

Enhancement of systemic acquired resistance in rice by F-box protein D3-mediated strigolactone signaling

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Abstract

Strigolactones (SLs) are butenolide-type plant hormones that play several roles in plants, such as suppressing shoot branching and promoting arbuscular mycorrhizal symbiosis. Recently, SLs have been reported to positively regulate disease resistance in plants. In this study, we analyzed the effect of the synthetic SL analog *rac*-4-bromodebranon (*rac*-4BD) on systemic acquired resistance (SAR) in rice. First, we demonstrated *in vitro* that *rac*-4BD, similar to the common SL analog *rac*-GR24, promotes the interaction of SL and karrikin receptor, D14 and D14-like (D14L), respectively, with signaling factor D3. Gene expression analysis and inoculation tests indicated that pretreatment with *rac*-4BD promotes the effect of the SAR inducer BIT. Activation of SAR was also significantly observed in the SL and karrikin signal-deficient rice mutant *d3*. These results suggest that D3-mediated SL signaling by *rac*-4BD treatment does not directly activate rice immunity but induces a priming state in the plant that enhances SAR induction.

Introduction

Rice is an economically important crop because it is the staple food for more than half of the world's population [1]. Future population growth will necessitate increased rice production, for which cultivation techniques must be adapted to biotic and abiotic stresses [2, 3]. Previous studies have estimated that up to 40% of rice production is lost annually owing to diseases [4]. Rice blast disease, caused by *Pyricularia oryzae* (synonym of *Magnaporthe oryzae*), is a critical disease in rice cultivation that must be controlled. Numerous fungicides are used in agricultural production to control diseases. However, the emergence of fungicide-resistant strains has become a serious problem, necessitating the development and application of technologies that increase disease resistance in plants. To protect themselves from pathogenic attacks, plants have evolved unique defense mechanisms that are governed by plant hormones, such as salicylic acid (SA) and jasmonic acid (JA) [5, 6]. SA is synthesized at the site of pathogen infection and functions as a signal for inducing systemic acquired resistance (SAR) throughout the plant body [7–10]. SAR is effective against various types of pathogens and protects plants from further attacks. Chemicals that activate SAR have been developed and are widely used to control rice blast disease in rice paddy fields. Probenazole and its active metabolite, 1, 2-benzisothiazol-3(2*H*)-one-1,1-dioxide (BIT), are SAR-inducing agents that activate SA biosynthesis in plants [11–13]. One of the key genes for rice SAR, *OsWRKY45*, has been identified to act downstream of SA and is used as an SAR marker gene [14, 15]. Overexpression of the transcription factor *OsWRKY45* confers strong disease resistance for rice [16].

Strigolactones (SLs) and karrikin are butanolide-type compounds (Fig. 1) with diverse effects on plant growth and development [17, 18]. SLs regulate shoot branching and root morphology in plants [19–21]. SLs are involved in biotic interactions, promoting symbiosis with mycorrhizal fungi and the settlement of parasitic weeds [22, 23]. Karrikin, a compound found in smoke emitting from burning wood, affects seed germination rates, seedling photomorphogenesis, and stress tolerance [24].

Various SL analogs have been characterized to elucidate SL functions and develop strategies for controlling parasitic weeds [25, 26]. The canonical, natural SL structure includes a tricyclic lactone (ABC ring) and a methylfuranone ring (D ring) connected by an enol ether. The representative synthetic SL analog, *rac*-GR24, has a four-ring structure similar to canonical SLs (Fig. 1). The smaller compound, *rac*-4-bromodebranone (*rac*-4BD), which is an ether linkage of a substituted phenol and D ring, is easier to synthesize than *rac*-GR24 [27]. Furthermore, *rac*-4BD was found to have stronger activity for shoot branching inhibition in rice than *rac*-GR24 [27].

The mechanism of SL signaling and biosynthesis in rice has been studied through the analysis of dwarf (*d*) and high-tillering dwarf mutants [28, 29]. The causative gene for the SL-insensitive mutant *d14* encodes the SL receptor, an α/β -fold hydrolase family protein. The D14 protein accepting SL interacts with the F-box protein D3 [30, 31] to form the Skp1-Cullin-F-box (SCF) E3 ubiquitin ligase complex with the SKP1 protein OSK1 [32, 33]. The D14-SCF^{D3} complex transmits the SL signal by inducing the ubiquitination and degradation of the D53 protein by the proteasome system. Additionally, the D3 protein interacts with the karrikin receptor D14L in a karrikin-dependent manner, whereas the internal ligands for D14L have not been identified [34]. Thus, D3 is a common pivotal factor in the SL- and karrikin-mediated signaling pathways.

The effects of SL on plant-pathogen interactions have been reported in recent years [35, 36]. In rice, SL biosynthesis mutant *d17* and SL receptor mutant *d14* showed higher susceptibility to *P. oryzae* than the wild type [35]. We previously demonstrated that SL signaling enhanced the expression of SAR marker genes in response to bacterial infection and enhanced disease resistance in Arabidopsis using *rac*-GR24 and an SL-biosynthesis inhibitor [36]. Therefore, SL signaling has positive effect on disease resistance and treatment with an SL analog is a promising tool for plant disease control. However, further research is necessary to determine whether activation of the SL-mediated signal influences rice immune systems. On the other hand, karrikin has been found to induce disease resistance in Arabidopsis [37]. Therefore, F-box protein MAX2, Arabidopsis D3 ortholog, is crucial for SL- and karrikin-mediated immune systems in Arabidopsis, which is also presumed to function in rice. Although D3- and MAX2-related signalings by SLs and Karrikins in growth and development have been extensively studied, their function and underlying mechanisms in the immune system are poorly understood. In this study, we analyzed the effects of the SL analog *rac*-4BD on defense gene expression and disease resistance in rice, with the aim of demonstrating the importance of the D3- and MAX2-related immune systems and providing a chemical tool to elucidate their mechanisms.

Results

Promotion of interaction between D14 or D14L and D3 by *rac*-4BD

We examined the effects of *rac*-4BD on interactions between the receptor protein D14 and F-box protein D3 using the yeast three-hybrid (Y3H) system to determine whether *rac*-4BD acts as an SL-analog in the SL receptor-F-box protein system. The receptor proteins D14L instead of D14 were used to determine

whether *rac*-4BD acts as a karrikin analog. The vector pGADT7-D14, -D14L, or -EV (empty) used as prey expressed the D14 or D14L protein, or nothing, respectively (Fig. 2). The vector pBridge-D3-OSK1 used as bait co-expressed D3 and the SKIP1 protein OSK1, a component of the SCF complex (Fig. 2). The yeast AH109 strain was transformed with pGADT7-D14, -D14L, or -EV and pBridge-D3-OSK1 and cultured in media containing *rac*-GR24 or *rac*-4BD. All transformants grew in the control medium (SD-Leu, Trp, Met). In the selection medium (SD-Leu, Trp, Met, His, Ade) containing *rac*-GR24 or *rac*-4BD, the transformants with pGADT7-D14 or pGADT7-D14L and pBridge-D3-OSK1 grew, whereas the transformant with pGADT7-EV and pBridge-D3-OSK1 failed to grow (Fig. 2). All transformants did not produce colonies on selection medium without synthetic SL (Mock). These results indicate that D14 or D14L interacts with D3 only when *rac*-GR24 or *rac*-4BD exist (Fig. 2). In rice, *rac*-GR24 enhances the interaction of D3 with D14 or D14L (Fig. 2) [31, 45], while in Arabidopsis the optical isomers GR24^{5DS} and GR24^{ent-5DS} enhance the interaction of D3 homolog MAX2 with AtD14 or KAI2, respectively [46, 47]. However, the effect of the optical isomers of *rac*-4BD on the interaction of D3 with D14 or D14L remains to be determined. Collectively, *rac*-4BD acts as both SL analog and karrikin analog in rice and can be used to assess the role of D3-mediated signaling in the rice immune system.

***rac*-4BD has no effect on the growth of rice blast fungus**

To properly evaluate the effect of *rac*-4BD on rice blast disease development, its antimicrobial activity against *P. oryzae* should be determined. To assess the direct effect of SL analogs on fungal growth, in vitro paper disk assays were performed using *rac*-GR24 or *rac*-4BD with *P. oryzae*. None of the SL analogs at concentrations up to 1 mg/ml had a significant inhibiting effect on the growth of *P. oryzae* colonies (supplementary Fig. 1). These results suggest that *rac*-4BD has no direct effect on the development of blast fungus.

Promotion of SAR response in rice by *rac*-4BD

We examined the effects of treatment with *rac*-4BD on BIT-induced SAR using gene expression analysis and pathogen infection assay. Expression levels of the rice SAR marker gene, *OsWRKY45*, in rice leaves were analyzed 1 and 3 days after 0.5 mg/pot BIT treatment, following pretreatment with *rac*-4BD. Whereas *rac*-4BD did not affect the expression of *OsWRKY45*, BIT induced the expression of *OsWRKY45* in both *rac*-4BD-pretreated and non-pretreated plants (Fig. 3a). The induction of *OsWRKY45* expression 3 days after BIT treatment in the *rac*-4BD-pretreated plant was significantly higher than that of non-pretreated control plants, which was unclear 1 day after BIT treatments (Fig. 3a). These results indicate that pre-treatment with *rac*-4BD alone does not affect *OsWRKY45* expression but promotes *OsWRKY45* induction by BIT, suggesting that *rac*-4BD enhances the SAR-inducing activity of BIT (Fig. 3a). Subsequently, we analyzed the effects of *rac*-4BD on SAR induction by BIT using the rice blast inoculation assay. Similar to *OsWRKY45* expression levels, the control and *rac*-4BD treatment did not differ significantly, indicating that *rac*-4BD treatment alone did not affect disease resistance against rice blast (Fig. 3b). Treatment with 0.5 or 0.1 mg/pot BIT reduced the number of lesions of both the *rac*-4BD-pretreated and non-pretreated plants (Fig. 3b). Disease resistance induced by 0.1 mg/pot BIT was

significantly higher in *rac*-4BD-pretreated plants than in non-pretreated plants; however, the results were ambiguous after treatment with 0.5 mg/pot BIT (Fig. 3b). These data indicate that *rac*-4BD pre-treatment does not affect rice blast resistance but promotes resistance induction by BIT. These results suggest that *rac*-4BD does not activate SA-mediated defense signaling and resistance but promotes SAR induction by BIT.

Defects of D3-mediated signaling do not affect SAR induction.

The fact that *rac*-4BD promoted SAR induction by BIT suggests that SAR induction depends on SL signaling or is regulated in intensity by SL signaling. To determine whether SL or karrikin-mediated signaling is required for SAR induction by BIT in rice, we examined the effects of BIT treatment in *d3* mutants deficient in those signals. Expression of defense-gene *OsWRKY45* and disease resistance against rice blast in *d3* mutant and its background wild type 'Shiokari' were analyzed 4 days after treatment with 0.5 mg/pot BIT. Treatment with BIT significantly induced the *OsWRKY45* expression in leaf tissues of *d3* and wild type plants (Fig. 4a). In the blast inoculation assay, the number of lesions on the leaves of *d3* plants without BIT-treatment was about 2-fold more than that wild type plants, indicating that D3-mediated signaling take part in disease resistance against rice blast. The number of lesions on the leaves of the BIT-treated wild-type plants was reduced to approximately one-fourth of the non-treated control plants (Fig. 4b). Similarly, in the *d3* mutants, the number of lesions of the BIT-treated plants was approximately one-third of that of the non-treated control plants (Fig. 4b). These results indicate that the deficiency of D3-mediated signaling reduces disease resistance against rice blast but does not affect SAR induction by BIT.

Discussion

Considering future food problems due to population growth, the use of plant immune mechanisms is important. However, SAR, the main technology for this purpose, is affected by environmental factors and is difficult to use for crops other than rice, so a technology to efficiently use plant immunity is needed. Since SL signaling has been shown to enhance SA signaling in Arabidopsis [36], we analyzed the effect of SL signaling on SAR and its mechanism in rice. The SL analog *rac*-4BD was shown by Y3H analysis to be able to act on both SL and karrikin receptors in rice, both of which lead to activation of the D3-mediated signaling pathway, and by gene expression analysis in rice plants and rice blast inoculation tests to enhance induction of SAR. Taken together with the priming effect of SL signaling on SA-mediated defense signaling, activation of SL signaling by *rac*-4BD treatment had the effect of priming the rice immune system, resulting in enhanced activation of SA signaling for SAR induction.

Interactions of D3-OSK1 with D14 or D14L were promoted by *rac*-4BD (Fig. 2), however, which optical isomer act on the interaction with D14 is unknown. Since *rac*-4BD has strong inhibitory activity against shoot branching in rice as functional analog of SL [27], we can assume that either or both optical isomers of *rac*-4BD activate signaling through SL in rice. Further analyses using optical isomers will confirm this idea and reveal the detailed mechanisms of priming of rice immune system by SL signaling.

Analysis of SL signaling-deficient rice mutants showed that the number of blast lesions in the *rac*-4BD-untreated *d3* mutant was about twice that of the wild type, suggesting that D3-mediated signaling positively regulates basal disease resistance to rice blast (Fig. 4b). On the other hand, treatment with BIT induced *OsWRKY45* expression and rice blast resistance in the *d3* mutant as well as in wild type, obviously indicated that SL and karrikin signaling are not essential for SAR induced by SA-mediated signaling. These suggest that D3-mediated signaling activated by *rac*-4BD plays a role in priming the plant immune system and accelerating the activation of SA-mediated signaling upon BIT treatment. In the case of Arabidopsis primed by *rac*-GR24 resulted in enhanced disease resistance against a bacterial pathogen [36], whereas priming effect of *rac*-4BD in rice was effective to SAR induction but not to disease resistance against rice blast. The reason for this difference is presumably due to the physiological properties of the plants or the combination of plants and pathogens, rather than to differences in compounds. The high level of endogenous SA in rice is possible reason why *rac*-4BD alone cannot successfully induce resistance to rice blast [48]. Another reason is that the priming state of plant immunity may be effective against infection with pathogenic bacteria but ineffective against intracellularly invading filamentous fungal pathogens. Further analyses using pathogenic filamentous fungi in Arabidopsis or pathogenic bacteria in rice would reveal the details of the effects of priming plant immunity by D3-mediated signals.

The positive roles of SL and karrikin in disease resistance have been demonstrated in Arabidopsis [36, 37]. The contribution of SL-mediated signaling to blast resistance in rice was demonstrated by increased susceptibility in the SL biosynthesis mutant *d17* and SL receptor mutant *d14* [35]. Conversely, it has been reported that suppression of SL signaling with the *d14* mutant, the SL biosynthesis mutant *d10*, or SL biosynthesis inhibitors activates JA signaling and enhances resistance to blast fungus [49]. These conflicting results about the function of SL signaling in the rice immune system may be caused by regulatory mechanisms of SA and JA signaling. For the semi-biotrophic pathogen *P. oryzae*, the SA signal is effective during the early biotrophic phase of infection, while the JA signal mainly acts on disease resistance during the late necrotrophic phase [50]. Furthermore, JA and SA signaling are mutually antagonistic in the pathway of inducing disease resistance in rice [51]. Unlike the suppression of SL signaling in those reports, this study demonstrated that activation of SL signaling by *rac*-4BD had no effect on rice blast infection but promote SAR induction by BIT. However, it is unknown whether SL or karrikin-mediated signaling is more important for priming of rice immune system by *rac*-4BD. Further investigation using optical isomers of 4BD and mutants defective in D14 or D14L will reveal the roles of SL and karrikin in D3-mediated immunity in rice.

Several SAR inducers, such as probenazole, tiadinil, and isotianil, are widely used to control rice blast disease in rice paddy fields [52, 53]. However, SAR is suppressed by environmental stresses, such as cold and drought, via the antagonistic crosstalk between SA- and ABA-mediated signaling pathways [54–56]. In addition, SAR inducers are difficult to use on other crops, including vegetables, because they affect plant growth. The data presented in this study demonstrate that the activation of D3-mediated signaling is beneficial for increasing the efficacy of SAR inducers, which would contribute to future disease control technologies that exploit plant potentials and reduce the use of SAR inducers as well as

antimicrobial agents. Furthermore, the manufacturing cost of *rac*-4BD is lower than that of SL and its analog *rac*-GR24 because the synthesis method is less complex. Therefore, the activity of *rac*-4BD demonstrated in this study will be a useful reference for the development of new crop management technologies in the future and, more importantly from a plant science perspective, will lead to a detailed mechanistic understanding of the priming by D3-mediated signals that regulate the plant immune system.

Materials and methods

Chemicals

Chemicals, (3aR,8bS,E)-3-((((R)-4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)-3,3a,4,8b-tetrahydro-2*H*-indeno[1,2-*b*]furan-2-one (*rac*-GR24) and 5-(4-bromophenoxy)-3-methylfuran-2(5*H*)-one (*rac*-4BD), were synthesized as previously described [38, 39] and dissolved in acetone; 1,2-Benzisothiazol-3(2*H*)-one 1,1-Dioxide (BIT) was dissolved in water.

Yeast three-hybrid assay

The yeast three-hybrid assay using the Matchmaker Three-Hybrid System (Takara Bio, Shiga, Japan) was performed as previously described [40, 41]. pGADT7-D14, pGADT7-D14L or pGADT7-EV served as prey and pBridge-D3-OSK1 as bait.

Plant materials and chemical treatment

Rice seeds (*Oryza sativa* cv. Nipponbare, cv. Shiokari and *d3* (cv. Shiokari background)) were surface-sterilized before sowing by soaking in hot water at 60°C for 10 min, followed by soaking in 25 °C water for 2 days. Rice was grown in potting soil Honens 1 (Honen Agri, Niigata, Japan) in pots (2.5 cm × 2.5 cm × 4 cm) inside a growth chamber under a 12 h:12 h light: dark regimen at 28 °C with 60% humidity. Seven days after sowing, young seedlings were transferred to 20 mL 0.25% fertilizer solution (Chiyoda Kasei; SunAgro Co., Ltd, Tokyo, Japan) in 50 mL polypropylene tubes. Rice seedlings were treated with *rac*-4BD (30 μM in tube) and/or BIT (0.5 mg or 0.1 mg in tube) using a soil drenching method 3 days before challenge inoculation.

Pathogen inoculation assay

P. oryzae compatible race hoku-1 (race 007) [42, 43] was cultured on oatmeal agar medium at 25°C. Spore formation of *P. oryzae* was induced by culturing under black light blue fluorescent lamp for 4 days. Rice seedlings were sprayed with a spore suspension (1×10^5 conidia/ml, 0.02% (w/v) Tween 20), maintained under high humidity and dark conditions for 20 h, then grown for 4 days under normal conditions. The number of lesions on the fourth leaf of the rice plants was recorded.

RNA isolation and RT-qPCR

Total RNA isolation from leaf samples, cDNA synthesis, and RT-qPCR were performed as previously described [44]. The gene-specific primer pairs used are as follows: *OsWRKY45* (Os05g0322900), forward 5'-CGGGTAAAACGATCGAAAGA-3', reverse 5'-TTTCGAAAGCGGAAGAACAG-3'; *OsUBQ* (Os03g0234200), forward 5'-AACCAGCTGAGGCCCAAGA-3', reverse 5'-ACGATTGATTTAACCAGTCCATGA-3'. Thermal cycling conditions included 30 s at 95°C, 40 cycles for 5 s at 95°C, and 20 s at 60°C. Transcript levels were normalized to the expression of *OsUBQ* measured in the same samples.

Statistical analysis

The data are presented as the mean \pm standard error. Statistical analysis was performed using GraphPad Prism 9 software (GraphPad, CA, USA). Multiple comparisons were performed using Tukey's test after analysis of variance (ANOVA). The Student's *t*-test was used to analyze the significant differences among groups. All statistical significance was set at $p < 0.05$.

Declarations

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Author contributions

Conceptualization, M.K., T.A. and H.Nakashita; methodology, M.K., I.T., H.Nakamura, and H.Nakashita; validation, M.K., M.F., K.Y., K.A., N.B., T.A. and H.Nakashita; investigation, M.K., M.F., T.M., I.T. and T.T.; writing, M.K. and H.Nakashita; visualization, M.K.; project administration, T.A. and H.Nakashita; funding acquisition, K.Y., K.A., T.A. and H.Nakashita. All authors have read and agreed to the version of the manuscript submitted for publication.

Data availability

All data presented in this study are included in this article. However, raw data of this manuscript can be provided by corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Figures

Figure 1

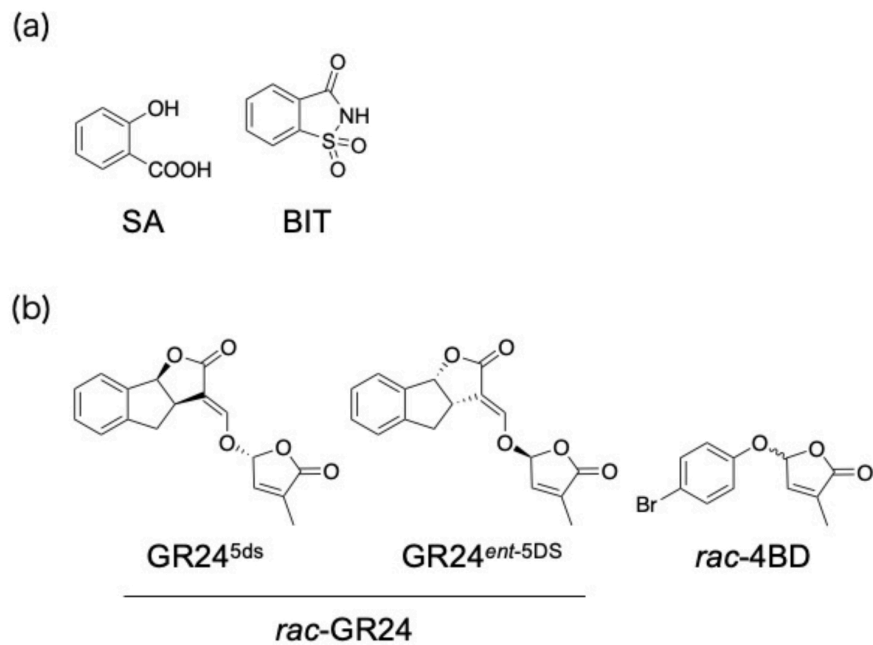


Figure 1

Chemical structures. (a) Structure of SAR inducers. SA, salicylic acid; BIT, 1,2-benzisothiazole-3(2*H*)-one 1,1-dioxide (b) Structure of SL analogs. *rac*-GR24 is a mixture of GR24^{5DS} and GR24^{ent-5DS}. *rac*-4BD, *rac*-4-bromodebranone

Figure 2

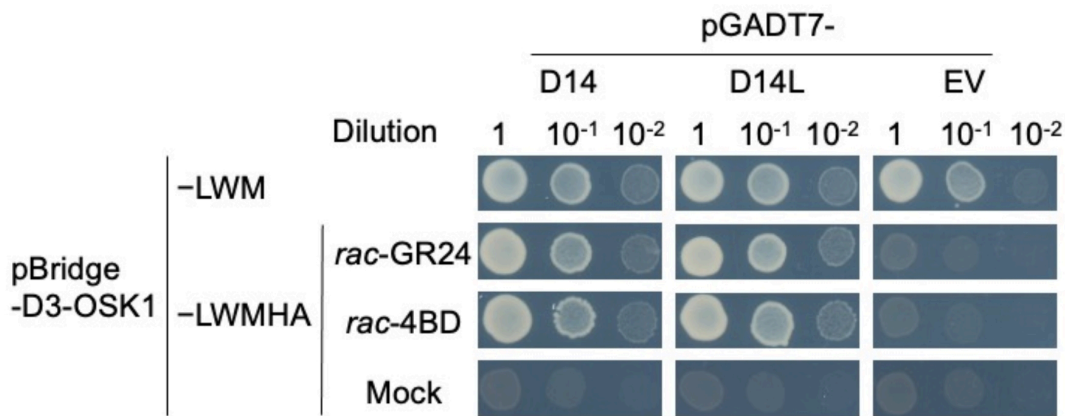


Figure 2

Effect of *rac*-GR24 and *rac*-4BD on the interaction between D14 or D14L and D3. Y3H analysis of the interaction between D14 or D14L and D3 in the presence of the third protein OSK1. Yeast (AH109) is transformed with pGADT7-D14, -D14L or -EV and pBridge-D3-OSK1. Transformants were spotted on control medium (SD -Leu, Trp, Met) (-LWM) and selection medium (SD-Leu, Trp, Met, His, Ade)

(-LWMHA) in the absence or presence of 10 μ M *rac*-GR24 or 10 μ M *rac*-4BD. Mock is the selection medium with acetone added.

Figure 3

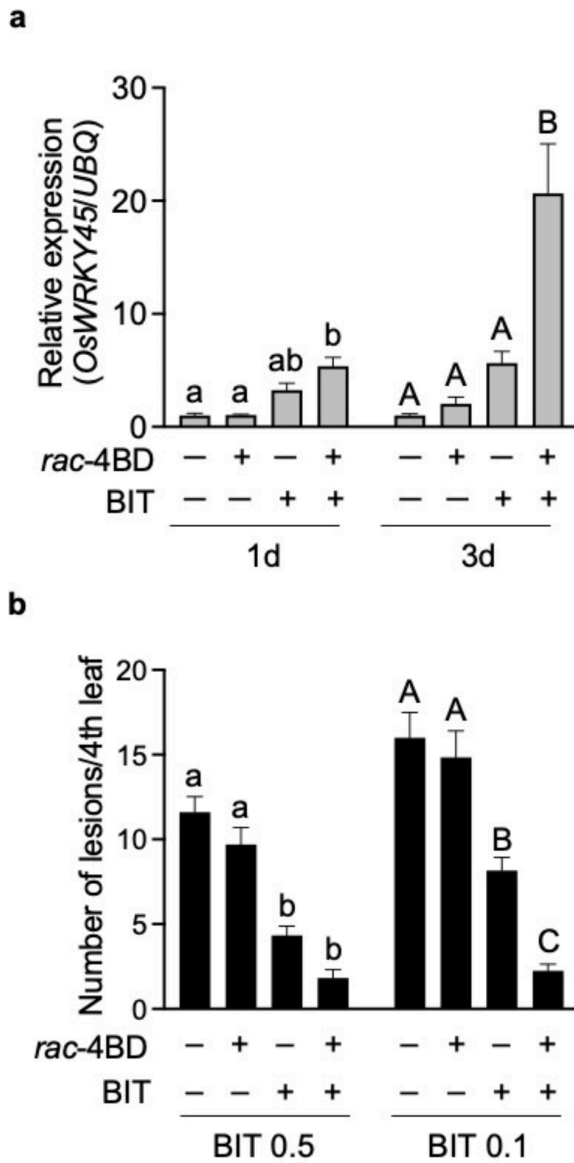


Figure 3

Effect of *rac*-4BD on BIT-induced disease resistance in rice. Rice plants at the 3.5 leaf stage were pretreated with *rac*-4BD (30 μ M in pot) and 1 day later with BIT (0.5 or 0.1 mg in pot) using the soil drenching method prior to RNA analysis and rice blast inoculation. (a) Expression of SAR marker gene.

Rice leaves were sampled 1 and 3 days after treatment with the BIT (0.5 mg in pot). The expression levels of *OsWRKY45* in the fourth leaves of plants were analyzed using RT-qPCR 1 or 3 days after treatment with compounds. The expression levels were normalized against the expression levels of *OsUBQ* measured in the same samples. Statistical analysis was performed for each sampling point. Different letters indicate significant difference. (ANOVA, Tukey's test, $p < 0.05$). (b) Rice blast inoculation assay. Spreading lesions on the fourth leaf were counted five days after inoculation. Statistical analysis was performed for each BIT concentration (0.5 mg or 0.1 mg/pot). Different letters indicate significant differences between samples (ANOVA, Tukey's test, $p < 0.05$)

Figure 4

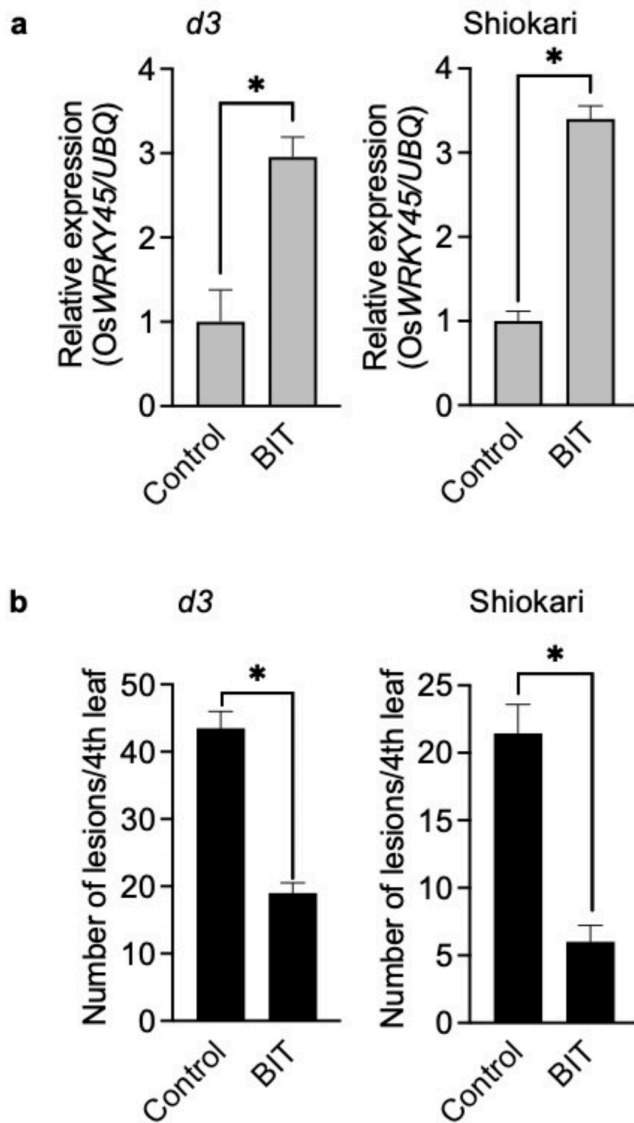


Figure 4

SAR induction in the D3-mediated signal deficient mutant. The *d3* and wild type Shiokari plants were treated with BIT (0.5 mg in the tube) using the soil-drenching method 3 days prior to gene expression analysis and challenge inoculation with rice blast fungus. (a) Expression of SAR marker gene. The expression levels of *OsWRKY45* were normalized against the expression level of *OsUBQ* measured in the same samples. The asterisks indicate significant differences compared to the control (Student's *t*-test, *,

$p < 0.05$). (b) Rice blast inoculation assay. Spreading lesions on the fourth leaf of rice plants were counted 5 days after inoculation. The asterisks indicate significant differences compared to controls (Student's t -test, *, $p < 0.05$).

Supplementary Files

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