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

Mari Narusaka, Satoshi Iuchi, and Yoshihiro Narusaka

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.

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

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.3,17 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/RRS1, and 3) specific amino acid polymorphisms in RPS4 play a role in the RPS4/RRS1 complex.
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 × 10⁴ 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, Ler-0, Ler-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 with 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 could not be assembled against the corresponding region of Col-0 reference sequence. Although Clark et al. 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. 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 are paralogous and functionally similar to RPS4/RRS1 and RPS4-WsN195D, RPS4-WsY950H, respectively. Subsequently, these clones were complemented into rps4-21 mutants (Ws-2 background). To determine whether N195 and Y950 are required for resistance to C. higginsianum, fungal infection levels were monitored in 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-08,17. Therefore, the mutated RPS4-Ws clones (Ws-2 background) with the amino acid changes, N195D and Y950H, were designated as RPS4-WsN195D and RPS4-WsY950H, respectively. Subsequently, these clones were complemented into rps4-21 mutants (Ws-2 background).6

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 with 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
Ws-2 and in the mutants, 5 days post-inoculation (Fig. 6A). The RPS4-WsY950H complemented rps4-21 plants were susceptible to C. higginsianum. On the contrary, RPS4-WsN195D 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-WsN195D or RPS4-WsY950H 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. 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.
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 non-functionality 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*.\(^6,22\) 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. Y950H 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, Ler-1, Lov-5, Nfa-8, Rrs-7, Sla, Tamm-2, Ts-1, Tsu-1, and Van-0 were obtained from the *Arabidopsis* Biological Resource Center (ABRC; USA). Col-0, Ler-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 Japan).
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 Arabidopsis 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. The quantification of Pst-avrRps4 was performed as described previously.

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, and harvested at 5 dpi for qRT-PCR analysis. The quantification of C. higginsianum was performed as described previously. Fungal hyphae within the resulting lesions and dead cells were stained with lactophenol-trypan blue, as described previously.

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. 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 pBlI101-SK’. Site-directed mutagenesis of genomic RPS4 was performed by a custom cloning service (Takara Bio Inc.) to generate RPS4-WsN195D and RPS4-WsY950H, 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 (pMJP90). 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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