Plant Signaling & Behavior 9, e29130; May; © 2014 Landes Bioscience

Arabidopsis dual resistance proteins, both RPS4 and RRS1, are required for resistance to bacterial wilt in transgenic *Brassica* crops

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Keywords: *R* gene, RPS4, RRS1, *Ralstonia solanacearum*, Brassicaceae

Bacterial wilt phytopathogen *Ralstonia solanacearum* is a serious soil-borne disease that attacks several economically important plants worldwide, including Brassicaceae. Previous studies indicate that recognition of avirulence (Avr)-effector PopP2 by resistance (R) protein, RRS1-R, and physical interaction between RRS1-R and PopP2 in the nucleus are required for resistance. Of late, we showed that a pair of *Arabidopsis thaliana* TIR-NLR proteins, RRS1 and RPS4, function together in disease resistance against multiple pathogen isolates. Here, we report that dual R proteins, RRS1 and RPS4, from *A. thaliana* accession Wassilewskija confer resistance to bacterial wilt in transgenic *Brassica* crops. For practical applications, this finding may provide a new strategy for developing disease resistant plants that express *R* genes from other plants.

Plant diseases cause serious agricultural losses worldwide. Breeding disease resistant crops help increase the world food supply. Among the various plant disease control strategies, the introgression of disease resistance (*R*) genes has been used in traditional plant breeding for decades. R proteins mostly carry a C-terminal leucine-rich repeats and a central the nucleotide-binding adaptor shared by Apaf-1, Resistance proteins, and CED-4 domain (NLR).[1] R proteins detect avirulence (Avr) proteins secreted by the pathogen and confer resistance to various pathogens. In addition, the hypersensitive reaction mediated by the R protein is the most powerful defense system in plants. Therefore, cloning of *R* genes from a variety of crops or their wild relatives and transferring them into susceptible cultivars is being attempted.[2] However, the transfer of NLR type *R* genes into crops is limited because the *R* genes often fail to function, i.e., either no responses or inappropriate auto-immune responses are obtained, when they are transferred between different plant families or even between related species in the same family. For example, a single NLR-type R protein, Mi-1.2, in tomato confers resistance to the following 3 different pathogens: root-knot nematodes, potato aphid, and sweet potato whitefly.[3] Goggin et al.[3] indicated that Mi-1.2-mediated resistance could not completely function in a heterologous background. Therefore, this suggests that the signaling components in NLR-type R protein-mediated defense system are highly conserved in family or genus.[4]

Of late, it was reported that some pairs of *R* genes, such as *RPP2A*/*RPP2B*, *N*/*NRG1*, *RPM1*/*TAO1*, *Pikm1-TS*/*Pikm2-TS*, *Lr10*/*RGA2*, and *Pi5–1*/*Pi5–2*,[5-11] may function in concert. Previously, we also demonstrated that a pair of Toll/interleukin-1 receptor (TIR)-NLR genes, *RRS1* and *RPS4,* localize in *Arabidopsis thaliana* accession Wassilewskija Chromosome V (**Fig. 1**), and function together in disease resistance against 3 different pathogens: anthracnose (*Colletotrichum higginsianum*), bacterial speck (*Pseudomonas syringae* pv. *tomato* strain DC3000 expressing *avrRps4*), and bacterial wilt (*Ralstonia solanacearum*).[12,13] Thus, both R proteins, RRS1 and RPS4, are required for effector-triggered immunity in *A. thaliana*.

The bacterial wilt caused by *R. solanacearum* is an serious soilborne disease affecting important agricultural crops. *R. solanacearum* infects more than 200 plant species belonging to 50 plant families, including Brassicaceae.[14,15] Previous studies have shown that RRS1-R in *A. thaliana* accession Nd-1 recognized Avr-protein secreted by *R. solanacearum* and conferred resistance to the pathogen.[16,17] Linden et al.[18] suggested that RRS1, but not RPS4, in *A. thaliana* Kil-0 is the key factor of a tolerant phenotype against *R. solanacearum* BCCF402. However, the bacterial wilt resistance mechanism is poorly understood at the molecular level.

The primary aim of present study was to clarify whether a pair of NLR-type R proteins, RRS1 and RPS4, from *A. thaliana* confer resistance to bacterial wilt in a heterologous background,

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Submitted: 04/16/2014; Revised: 05/06/2014; Accepted: 05/06/2014; Published Online: 05/15/2014
Citation: Narusaka M, Hatakeyama K, Shirasu K, Narusaka Y. Arabidopsis dual resistance proteins, both RPS4 and RRS1, are required for resistance to bacterial wilt in transgenic Brassica crops. Plant Signaling & Behavior 2014; 9:e29130; PMID: 24832367; http://dx.doi.org/10.4161/psb.29130

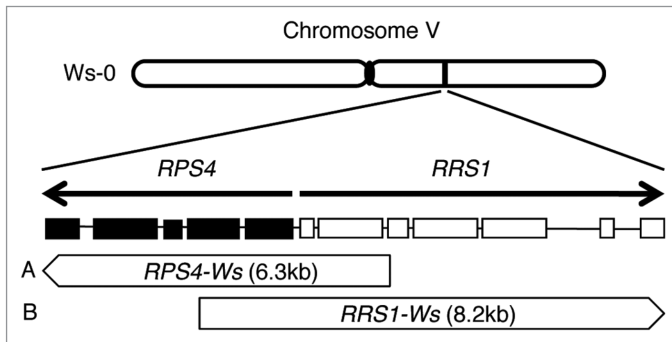


Figure 1. Construction of the *R* gene plasmid. *RPS4* and *RRS1* genes are arranged in a head-to-head configuration in *Arabidopsis thaliana* accession Wassilewskija (Ws-0) chromosome V. The genomic fragments containing *RPS4* or *RRS1* were amplified from Ws-0 genomic DNA using PCR. (**A**) The 6.3 kbp genomic *RPS4* fragment, including approximately 2.1 kbp upstream and 109 bp downstream regions, was cloned into pBI101-SK+.[12] (**B**) The 8.2 kbp genomic *RRS1* fragment, including approximately 1.8 kbp upstream and 176 bp downstream regions, was subcloned into the destination vector pGWB1 using the LR cloning reaction.[12] *Brassica rapa* var. *perviridis* (Japanese Mustard Spinach, Komatsuna cv. Osome, Takii and Co. Ltd, Kyoto, Japan) was transformed by inoculating hypocotyl sections with *Agrobacterium tumefaciens* (*Rhizobium radiobactor*) strain EHA101, harboring the binary vector with the fragments, i.e., both (**A**) and (**B**) (*RPS4* + *RRS1*), either (**A**) (*RPS4*) or (**B**) (*RRS1*) alone, as described above.[26]

i.e., *Brassica* crops, to facilitate molecular breeding using the *Arabidopsis* dual *R* gene. Therefore, we transferred genomic fragments of *Arabidopsis RRS1* and *RPS4* under the control of their native promoters into *Brassica rapa* plants, which belong to the same family (Brassicaceae). Furthermore, we also determined whether both RRS1 and RPS4 are essentially required for resistance to bacterial wilt in *Brassica* crops, i.e., *Brassica rapa*.

To determine whether *Arabidopsis* RRS1 and RPS4 function as a pair in Brassicaceae plants other than *A. thaliana*, we transformed *Arabidopsis RRS1* and *RPS4* genes into *Brassica rapa* var. *perviridis* (Japanese Mustard Spinach, Komatsuna). The transgenes, *RRS1* and *RPS4*, were then segregated in a 1:1 ratio in the T3 generation. We also generated *B. rapa* transformants that expressed only *RRS1* or *RPS4* genes. Under the normal condition, these transformants grew normally and did not constitutively induce the expression of defense-related marker gene *Br-PR1*. This suggests that a inappropriate auto-immune responses was not induced by transformation with both *RRS1* and *RPS4* genes (**Fig. 2**).

To analyze whether *RRS1* and *RPS4* could provide resistance to *R. solanacearum* strain 1002 (*Rs1002*), which produces the PopP2 effector that can be recognized by RRS1,[16] we performed a root inoculation assay with the pathogen. Wilting was observed 3 to 4 d after the inoculation (dai) in the wild type (WT), followed by complete wilting within 7 to 10 dai (**Fig. 3A**). In contrast, the transgenic *B. rapa* plants that expressed *RRS1* and *RPS4* were healthy after inoculation with the pathogen. The bacterial growth in the WT was approximately 10-fold higher than that in the dual *R* gene-transformed plants (**Fig. 3B**). To assess whether RRS1 and RPS4 function in concert, the *B. rapa* transformants that expressed only *RRS1* or *RPS4* were inoculated with *Rs1002*. As shown in **Figure 3B**, there are no significant difference in bacterial growth between the WT and the single *R* gene transformants. Therefore, these results show that both RRS1 and RPS4 are required for resistance to *Rs1002* and function cooperatively. *Rs1002* contains an *Avr*-gene *popP2*, which belongs to a YopJ-like family of effectors.[16] To determine whether PopP2 plays an important role in the interaction with RRS1, we used an *Rs1002-ΔpopP2* where *popP2* homolog was disrupted.[12] On the root inoculation assay with *Rs1002-ΔpopP2,* the level of susceptibility in the dual *R* gene transformed plants was similar to that in the WT (**Fig. 3C**). These results indicate that PopP2 specifically elicits RRS1/RPS4-mediated resistance in the dual *R* gene-transformed *B. rapa* plants.

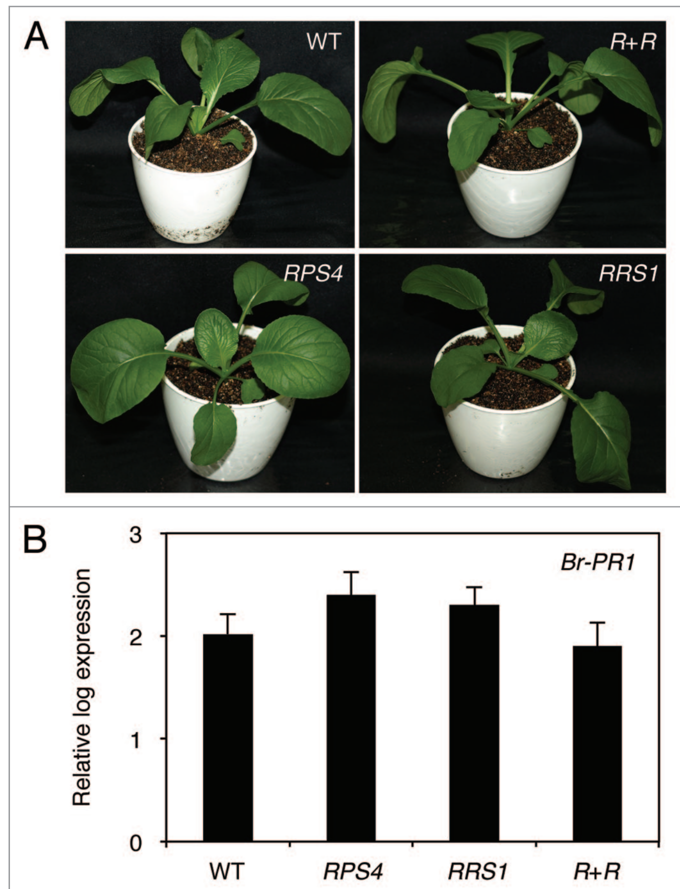
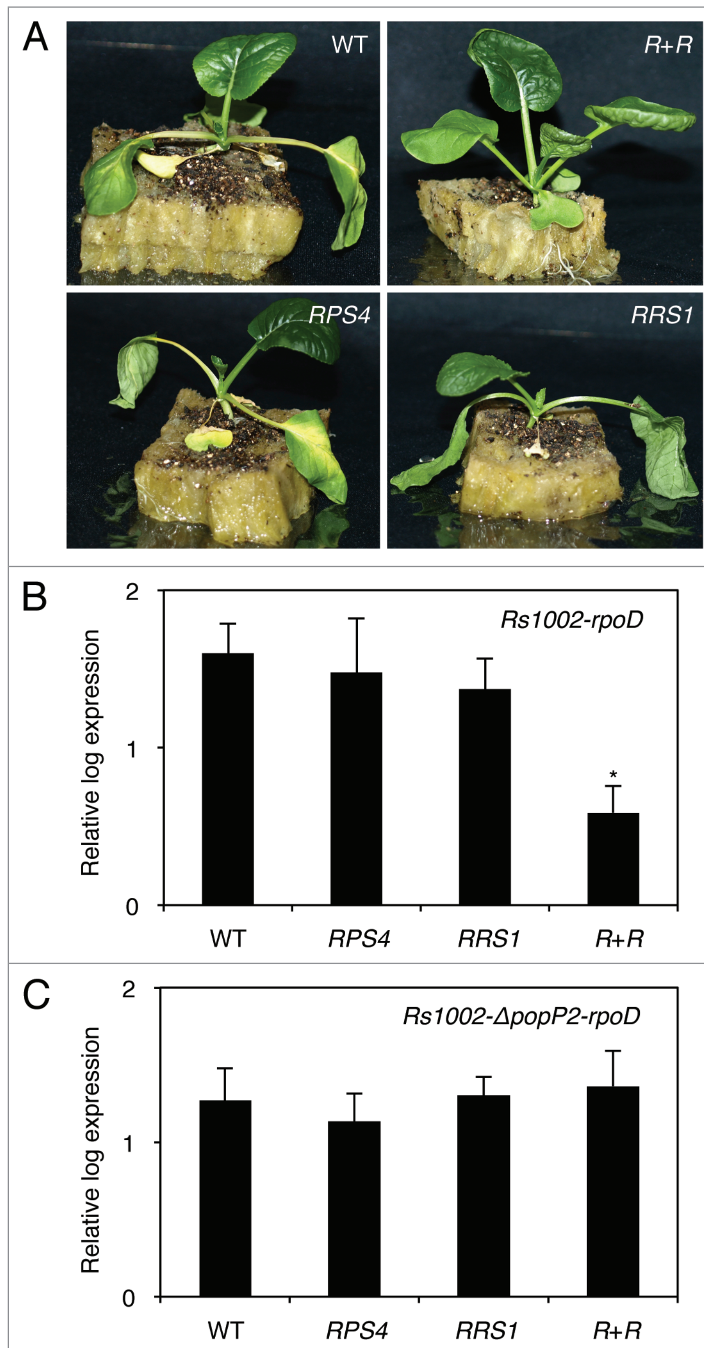


Figure 2. Growth and expression of defense-related genes (*Br-PR1*) in transgenic *Brassica rapa* plants under normal growth condition. (**A**) Each image shows 25-d-old T3 transgenic *B. rapa* that carried both *RPS4* and *RRS1* (*R* + *R*), *RPS4* alone, *RRS1* alone, and wild type control plants (WT). (**B**) Five leaf disks were cut from the leaves of WT and transgenic *B. rapa* plants, and total RNA was isolated for qRT-PCR analysis. Expression level of *Br-CBP20* in *B. rapa* was used for normalization.[27] *Br-PR1* expression is shown relative values.[27] Bars indicate SE. The data indicate no significant differences compared with the wild type controls (Dunnett's method, $P < 0.05$).[28] The experiment was repeated twice with similar results.

Figure 3. *Ralstonia solanacearum* resistance analysis in *RPS4* and *RRS1* dual *R* gene-transformed *Brassica rapa*. *R. solanacearum* strains 1002 (*Rs1002*) and 1002-*ΔpopP2* (*Rs1002-ΔpopP2*) were described previously.[12,29] T3 transgenic *B. rapa* lines that carried both *RPS4* and *RRS1* (*R* + *R*), either *RPS4* or *RRS1* alone, and wild type control plants (WT) were tested. *B. rapa* plants were grown in rock wool for 17 d (12 h light cycle) at 22 °C. Root inoculations were performed by cutting approximately 2 cm from the bottom of the rock wool, and the exposed roots of the plants were immersed in a bacterial suspension at a concentration of 5×10^8 colony-forming units (cfu) ml^{-1} for 24 h. The plants were then transferred to a growth chamber at 25 °C (12 h light cycle). (**A**) Infection phenotypes of plants inoculated with *Rs1002*. Images were taken 4 d after inoculation. Each image shows a representative example from 3 independent experiments. Quantification of *Rs1002* (**B**) and *Rs1002-ΔpopP2* (**C**) in planta was performed using qRT-PCR, as described by Narusaka et al.[30] Inoculated plants were harvested at 4 dpi and total RNA was isolated. qRT-PCR data for *R. solanacearum rpoD* (*Rs-rpoD*) and *Br-CBP20* expression from *B. rapa* were obtained from a standard curve of cycle times as a function of copy number. Abundance of *Rs-rpoD* was normalized with *Br-CBP20* in infected samples.[27,30] Bars indicate standard error (SE). Asterisks indicate significant differences compared with the wild type controls (Dunnett's method, $P < 0.05$).[28] This experiment was repeated at least twice with similar results.



In plant biology, *A. thaliana* is the most popular and important model species in the Brassicaceae (mustards or crucifers) family. *A. thaliana* is an excellent tool for the research into genetics, molecular basis of disease resistance, and molecular breeding of *Brassica* crops. *A. thaliana* is also an important model plant for the investigation of plant immune systems.[19] The reference *Arabidopsis* accession Col-0 genome sequence has been used to generate a list of defense-related genes against pathogens and subsequently identified 149 NLR type *R* genes.[20] If these *R* genes can function in a heterologous background, they will be useful for breeding disease resistant plants. Despite the discovery of *R* genes against *Brassica* crop pathogens, the direct use of *Arabidopsis R* genes has not progressed in other Brassicaceae plants.[21-25]

The present study showed that the NLR type R proteins, RRS1 and RPS4, from *A. thaliana* functioned as a pair in another Brassicaceae species. According to the guard hypothesis, the dual R protein pair, RRS1 and RPS4, may function as a guard–guardee pair where the R protein can interact with signaling components downstream of R protein in a heterologous background. If the functional expression of the *R* gene pair is required for *R* gene-mediated resistance in a heterologous background, each partner of the previously reported *R* genes will soon be discovered and *R* genes could be exploited in molecular breeding to enhance resistance to crop diseases. This suggests that all the necessary components of *R* gene-mediated signaling must be highly conserved among plant species. This solution will increase the range of potential resistance resources. For practical applications, this novel finding will provide strategies for development of disease-resistant transgenic plants and novel approaches for plant genome manipulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the science and technology research promotion program for agriculture, forestry, fisheries and food industry to K.S. and Y.N. and by a Grant-in-Aid for Scientific Research (KAKENHI) (25450523 to M.N.). We thank Mses. Yasuyo Katayama, Shoko Miyashita, Yukiko Kurosaki, Chiaki Watanabe, and Masami Miyamoto for their excellent technical assistance, and Tsuyoshi Nakagawa (Shimane University) for kindly providing pGWB1.

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