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Rpi-blb2-mediated late blight resistance in *Nicotiana benthamiana* requires *SGT1* and salicylic acid-mediated signaling but not *RAR1* or *HSP90*

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ABSTRACT

The Rpi-blb2 recognizes the presence of the *Phytophthora infestans* AVRblb2 and initiates effector-triggered immunity (ETI). We performed gain-of-function and loss-of-function studies in *Nicotiana benthamiana* to elucidate *Rpi-blb2*-mediated resistance to *P. infestans*. *Rpi-blb2* triggered a hyper-sensitive response through *SGT1*-mediated, but not *RAR*-mediated or *HSP90*-mediated, pathways. *NbSGT1* was also required for basal and ETI-mediated by Rpi-blb2 in *N. benthamiana*. Moreover, salicylic acid (SA) affected basal defense and *Rpi-blb2*-mediated resistance against *P. infestans*. The increased susceptibility of *Rpi-blb2*-transgenic plants in the *NahG*-background correlated with reduced levels of SA. These findings provide evidence for the roles of SGT1- and SA-signaling in *Rpi-blb2*-mediated resistance against *P. infestans*.

1. Introduction

Phytophthora infestans, the cause of one of the most destructive diseases in *Solanum* species, delivers to host cells effectors that interact with target proteins to reprogram host defenses [1]. Aviru-lence (Avr) effectors, such as RXLR proteins, can function in innate immunity (effector-triggered immunity; ETI) in plant varieties that carry the cognate disease-resistance (*R*) genes [2,3]. Many late blight *R* genes were introgressed into potato cultivars from the wild species *Solanum demissum* and *Solanum bulbocastanum*. Some genes, including *R3a*, *Rpi-blb1*, and *Rpi-blb2*, were cloned from wild potato cultivars [3]. Perception mechanisms based on *R*-gene prod-ucts that recognize cognate pathogen Avr proteins and a number of *R-Avr* gene interactions have been characterized [4]. Avr protein activity is recognized by intracellular R proteins in plant cells, culminating in defense signaling and pathogen resistance [4]. Most R-proteins have nucleotide-binding and leucine-rich-repeats (NB-LRRs), which recognize Avr effectors and activate a hypersen-sitive response (HR) to infection [4].

The gene encoding Rpi-blb2, which has a coiled-coil (CC)-do-main in its N-terminus region, recognizes *P. infestans* AVRblb2 [5,6]. AVRblb2 is expected to have Avr activity that directly or indi-rectly activates the HR and associated disease-resistance responses mediated by *Rpi-blb2* [7]. The HR includes both a local increase and a systemic increase in endogenous salicylic acid (SA) levels, the generation of reactive oxygen species (ROS), and the up-regulation of a large set of defense genes [8].

SA-mediated defense responses play an important role in the *R*-gene-mediated resistance leading to the expression of defense-related genes, which are presumed to contribute to resistance [9]. Moreover, SA is required for basal defense and systemic ac-quired resistance (SAR), which confers resistance against a broad spectrum of pathogens [8,10]. SAR is associated with the expres-sion of pathogenesis-related proteins and is accompanied by several defense responses [10]. Basal defense responses are activated in susceptible plants by pretreatments with pathogen-derived molecules or SA [10], but they are not sufficient to arrest pathogen growth. The *NahG* gene encodes a salicylate hydroxylase that catalyzes the conversion of SA to catechol and prevents SA

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accumulation when *NahG*-transgenic plants are challenged by pathogens [11,12]. Likewise, SA is required for the function of many *R*-genes.

Knockdown analyses led to the identification of signaling components downstream of the R proteins [13]. The enhanced disease susceptibility-1 protein (EDS1) is a central regulator of basal resistance and the ETI mediated by Toll-interleukin-1 (TIR)-NB-LRR proteins [14]. In contrast, CC-NB-LRR proteins require SGT1 (a suppressor of the G2 allele of *skp1*), RAR1 (required for Mla12 resistance), and HSP90 [15]. Among those, the ubiquitin ligase-associated protein SGT1 is essential for the function of several *R* genes. In *N. benthamiana*, *SGT1* homologues are indispensable for *Rx*-dependent resistance to Potato virus-X [16] and for *R3a*-dependent resistance to *P. infestans* strains carrying *avr3a*[EM] [17]. The silencing of *SGT1* in potato plants interferes with the *RB* gene required for *RB*-mediated broad-spectrum resistance to potato late blight [18]. In addition, SGT1 is required for the resistance conferred by some *R* genes that depend on RAR1 and HSP90 [19,20]. The knockdown of *NbHSP90* resulted in the loss of *R3a*-mediated resistance to *P. infestans* in *N. benthamiana* [17]. There is no information available, however, about *Rpi-blb2*-mediated resistance pathways.

To elucidate the signaling pathway mediated by *Rpi-blb2*, we examined the molecular and physiological functions of *Rpi-blb2* in *N. benthamiana*, a Solanaceous model plant that is well established for the functional analysis of *P. infestans* infections using overexpression- or VIGS-technology. Here, we demonstrate the requirement for *SGT1*- and the SA-signaling pathway, but not for *RAR1* or *HSP90*, in eliciting *Rpi-blb2*-mediated resistance against *P. infestans* and its effector *AVRblb2*.

2. Materials and methods

2.1. Bacteria and plants

Escherichia coli and *Agrobacterium tumefaciens* were grown in LB media with appropriate antibiotics at 37 °C and 28 °C, respectively. *P. infestans* 88 069 was cultured on rye agar medium at 20 °C. Transgenic *N. benthamiana* plants were grown in a growth chamber and maintained under a 16-h photoperiod at 22–25 °C.

2.2. RT-PCR analysis

Total RNA was extracted from *N. benthamiana* plants using TRI reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RT-PCR was performed to detect the transcript levels of several genes using the primer sets listed in Supplemental Table S1. The expression of *PiEF2α* was controlled with a primer pair specific for the constitutively expressed *NbActin* gene [21].

2.3. In planta HR assays

For agroinfiltration assays, recombinant *A. tumefaciens* was grown as described previously [6]. Agrobacterium solutions were infiltrated at an adjusted OD$_{600}$ of 0.4. For HR assays, *Agrobacterium* expressing the pGR106-*AVRblb2* or control (pGR106-d*GFP*) were infiltrated with a final OD600 of 0.3 into ten-transgenic *N. benthamiana* plants. Five plants were inoculated with the pGR106-*AVRblb2*, and five plants were inoculated with the control. The assays were repeated at least three times with similar results (20 inoculation sites per five plants) [6].

2.4. Evaluation of disease resistance

Four-week-old homozygous *N. benthamiana* plants were inoculated with agar plugs containing *P. infestans* or with *P. infestans* zoospores (5×10^5 zoospores/mL) [5]. Transgenic plants were

infected, and disease symptoms appeared within 4–6 days after infection (dai). The index of *Phytophthora* infection was determined visually based on necrotic leaf areas. All experiments were repeated at least twice [21].

2.5. VIGS assay

For the VIGS assay, cultures containing *Agrobacterium* with a TRV-derivative vector (TRV2-d*GFP*, TRV-*NbSGT1*, TRV-*NbHSP90*, or TRV-*NbRAR1* constructs; provided by the Dr. Dinesh-Kumar Lab.) and *Agrobacterium* GV3101 (*TRV1*) were transferred into LB media with antibiotics (50 mg/L kanamycin and 25 mg/L rifampicin) and grown at 28 °C to an A$_{600}$ of 0.8. The cultures containing *Agrobacterium* (with the TRV2-d*GFP* and TRV2-*NbSGT1* constructs) were mixed at a 1:1 ratio (OD$_{600}$ = 0.3) with GV3101 (TRV1) and infiltrated into the expanded leaves of 4-week-old wild-type (WT) and transgenic *N. benthamiana* plants, respectively. On day 21 after infiltration, transient expression of pGR106-d*GFP* or pGR106-*AVRblb2* was induced by mixed infiltration of appropriate *A. tumefaciens* strains carrying each individual gene in an induction buffer at a ratio of 1:1 (final OD$_{600}$ = 0.6) [6]. For RT-PCR analysis, total RNA was extracted from control (d*GFP*), *SGT1*-silenced, *HSP90*-silenced, and *RAR1*-silenced *N. benthamiana* leaves using TRIZOL solution (Invitrogen, Carlsbad, CA). Specific primers were used to amplify several genes annealed outside the VIGS target region (Table S1).

2.6. Measurement of ion leakage

Ion leakage was measured as described by Oh et al. [21]. After pathogen inoculation, leaf discs (1-cm diameter) were incubated in 5 ml distilled water for 2 h at room temperature. The electrolyte leakage from the leaf discs was measured using a conductivity meter (model 455C; Istek, http://www.istek.co.kr/).

2.7. Trypan-blue staining

Trypan-blue staining (TBS) was performed to detect dead *N. benthamiana* cells and *P. infestans* mycelia [21]. A trypan-blue stock solution (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water, and 0.02 g trypan blue) was prepared, and a working solution was then prepared by diluting the stock solution with ethanol (96%, 1:2 [v/v]). Infected *N. benthamiana* leaf discs (18-mm diameter) were transferred into a lidded 50-ml Falcon tube and covered with diluted trypan-blue solution. The Falcon tubes were then placed in a heated water bath, and the staining solution was boiled for 2 min. The leaf discs were left overnight in the TBS solution and destained overnight in a chloral hydrate solution (2.5 g ml^{-1}) [21].

2.8. DAB staining for H$_2$O$_2$ detection

To detect plant cell death and H$_2$O$_2$ levels after the appropriate assays were performed, leaves were stained with 1 mg ml^{-1} 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO), as described previously [21]. DAB staining intensity indicating H$_2$O$_2$ levels was analyzed in arbitrary units with ImageJ 1.42q (http://rsb.info.nih.gov/ij/) [21].

2.9. SA and SAG determinations

SA and SA glycoside were extracted from *N. benthamiana* leaves (0.5 g tissue) and separated and quantified by HPLC, as described previously [21]. Briefly, SA and SAG concentrations were expressed in µg g^{-1} fresh weight. As an internal standard for SA, 50 mg 3,4-dihydroxybenzoic acid (3,4-DHBA, Sigma, St. Louis, MO) was added per gram of leaf fresh weight. SA was quantified by fluorescence