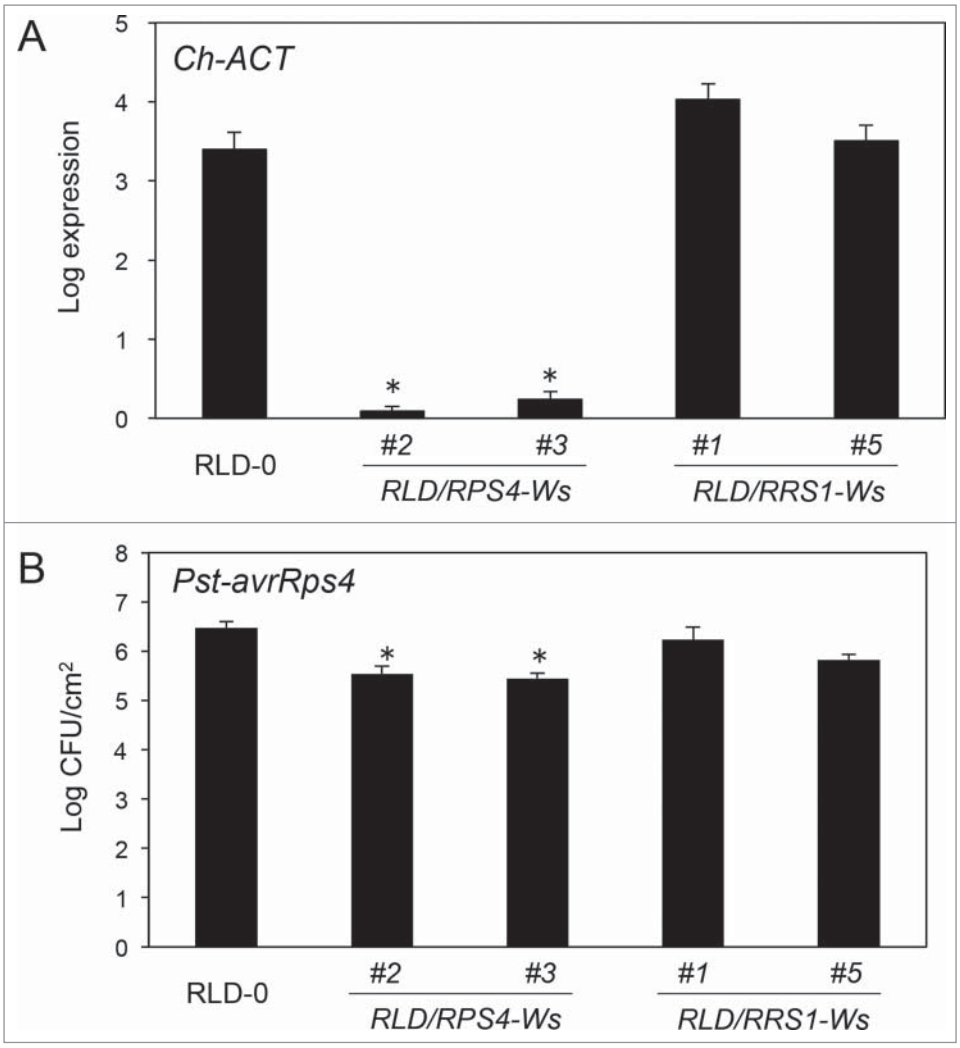

Figure 4. *C. higginsianum* and *Pst-avrRps4* resistance analysis in the transgenic lines. *RLD/RPS4-Ws-#2* and *-#3* lines represent independent transgenic RLD-0 plants harboring the genomic *RPS4-Ws* fragment. *RLD/RRS1-Ws-#1* and *-#5* lines represent independent RLD-0 transgenic plants harboring the genomic *RRS1-Ws* fragment. (A) Quantification of *C. higginsianum in planta* by qRT-PCR. Twenty eight-day-old plants were spray-inoculated with *C. higginsianum*. The inoculated leaves were harvested at 5 dpi and total RNA was isolated. QRT-PCR was performed with *Ch-ACT* primers for each sample. (B) Quantification of *Pst-avrRps4 in planta*. Leaves of 5-week-old plants were infiltrated with bacterial suspensions (5×10^4 cfu ml^{-1}). The leaves were harvested 3 days after inoculation. Bacterial growth (cfu cm^{-2}) was assessed using five leaf disks by cell counting. Bars indicate SE. The asterisks indicate statistical significance from the RLD-0 WT controls (Dunnett's method,[28] $P < 0.01$). This experiment was repeated at least two times with similar results.

Ws-2 and in the mutants, 5 days post-inoculation (Fig. 6A). The *RPS4-Ws*^{Y950H} complemented *rps4-21* plants were susceptible to *C. higginsianum*. On the contrary, *RPS4-Ws*^{N195D} complemented *rps4-21* plants were resistant to *C. higginsianum*.

To determine whether N195 and Y950 are required for resistance to *Pst-avrRps4*, the bacterial infection levels were monitored in Ws-2 and in the mutants, 3 days post-inoculation (Fig. 6B). *Pst-avrRps4* count was about three- or eight-times higher in *RPS4-Ws*^{N195D} or *RPS4-Ws*^{Y950H} complemented *rps4-21* plants, respectively, than in wild-type Ws-2.

Discussion

In the present study, we analyzed 14 *A. thaliana* accessions for understanding the natural variation in *C. higginsianum* and *Pst-avrRps4* susceptibility, and found new compatible and incompatible *Arabidopsis*-pathogens interactions. We first found that *A. thaliana* accession Cvi-0 is susceptible to *Pst-avrRps4*. RPS4 and RRS1 together recognize bacterial effector AvrRps4 in *Pst-avrRps4* and subsequently induce resistance to the pathogen. However, the genome sequence assembly indicated that Cvi-0 lacks both *RPS4* and *RRS1*; however, *RPS4B* and *RRS1B*, which are closely linked to *RPS4* and *RRS1*, respectively are present in the genome. It suggests that RRS1B and RPS4B in Cvi-0 might recognize AvrRps4 from *Pst-avrRps4*, as susceptibility to *Pst-avrRps4* in Cvi-0 was slightly lower than that in RLD-0, which is super susceptible to the pathogen. Clark *et al.*[19] reported that most of the NLR genes harbor at least one 'major-effect change', i.e. SNP with large-effects on gene integrity and/or polymorphic region prediction. One hypothesis is that the major-effect change arose in putative *RPS4-Cvi* and *RRS1-Cvi* alleles resulting in trade-offs between plant growth and defense against the pathogen.[21] *A. thaliana* Cvi-0 accession might evolve in an environment without exposure to the pathogens that could be recognized by the RPS4 and RRS1 pair. As we do not understand which of the two *R*-gene pairs, *RPS4/RRS1* or *RPS4B/RRS1B*, is an ancestor, it is also interesting for understanding the branching process in the evolution of RRS4 and RRS1 among the *A. thaliana* accessions.

		TIR	NBS				LRR	C-term				
Resistant/Susceptible (*C. higginsianum*)		153	195	282	291	518	649	950	1004	1033	1170	1189
S	Col-0	I	N	L	H	V	M	Y	V	A	N	G
S	Bur-0	I	N	L	N	I	M	Y	V	P	N	E
S	Cvi-0	I	N	L	H	V	M	Y	V	A	N	G
S	L*er*-1	I	N	L	N	V	M	Y	V	P	N	G
S	Lov-5	I	N	L	N	V	T	Y	V	P	N	G
S	C24	I	N	L	N	V	T	Y	I	P	N	G
S	RLD-0	I	D	L	N	V	M	H	V	P	K	G
R	Ts-1	I	D	L	N	V	M	Y	V	P	N	G
R	Fei-0	I	D	L	N	V	M	Y	V	P	N	G
R	Ws-2	T	N	L	N	I	M	Y	V	P	K	G
R	Bay-0	I	N	L	N	V	M	Y	V	P	N	G
R	Br-0	I	N	L	H	V	M	Y	V	A	N	G
R	Est-1	I	N	L	H	V	M	Y	V	A	N	G
R	Nfa-8	I	N	L	N	V	M	Y	V	P	N	G
R	Rrs-10	I	N	L	H	V	M	Y	V	A	N	G
R	Rrs-7	T	N	V	N	V	M	Y	V	P	N	G
R	Sha	T	N	V	N	V	M	Y	V	P	N	G
R	Tamm-2	I	N	L	N	V	T	Y	I	P	N	G
R	Tsu-1	I	N	L	N	V	T	Y	I	P	N	G
R	Van-0	I	N	L	N	V	M	Y	V	P	N	G

Figure 5. Amino acid sequence variations in RPS4 proteins among 20 *Arabidopsis* accessions. Only amino acid differences from 20 *Arabidopsis* accessions are shown, without identical residues. Resistance and susceptibility to *C. higginsianum* are indicated by 'R' and 'S', respectively.

Previously, Gassmann *et al.*[8] also reported that RLD-0 was susceptible to *Pst-avrRps4* and Col-0 showed resistance against the pathogen. A previous study indicated that the two amino-acid changes, N195D and Y950H, in RLD-0 are sufficient for the nonfunctionality of RPS4-RLD and for the loss of resistance to *Pst-avrRps4*[17]. Similarly, we found that RLD-0 accession is also susceptible to *C. higginsianum*. The *C. higginsianum* strain MAFF305635 causes anthracnose disease symptoms in Col-0 and Cvi-0 plants.[6,22] In contrast, incompatible accession, Ws-2 (moderate resistance), formed restricted brown necrotic lesions at the inoculation sites, which did not expand.[6,22] Moreover, we also found that two accessions, Fei-0 and Ts-1, which contain RPS4 with N195D but not Y950H, are resistant to *C. higginsianum*. In addition, Fei-0 showed resistance to *Pst-avrRps4*. The analyses of natural variation showed that one amino acid change, Y950H, in RLD-0 is unique among all the accessions used here. As the *RPS4-Ws*^{Y950H} complemented *rps4-21* plants were susceptible to *Pst-avrRps4*, Y950H in RPS4 is likely to be responsible for susceptibility to the pathogen and also possibly to *Pst-avrRps4* in RLD-0.

We also showed that *RPS4-Ws*^{Y950H} transgenic plants were susceptible to *C. higginsianum*; therefore, one amino acid change compromised the RPS4 function. Y950 is located in the C-terminal end after LRR; the consequence of the amino acid change on the protein activity and stability is unknown. It is known that proteins can bind to one another by using phosphorylated tyrosines.[23,24] The replacement of Y950 may cause an unstable LRR domain structure. Our recent report showed that the C-termini of RPS4 and RRS1 are responsible for resistance signaling against *C. higginsianum*.[13] Therefore, the C-terminal region of RPS4, containing Y950, likely plays an important role in the activation of RPS4/RRS1-dependent defense responses. On the other hand, Saucet *et al.*[20] suggested that *A. thaliana* accession RLD-0 lacks function of both RPS4/RRS1 and RPS4B/RRS1B. In this study, we showed that *RPS4-Ws* transferred RLD-0 plants, containing nonfunctional RPS4B/RRS1B, were resistant to the pathogens and *RPS4-Ws*^{Y950H} complemented *rps4-21* plants (Ws-2 background, containing functional RPS4B/RRS1B) were compatible to the pathogen. Therefore, some structural domains in RPS4 and RRS1 are essential for disease resistance and contribute to the interaction of RPS4 with RRS1. On the other hand, we found that a pair of RRS1B and RPS4B in RLD-0 and Cvi-0 was not required for resistance to *C. higginsianum*.

In conclusion, our data indicated that the amino acid change, Y950H, in RPS4 resulted in the loss of both the RPS4 and RRS1 functions and resistance to pathogens. Further experiments will be required to address the evolutionary genetic and functional studies in RPS4 and RRS1 pair using Cvi-0 as an important tool.

Material and methods

Plant materials and growth

Arabidopsis ecotypes Bay-0, Br-0, Bur-0, C24, Cvi-0, Est-1, Fei-0, L*er*-1, Lov-5, Nfa-8, Rrs-7, Rrs-10, Sha, Tamm-2, Ts-1, Tsu-1, and Van-0 were obtained from the *Arabidopsis* Biological Resource Center (ABRC; USA). Col-0, L*er*-0, RLD-0, and Ws-2 were obtained from the RIKEN BRC, Japan. The *rps4-21* mutant has been described previously.[6] *Arabidopsis* plants were grown in soil mix (Sakata Seed Corp.) and expanded vermiculite (2–5 mm granules) at a 1:1 ratio for 28 days in a growth chamber at 22°C under a 12-h light/12-h dark cycle.

Genome sequence

Genome sequence data sets for *Arabidopsis thaliana* accession Ws-2 (SRR492407) and Cvi-0 (SRR492239) were downloaded from the sequence read archive of DDBJ (DNA Data Bank of

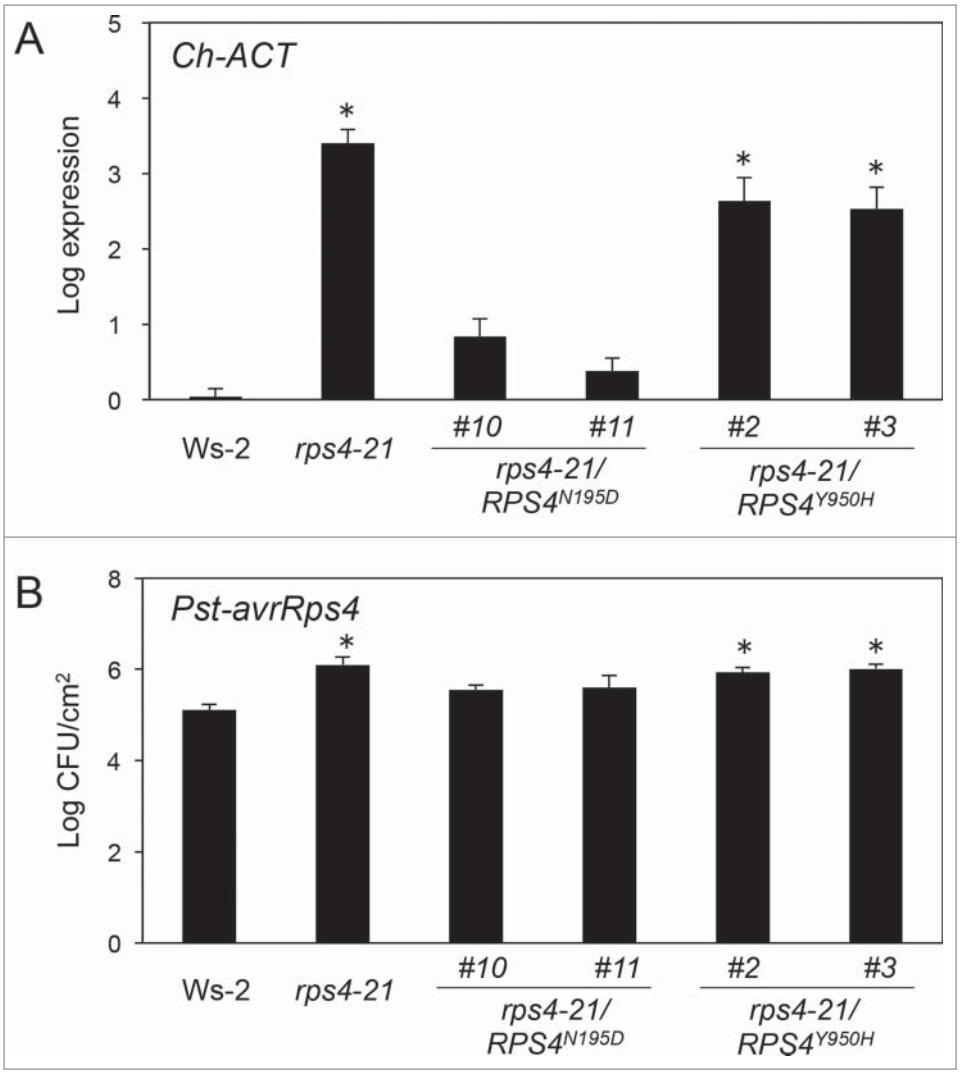

Figure 6. Complementation of mutated RPS4 (N195D or Y950H) to *Arabidopsis rps4-21* mutants. The *rps4-21/RPS4-Ws*^{N195D} *-#10*, and *-#11*, and *rps4-21/RPS4-Ws*^{Y950H} *-#2*, and *-#3* lines represent independent transgenic *rps4-21* plants harboring the genomic *RPS4-Ws*^{N195D} and *RPS4-Ws*^{Y950H} fragments, respectively. (A) Quantification of *C. higginsianum in planta* by qRT-PCR. Twenty eight-day-old plants were spray-inoculated with *C. higginsianum*. The inoculated leaves were harvested at 5 dpi and total RNA was isolated. QRT-PCR was performed with *Ch-ACT* primers for each sample. (B) Quantification of *Pst-avrRps4 in planta*. The leaves of 5-week-old plants were infiltrated with bacterial suspensions (5×10^4 cfu ml^{-1}). The leaves were harvested at 3 days after inoculation. Bacterial growth (cfu cm^{-2}) was assessed using five leaf disks by cell counting. Bars indicate SE. The asterisks indicate statistical significance from the Ws-2 WT controls (Dunnett's method,[28] $P < 0.01$). This experiment was repeated at least two times with similar results.

Japan, https://trace.ddbj.nig.ac.jp/index_e.htm). These sequence data, originally open to public from the 1,001 *Arabidopsis* Genomes project, were contributed by the Salk Institute (http://1001genomes.org/, http://signal.salk.edu/atg1001/accessions.php). The reference genome sequence and gene annotation data of *A. thaliana* (TAIR10) were obtained using the download function at the sequence analysis software, CLC genomic workbench (QIAGEN Bioinformatics). These genome sequence data from Ws-2 and Cvi-0 were independently mapped to the reference genome sequence of *Arabidopsis thaliana* by the CLC genomic workbench.

Pst-avrRps4 infections

Arabidopsis plants were inoculated as described previously.[6] The quantification of *Pst-avrRps4* was performed as described previously.[6]

C. higginsianum inoculation

C. higginsianum Saccardo isolates (MAFF305635) were obtained from the Ministry of Agriculture, Forestry and Fisheries (MAFF) Genebank, Japan. The *Arabidopsis* plants were inoculated as described previously,[6,25] and harvested at 5 dpi for qRT-PCR analysis. The quantification of *C. higginsianum* was performed as described previously.[25] Fungal hyphae within the resulting lesions and dead cells were stained with lactophenol-trypan blue, as described previously.[26]

Construction of the R-gene plasmid

All the DNA fragments containing *RRS1* and/or *RPS4* used in this study were derived from the genome of *A. thaliana* Ws-2 accession. The plasmids used in this study have been described in a previous study.[6] All the clones were verified by DNA

sequencing. The 6.3-kbp genomic *RPS4* fragment, including approximately 2.1-kbp upstream and 109-bp downstream regions, was cloned into pBI101-SK$^+$.[6] Site-directed mutagenesis of genomic *RPS4* was performed by a custom cloning service (Takara Bio Inc.) to generate *RPS4-Ws*^{N195D} and *RPS4-Ws*^{Y950H}, carrying Asn to Asp at 195 a.a. and Tyr to His at 950 a.a. mutations, respectively.

Arabidopsis transformation

Arabidopsis transformation was carried out by the floral inoculation method using *Agrobacterium tumefaciens* strain GV3101 (pMP90).[27] T3 homozygous lines were used for further analysis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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