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Enhanced disease resistance against *Botrytis cinerea* by strigolactone-mediated immune priming in *Arabidopsis thaliana*

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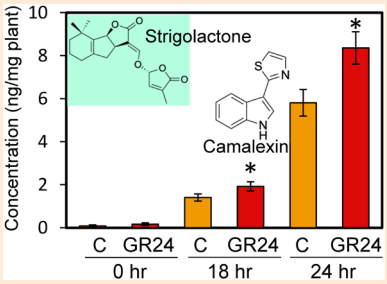
Supplementary material

Strigolactones (SLs) are a class of plant hormones that play several roles in plants, such as suppressing shoot branching and promoting arbuscular mycorrhizal symbiosis. The positive regulation of plant disease resistance by SLs has recently been demonstrated by analyses using SL-related mutants. In Arabidopsis, SL-mediated signaling has been reported to modulate salicylic acid-mediated disease resistance, in which the priming of plant immunity plays an important role. In this study, we analyzed the effect of the synthetic SL analogue *rac*-GR24 on resistance against necrotrophic pathogen *Botrytis cinerea*. In *rac*-GR24-treated plants, disease resistance against *B. cinerea* was enhanced in an ethylene- and camalexin-dependent manners. Expression of the ethylene-related genes and the camalexin biosynthetic gene and camalexin accumulation after pathogen infection were enhanced by immune priming in *rac*-GR24-treated plants. These suggest that SL-mediated immune priming is effective for many types of resistance mechanisms in plant self-defense systems.



Keywords: priming, disease resistance, strigolactone, camalexin, *Botrytis cinerea*.

Introduction

Control of various diseases is necessary for stable food production, but in order to establish a sustainable agricultural production system, it is desirable to utilize not only antimicrobial agents but also plant immunity. Among plant self-defense mechanisms against pathogens, the utilization of systemically induced disease resistance will help achieve such goals. Systemically induced resistances activated by various types of stimuli are able to protect the plant for long periods from a broad range of attackers. The mechanisms of these systemic resistance are governed by defense-related plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET).[1–3] In general, SA-mediated defense responses are effective against biotrophic pathogens, while JA-mediated defense signaling, in concert with ET-mediated signaling, plays an important role in resistance against necrotrophic pathogens. Systemic acquired resistance (SAR), induced through SA-mediated signaling after pathogenic infection, is a relatively strong resistance against pathogenic attacks; therefore, it has been practically used in the rice paddy field by exploiting plant activators that activate SA-mediated signaling.[4–8] They are probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide),[9,10] tiadinil (5-(3-Chloro-4-methylanilinocarbonyl)-4-methyl-1,2,3-thiadiazole),[11] isotianil (3,4-dichloro-2′-cyano-1,2-thiazole-5-carboxanilide),[12] and acibenzolar-*S*-methyl (BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester),[13] some of which are characterized and used also for investigation of plant immune systems.[14–16] They are widely used in rice, but are difficult to use in other crops, including vegetables, because of the trade-off between SAR induction and growth.[17]

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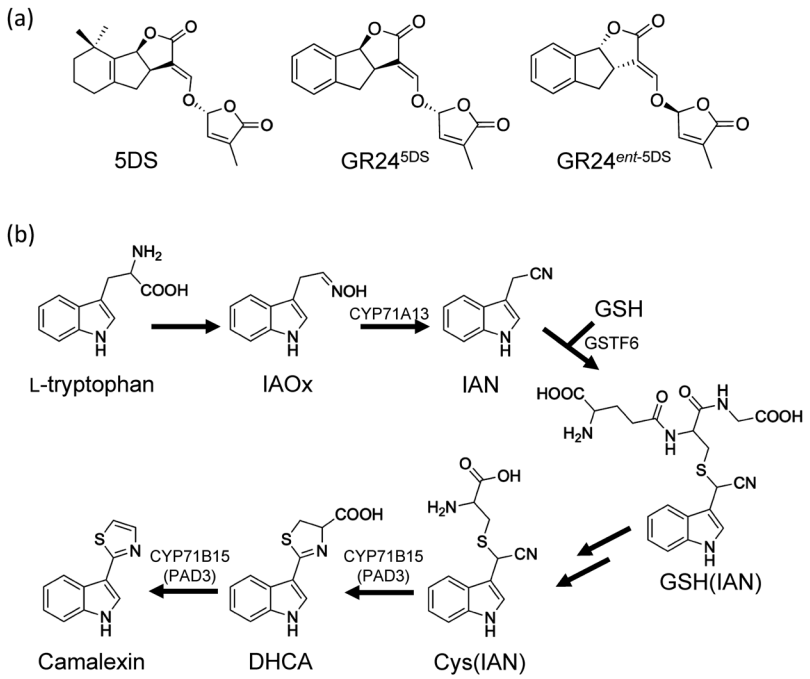

Fig. 1. Structure of chemicals. (a) Strigolactone and its analogues. 5DS, 5-deoxystrigol; *rac*-GR24, 1 : 1 mixture of GR24^{5DS} and GR24$^{ent\text{-}5DS}$. (b) Camalexin biosynthetic pathway. IAOx, indole-3-acetaldoxime; IAN, indole-3-acetonitrile; GSH(IAN), glutathione-indole-3-acetonitrile; Cys(IAN), cysteine-indole-3-acetonitrile; DHCA, dihydrocamalexic acid; GSH, glutathione; GSTF6, glutathione-*S*-transferase 6; PAD3, phytoalexin deficient 3; CYP71A13 and CYP71B15, cytochrome P450 enzymes.

On the other hand, priming of the plant immune system has the protecting effects against pathogens without inhibiting plant growth.[18] In the primed plants, major defense-related signals such as SA- or JA-mediated signals are not activated before pathogen infection but are activated more rapidly and strongly upon pathogen infection. In nature, priming is known to be induced in host plants by symbiotic relationships with certain microorganisms, such as the arbuscular mycorrhizal fungi *Rhizophagus irregularis*,[19,20] *Funneliformis mosseae*,[21] and *Gigaspore margarita*,[22] the endophytic bacterium *Azospirillum* sp. B510,[23,24] and the non-pathogenic rhizobacteria *Pseudomonas simiae* WCS417r,[25,26] *Bradyrhizobium* sp. ORS278,[27] and *Pseudomonas aeruginosa* 7NSK2.[28] Priming has been also reported to be activated by treatment with chemicals, such as a synthetic strigolactone (SL) *rac*-GR24 (Fig. 1a),[29] β-aminobutyric acid (BABA),[30] (*R*)-β-homoserine (RBH),[31] and green leaf volatiles (GLVs).[32]

SLs (Fig. 1a) are a class of plant hormones that play important roles in developmental processes, such as the regulation of branching,[33] and also functions in interactions with different species, such as promoting symbiosis with mycorrhizal fungi and germination of parasitic weeds.[34,35] Analyses using SL biosynthetic mutants of several plant species have recently shown that SL is required for disease resistance. Rice *d17* mutant and Arabidopsis mutants *max1*, *max3* and *max4* are more susceptible to hemibiotrophic pathogens *Pyricularia oryzae* (synonym *Magnaporthe oryzae*) and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*),[36,37] respectively. Similarly, the tomato *ccd8* mutant is more susceptible to the necrotrophic fungi *Botrytis cinerea* and *Alternaria alternata*.[38] As a mechanism by which SL enhances disease resistance, we have previously reported that SL signaling modulates the activation level of SA signaling in Arabidopsis; treatment with the SL analogue *rac*-GR24 promotes activation of SA signaling after infection with the bacterial pathogen *Pst* and enhances resistance, while suppression of SL signaling by the SL biosynthesis inhibitor TIS108 reduces resistance to *Pst* infection.[29] Priming of plant immunity by SL signaling has been shown to be effective in enhancing disease resistance *via* SA signaling, but its effects on other defense-related signals remain unknown.

B. cinerea is a necrotrophic fungal pathogen that causes gray mold on a variety of plants. The tomato SL biosynthetic mutant *ccd8* has reduced resistance to *B. cinerea*, while in Arabidopsis, JA-[39] and ET-mediated[40] signals and the phytoalexin, camalexin,[41,42] are known to function in resistance to this pathogen. Camalexin is a sulfur-containing phytoalexin that is synthesized from tryptophan in response to pathogen attack (Fig. 1b). In its biosynthetic pathway, indole-3-acetonitrile (IAN), produced from indole-3-acetaldoxime (IAOx) by CYP71A13, is conjugated to glutathione (GSH) to generate glutathione-indole-3-acetonitrile (GSH(IAN)), which is then converted to cysteine-indole-3-acetonitrile (Cys(IAN)) (Fig. 1b).[43] Finally, Cys(IAN) is catalyzed by PAD3/CYP71B15[44] to produce camalexin (Fig. 1b).

In this study, to clarify the significance of SL-induced immune priming in plant self-defense systems, we investigated whether this priming is effective against JA/ET-mediated defense signaling in Arabidopsis by analyzing the responses to the necrotro-

phic pathogen *B. cinerea*.

Materials and methods

1. Chemicals and Plant materials

rac-GR24 was purchased from Chiralix B. V. (Nijmegen, Netherlands) and used for the preparation of 20 μM solution in 0.2% acetone. Mock treatment was performed with 0.2% acetone. Camalexin was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Arabidopsis (*Arabidopsis thaliana*) wild-type (Col-0) and its mutants *ein2* (*ein2-1*) (*ethylene insensitive 2*), *jar1* (*jar1-1*) (*jasmonate resistant 1*), and *pad3* (*pad3-1*) (*phytoalexin deficient 3*) were obtained from Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA).

2. Culture of fungal pathogen

B. cinerea TV335 was grown and maintained on a potato dextrose plate (1.2% potato dextrose broth (BD, NJ, USA) and 1.5% agar) at 20°C. Sporulation was induced by irradiating the culture plates with UV-A light (FL15BLB, Toshiba Lighting & Technology Co., Tokyo, Japan) for 3 days. Spores were collected by washing the surface of culture plate with sterilized distilled water. After counting spores using a Thoma counting chamber (Sunlead Glass Corp, Saitama, Japan), spore suspension (1×10^6 or 3.5×10^5 spores/mL) was prepared in 1.2% potato dextrose broth.

3. Plant growth condition

Arabidopsis seeds were sterilized with 4% sodium hypochlorite for 5 min and rinsed five times with sterilized distilled water. Sterilized seeds were sown and cultured in sterilized potting soil Kumiai Nippi 1 (Nihon Hiryo, Tokyo, Japan) in plastic pots (5×5×5 cm) inside a growth chamber under a 16 : 8 hr light : dark cycle at 23°C with 60% humidity.

4. Pathogen inoculation assay

Three-week-old Arabidopsis plants were treated with 20 μM *rac*-GR24 or mock by spraying 4 days prior to inoculation. Leaves were cut from *rac*-GR24-treated or mock treated plants and placed face up in a plastic box (21×29×6 cm). Drop-inoculation with *B. cinerea* was performed by placing 3 μL of spore suspension (1×10^6 spores/mL) on the surface of the excised leaves. The infected leaves were incubated at 22°C for 60 hr in the dark with 100% humidity. The size of lesions that appeared on the infected leaves was measured using imageJ 1.53t software (National Institute of Health, USA).

5. RNA extration and RT-qPCR analysis

For the gene expression analyses, leaves of wild-type or mutant plants were harvested 4 days after foliar treatment with 20 μM *rac*-GR24 or mock. Leaf tissues were powdered in liquid nitrogen and used for total RNA extraction using Sepasol-RNA I super reagent (Nacalai Tesque, Kyoto, Japan). Then RNA samples were used for cDNA synthesis using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) with gene-specific primers and cDNA as a template was performed using a LightCycler 96 System (Roche, Basel, Switzerland). PCR conditions were 40 cycles of 5 sec at 95°C and 20 sec at 60°C. The PCR reaction mixture contained 2 μL of 10-fold diluted cDNA template, 0.8 μL of primer solution (containing 10 μM each of forward and reverse primers), 6.4 μL Milli Q water, and 10 μL of SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan). All RT-qPCR data were from six biological replicates and normalized to *UBQ2* as an internal control. The gene-specific primer pairs used are as follows: for *UBQ2*, forward 5′-CAAGAGCTGTGAACTGCAGGA-3′ and reverse 5′-AAGGTTTGTGTCAGAACAATAGAGGAG-3′; for *ACS6*, forward 5′-AACGCAGCATTTGATCGCTA-3′ and reverse 5′-TGGTTATCTCAGCGTGCCTTG-3′; for *ERF1*, forward 5′-GAGCCGATACTCAGTGAGTCGA-3′ and reverse 5′-GCTCTCGGTGAAGCAAGGATA-3′; for *PAD3*, forward 5′-AGTGTTGTAGTCGACCAGAGGC-3′ and reverse 5′-CCG CATCAGACTCCACTCGT-3′; for *PDF1.2*, forward 5′-TTT GCTGCTTTCGACGCAC-3′ and reverse 5′-CGCAAACCC CTGACCATG-3′.

6. Analysis of defense responses to pathogen infection

Foliar treatment of three-week-old wild-type plants with 20 μM *rac*-GR24 or mock was performed 4 days before inoculation with *B. cinerea*. After spraying the spore suspension (3.5×10^5 spores/mL), plants were incubated at 22°C in the dark with 100% humidity, followed by sampling leaves at 12, 16, and 20 hr post inoculation. Leaf samples were used for RNA extraction and RT-qPCR as described above.

7. Measurement of camalexin

Three-week-old Arabidopsis plants were treated with 20 μM *rac*-GR24 or mock by spraying 4 days prior to pathogen inoculation. Leaves were cut from *rac*-GR24-treated or mock treated plants and placed face up in a plastic box (21×29×6 cm). Inoculation with *B. cinerea* was performed by spraying the spore suspension (1×10^6 spores/mL) on excised leaves. Inoculated leaves were incubated at 22°C for 18 or 24 hr in the dark with 100% humidity. Approximately 100 mg of leaves were put in a plastic tube and powdered in liquid nitrogen for extraction and analysis. Extraction was performed with 1 mL of 50% methanol in water (v/v) and then twice with 100% methanol. These extracts were combined and dried *in vacuo*. The pellets were dissolved in 1 mL of 80% methanol in water (v/v) and 20 μL of each sample was injected onto a reversed-phase TSKgel ODS-120T column (4.6 mm×250 mm, 5 μm, Tosoh Bioscience, Tokyo, Japan) in the HPLC system. Camalexin was eluted with a mixture of methanol : water (gradient from 60 to 100% methanol for 20 min) at a flow rate of 0.7 mL/min at 35°C, detected and fluorometrically quantified at excitation wavelength of 310 nm and emission wavelength of 390 nm. Camalexin content was estimated using a calibration curve prepared by analyzing leaf tissue samples of *pad3* mutants to which various concentrations of standard camalexin were added.

Results

1. *Effects of rac-GR24-treatment against B. cinerea in Arabidopsis thaliana*

To determine the effect of SL-mediated signaling on defense signaling pathways other than the SA-mediated signaling pathway, we analyzed the effect of SL-mediated signaling on resistance against gray mold caused by the necrotrophic fungal pathogen *B. cinerea*. The three-week-old wild-type Arabidopsis plants were foliar treated with 20 μM *rac*-GR24 and was drop-inoculated with *B. cinerea* 4 days later. Water-soaked lesions appeared on the leaves 48 hr after *B. cinerea* inoculation, spread gradually from the inoculated points, and reached the entire leaf after 4–5 days. The area of lesions was measured 60 hr post inoculation, using imageJ software. The degree of disease susceptibility was evaluated by comparing the average lesion area of *rac*-GR24-treated and mock-treated control plants. The lesion area of *rac*-GR24-treated plants (20.1±3.55 mm^2) was significantly reduced by 26% compared to that of the control plants (27.3±2.35 mm^2). *rac*-GR24 at concentration of up to 20 μM did not affect the growth of *B. cinerea* on PDB medium (Supplemental Fig. S1). These results suggest that *rac*-GR24-treatment enhances resistance to *B. cinerea* in wild-type Arabidopsis plants (Fig. 2a).

To know the mechanism of *rac*-GR24-induced resistance against *B. cinerea*, we examined the effects of *rac*-GR24-treatment on disease resistance using defense-related Arabidopsis mutants defective in plant hormone-mediated signaling or phytoalexin biosynthesis. Because JA-mediated signaling plays an important role in the response to necrotrophic pathogens, we examined the effect of *rac*-GR24-treatment on resistance to *B. cinerea* in the *jar1* mutant, which lacks JA-mediated signaling due to a deficiency in jasmonoyl--L-amino acid synthetase. Whereas the average lesion area in the mock-treated control *jar1* plants was 19.6±1.39 mm^2, that in *rac*-GR24-treated plants was 14.3±1.18 mm^2, indicating a significant 27% decrease in the average lesion area by *rac*-GR24-treatment (Fig. 2b). These suggest that the JA-medicated signaling pathway is not required for the enhancement of resistance against *B. cinerea* by *rac*-GR24.

Since camalexin, an antibacterial substance, takes an important part in the resistance against *B. cinerea*, we examined the effect of *rac*-GR24 on resistance to *B. cinerea* in the camalexin biosynthesis-deficient mutant *pad3*. The lesion area of *rac*-GR24-treated plants and mock-treated control plants were 19.8±1.31 mm^2 and 22.2±2.13 mm^2, respectively, indicating that *rac*-GR24-treatment had no effect on disease resistance in *pad3* mutant (Fig. 2c). Since the biosynthesis of camalexin is known to be regulated by ET, the effect of *rac*-GR24 against *B. cinerea* was examined in ET-mediated signal deficient mutant *ein2*. In *ein2* mutant, the average lesion area of the *rac*-GR24-treated plants and mock-treated control plants were 66.9±3.43 mm^2 and 65.5±5.97 mm^2, respectively, indicating that disease resistance against *B. cinerea* was not enhanced by *rac*-GR24 in *ein2* mutants (Fig. 2d). These results suggest that the enhancement of resistance against *B. cinerea* by *rac*-GR24 requires functional camalexin biosynthesis and proper activation of the ET-mediated signaling pathway.

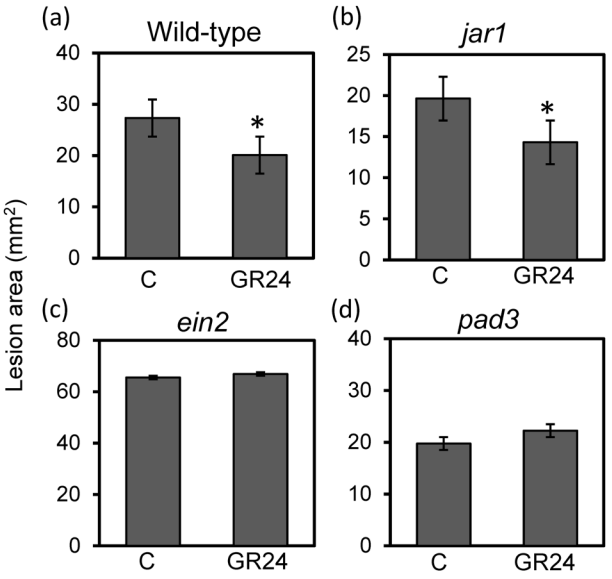

Fig. 2. Effects of *rac*-GR24 treatment on gray mold in Arabidopsis plants. Foliar treatment with mock (C) or 20 μM *rac*-GR24 (GR24) was performed to three-week-old wild-type (a), *jar1* (b), *pad3* (c), and *ein2* (d) plants for 4 days. Excised leaves were used for inoculation with *B. cinerea* spore suspension (1×10^6 cfu/mL). Lesions were measured 60 hr after inoculation. Values are shown as the means±S.E. (n>30). Asterisks indicate significant difference between control and *rac*-GR24-treated plants (Unpaired student's t-test, *p<0.05). The experiment was repeated three times with similar results.

2. *Effects of rac-GR24-treatment on expression of defense-related genes*

To characterize the disease resistance induced by *rac*-GR24 against *B. cinerea*, we investigated physiological changes in *rac*-GR24-treated wild-type plants. Because pathogen inoculation assays indicated that ET-mediated signaling and camalexin biosynthesis are involved in the resistance by *rac*-GR24-treatment, we examined the effects of *rac*-GR24-treatment on expression of ET-related and camalexin biosynthesis-related genes in Arabidopsis wild-type plants using RT-qPCR. The expression levels of the ET biosynthetic gene *ACS6* (*1-Aminocyclopropane-1-carboxylate* (*ACC*) *synthase 6*) and ET responsive gene *ERF1* (*Ethylene response factor 1*) were not significantly different between the *rac*-GR24-treated plants and the mock-treated control plants, indicating that ET-mediated signaling was not activated by *rac*-GR24-treatment (Fig. 3a, 3b). The expression levels of *PDF1.2* (*Plant defensin 1.2*), which is synergistically induced by the combination of ET- and JA-mediated signaling, was not affected by *rac*-GR24-treatment, indicating that both ET-and JA-mediated defense signaling pathways were not activated by *rac*-GR24-treatment (Fig. 3c). The expression of the camalexin biosynthetic gene *PAD3* (*Phytoalexin deficient 3*) was not significantly different between the *rac*-GR24-treated plants and the mock-treated control plants, indicating that camalexin biosynthesis was not

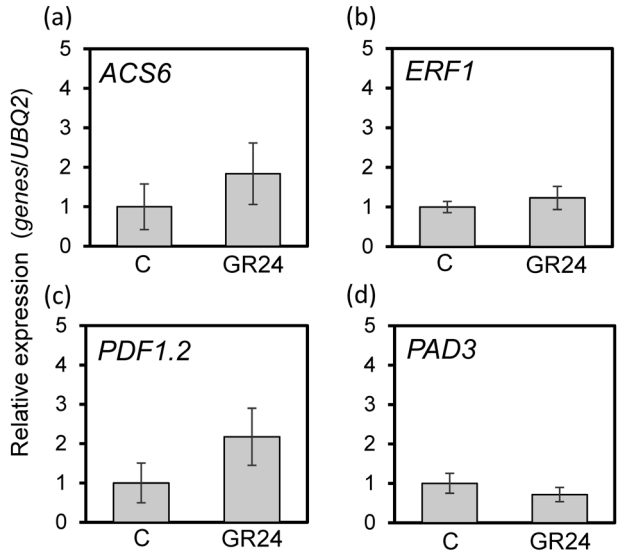

Fig. 3. Expression of defense-related genes in *rac*-GR24-treated wild-type plants. Three-week-old plants were foliar-treated with mock (C) or 20 μM *rac*-GR24 (GR24). The leaves were collected 4 days after treatment. RT-qPCR analysis was performed to evaluate the expression of the *ACS6* (a), *ERF1* (b), *PDF1.2* (c), and *PAD3* (d). Transcript levels were normalized to the expression of *UBQ2* measured in the same samples. Values are shown as the means±S.E. (n=6). No significant differences at $p<0.05$ level were detected in gene expression levels between the control and *rac*-GR24-treated plants. The experiment was repeated three times with similar results.

activated by *rac*-GR24-treatment (Fig. 3d). These results indicate that under our experimental conditions *rac*-GR24-treatment alone does not activate defense signaling mediated by ET or JA and camalexin biosynthesis in Arabidopsis.

3. Accelerated responses to pathogen infections in the rac-GR24-treated plants

In *rac*-GR24-treated plants, the ET-related genes and camalexin-biosynthetic gene were not activated before pathogen inoculation (Fig. 3), however EIN2 and PAD3 were required for disease resistance against *B. cinerea* (Fig. 2c, 2d). These suggest that ET-mediated signaling and camalexin biosynthesis play important roles in the resistance mechanisms activated by pathogen infection. To determine how *rac*-GR24 enhanced the resistance against *B. cinerea*, we analyzed the effects of *B. cinerea* infection on expression levels of *ACS6*, *ERF1*, *PDF1.2* and *PAD3* in wild-type plants. Time course analyses indicated that expression of ET-related genes increased from 12 hr post inoculation (hpi), peaked at 16 hpi, and then decreased to the similar levels as 12 hpi at 20 hpi (Fig. 4). Although the expression of *ERF1* and *PDF1.2* in mock-treated control plants slightly increased by pathogen infection compared to before pathogen infection, at 16 hpi the transcript levels of *ACS6*, *ERF1* and *PDF1.2* in *rac*-GR24-treated plants were about 5.8-, 1.7- and 3.8-fold higher than those of control plants (Fig. 4a–c). These indicate that *rac*-GR24-treatment promoted the activation of ET-mediated defense signaling by infection with *B. cinerea*.

Expression of *PAD3* increased gradually after infection with *B. cinerea* in control plants, but the transcript levels of *PAD3* in *rac*-GR24-treated plants were about 2- and 1.6-fold higher than those of control plants at 12 hpi and at 16 hpi, respectively (Fig. 4d). This result indicates that camalexin biosynthetic genes was more rapidly activated in *rac*-GR24-treated plants compared to in the mock-treated control plants. Taken together, *rac*-GR24-treatment had a priming effect on activation of ET-mediated defense signaling pathway and camalexin biosynthesis in response to infection by *B. cinerea*.

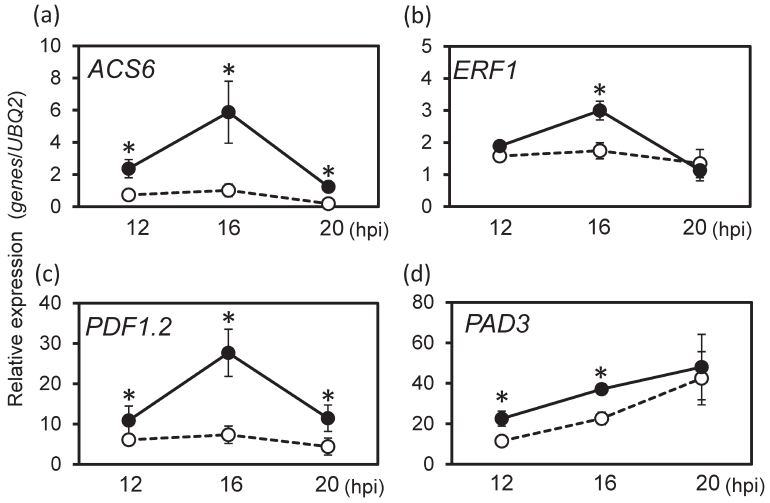

Fig. 4. Expression of defense-related genes after pathogen infection. Three-week-old plants were foliar-treated with mock or 20 μM *rac*-GR24 4 days before spray-inoculation with *B. cinerea* spores (3.5×10^5 cfu/mL). Leaves were collected 12, 16, and 20 hr after inoculation, followed by gene expression analyses of *ACS6* (a), *ERF1* (b), *PDF1.2* (c), and *PAD3* (d). The transcript levels were normalized to the expression of *UBQ2* measured in the same samples. Values are shown as the means±S.E. (n=6). Open circle, mock-treated control plant; Closed circle, *rac*-GR24-treated plant. Statistically significant difference between mock-treated and *rac*-GR24-treated plants at each time point is indicated by asterisk (unpaired t-test, *$p<0.05$). The experiment was repeated two times with similar results.

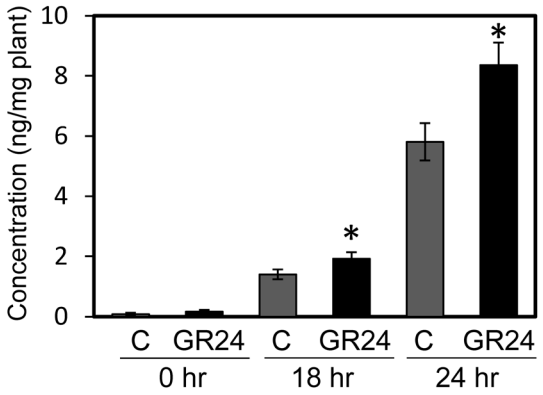

Fig. 5. Accumulation of camalexin in Arabidopsis after pathogen inoculation. Three-week-old plants were foliar-treated with mock (C) or 20 μM *rac*-GR24 (GR24) 4 days before spray-inoculation with *B. cinerea* (1×10^6 cfu/mL). Leaves were collected 0, 18 and 24 hr after pathogen inoculation. The levels of camalexin were measured using high-performance liquid chromatography. Values are shown as the means±S.E. ($n=6$). Significant differences between mock-treated control plants and *rac*-GR24-treated plants at each time point are indicated by asterisks (unpaired *t*-test, $*p<0.05$). The experiment was repeated two times with similar results.

4. Effects of rac-GR24-treatment on camalexin accumulation after B. cinerea infection

Since inoculation of *B. cinerea* induced expression of camalexin biosynthetic gene *PAD3* and which was promoted by *rac*-GR24-treatment, we examined whether *rac*-GR24-treatment affects camalexin accumulation in wild-type plants after pathogen inoculation. Before pathogen inoculation, accumulation levels of camalexin are not different between *rac*-GR24-treated and mock-treated control plants (Fig. 5). The camalexin levels increased gradually after inoculation in both *rac*-GR24-treated and mock-treated control plants but were significantly higher in *rac*-GR24-treated plants than in control at 18 and 24 hpi (Fig. 5). The camalexin levels in *rac*-GR24-treated plants were 36% and 44% higher than in control at 18 hpi and at 24 hpi, respectively. This increasing pattern of camalexin accumulation and its enhancement by *rac*-GR24-treatment correlated with the pattern of *PAD3* expression (Figs. 4 and 5). These suggest that the biosynthesis and accumulation of camalexin induced by infection with *B. cinerea* is accelerated in *rac*-GR24-treated plant compared to control.

Discussion

Regulating the strength of defense signals is a necessary and important physiological function for plants to adapt to various types of pathogens and environmental changes, as well as to adapt to the trade-off relationship between disease resistance and growth. Priming by SL-mediated signaling is such a regulatory mechanism, and elucidating its detailed function is important for crop protection as well as for understanding the immune mechanisms in plants. To determine how widely SL-induced priming can modulate plant immune systems, we analyzed its effects on JA/ET-mediated signals that function in resistance to necrotrophic pathogens. Activation of SL signaling by *rac*-GR24-treatment enhanced the disease resistance against *B. cinerea* in Arabidopsis wild-type plants and *jar1* mutant but not in *ein2* and *pad3* mutants (Fig. 2). Expression of ET-related genes and *PAD3* in wild-type plants was not altered by *rac*-GR24 treatment alone but was enhanced after subsequent infection with *B. cinerea*. (Figs. 3 and 4). Furthermore, accumulation of camalexin after pathogen infection was also enhanced by *rac*-GR24-treatment (Fig. 5). These data demonstrated that priming of plant immune system by activation of SL signaling is effective against ET-mediated defense signaling and camalexin biosynthesis, resulting in enhancement of disease resistance against the necrotrophic fungal pathogen *B. cinerea*. Taken together with the previous report of its effect on SA signaling,[29] it is suggested that SL-mediated immune priming is effective against many types of defense-related signaling, including both SA- and ET-mediated defense signaling pathways.

We used *rac*-GR24 consisting of optical isomers $GR24^{5DS}$ and $GR24^{ent\text{-}5DS}$ as the SL analogue (Fig. 1a), although $GR24^{5DS}$ and $GR24^{ent\text{-}5DS}$ enhance the interaction of F-box protein MAX2 with the SL receptor AtD14 and the karrikin receptor KAI2, respectively.[45,46] In the previous report, disease resistance against *Pst* was enhanced by *rac*-GR24-treatment and reduced by treatment with the SL biosynthesis inhibitor TIS108, whereas both *rac*-GR24 and TIS108 did not have any effect on expression of SA-related genes and SA accumulation before pathogen infection.[29] Thus, there is no doubt that SL-mediated signaling activated by *rac*-GR24-treatment positively regulates SA-mediated defense signaling and resistance against *Pst*. On the other hand, karrikin (KAR2) has recently been reported to induce disease resistance against *Pst* through KAI2-MAX2-mediated signaling.[47] These indicate that MAX2-mediated signaling functions to fuse both SL and karrikin signals[48] and ultimately enhance disease resistance. Therefore, the enhanced resistance to *B. cinerea* by *rac*-GR24-treatment shown in this study would be achieved by F-box protein MAX2-mediated signaling, in which both SL-mediated and karrikin-mediated signals were involved.

Camalexin biosynthesis in Arabidopsis is rapidly induced by infection with necrotrophic fungal pathogens *Alternaria brassicicola, A. alternata* as well as *B. cinerea*,[49] and the incompatible bacterial pathogen *Pseudomonas syringae* PSSD20,[50] and also by exposure to non-biological stresses like silver nitrate and UV-B.[51] Priming by SL signaling has the effect of promoting camalexin biosynthesis, which may contribute to disease resistance not only to *B. cinerea* but also to other necrotrophic pathogens.

Reportedly, 0.2, 0.1, and 0.05 mM camalexin inhibits mycelial growth of *B. cinerea* strains by 100%, 54–57%, and 28–29%, respectively.[52] In this study, the camalexin concentrations of *rac*-GR24-treated and control plants 24 hr after *B. cinerea* inoculation were approximately 0.04 mM and 0.03 mM, respectively, suggesting that much higher concentrations of camalexin around the infection site affected the growth of *B. cinerea*.[53] The primed plants had at least a 40% increase in camalexin concentration relative to the control at 24 hpi, and the difference between them was estimated to be greater than 0.01 mM at the

B. cinerea infection site, which may have contributed to the enhancement of resistance.

B. cinerea can detoxify camalexin by converting it to indole-3-thiocarboxamide, then to 3-indolecarboxynitrile, and finally to 3-indolecarboxylic acid. The antimicrobial activity of 3-indolecarboxylic acid is only about 14% of that of camalexin, but the metabolic intermediates, indole-3-thiocarboxamide and 3-indolecarboxynitrile, still retain 37–48% of the antimicrobial activity of camalexin.[52] In addition, the reaction in the final step to produce 3-indole carboxylic acid is quite slow.[52] Thus, priming of camalexin biosynthesis is suggested to be a very effective defense mechanism against *B. cinerea* and other necrotrophic pathogens because of the antimicrobial activity of the more rapidly accumulated camalexin and its degraded metabolites.

The reason for the trade-off relationship between disease resistance and growth is due to the distribution of energy that plants can acquire.[17,54] In addition, the amount and balance of metabolites that assimilate and utilize elements taken in from outside may also play a significant role, and this study suggests one aspect of their regulation. In the primed plant body, sulfur-containing camalexin, important for resistance, did not accumulate, but their rapid biosynthesis after pathogen infection was further enhanced. The sulfur-containing compound GSH is involved in the camalexin biosynthetic pathway. A biosynthetic intermediate IAN is conjugated to GSH by the glutathione-*S*-transferase GSTF6 to form GSH(IAN) (Fig. 1b).[43] This biosynthetic pathway is rapidly activated by pathogen infection to synthesize relatively large amounts of camalexin, which may affect sulfur metabolism in plants. Sulfur is an important element for plants and is used not only for GSH but also for proteins, amino acids such as cysteine and methionine, and the important coenzymes Co-A and *S*-adenosylmethionine, which function in a variety of biological processes.[55,56] Camalexin biosynthesis is likely to affect sulfur metabolism in plants to influence the amount of sulfur compounds in growth-related physiological functions, which may result in a trade-off relationship between disease resistance and growth. In primed plants, the controlled sulfur metabolism before pathogen infection contributes to maintaining the balance between disease resistance and growth. Furthermore, SL is a plant hormone that properly regulates growth,[48,57] but on the other hand it also positively acts on disease resistance, as shown here, so SL has very specific properties compared to other plant hormones.[29,36–38] We speculate that there is a biological rationale and significance for the positive effect of SL on both immune system and growth, but further analysis is needed to elucidate the detailed mechanism.

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Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Fig. S1), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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