Brassinosteroid functions in a broad range of disease resistance in tobacco and rice

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Summary

Brassinolide (BL), considered to be the most important brassinosteroid (BR) and playing pivotal roles in the hormonal regulation of plant growth and development, was found to induce disease resistance in plants. To study the potentialities of BL activity on stress responding systems, we analyzed its ability to induce disease resistance in tobacco and rice plants. Wild-type tobacco treated with BL exhibited enhanced resistance to the viral pathogen tobacco mosaic virus (TMV), the bacterial pathogen Pseudomonas syringae pv. tabaci (Pst), and the fungal pathogen Oidium sp. The measurement of salicylic acid (SA) in wild-type plants treated with BL and the pathogen infection assays using NahG transgenic plants indicate that BL-induced resistance does not require SA biosynthesis. BL treatment did not induce either acidic or basic pathogenesis-related (PR) gene expression, suggesting that BL-induced resistance is distinct from systemic acquired resistance (SAR) and wound-inducible disease resistance. Analysis using brassinazole 2001, a specific inhibitor for BR biosynthesis, and the measurement of BRs in TMV-infected tobacco leaves indicate that steroid hormone-mediated disease resistance (BDR) plays part in defense response in tobacco. Simultaneous activation of SAR and BDR by SAR inducers and BL, respectively, exhibited additive protective effects against TMV and Pst, indicating that there is no cross-talk between SAR- and BDR-signaling pathway downstream of BL. In addition to the enhanced resistance to a broad range of diseases in tobacco, BL induced resistance in rice to rice blast and bacterial blight diseases caused by Magnaporthe grisea and Xanthomonas oryzae pv. oryzae, respectively. Our data suggest that BDR functions in the innate immunity system of higher plants including dicotyledonous and monocotyledonous species.

Keywords: brassinosteroid, brassinolide, disease resistance, tobacco, rice.

Introduction

Plants have no motor apparatus that allows them to escape from environmental stresses originating from changes in the temperature, light and moisture, as well as from the attacks by pathogens and insects. Diseases caused by microorganisms are inevitable and constitute a serious stress for plants, which have evolved a unique self-protection system in addition to morphological adaptations. The primary response in this system involves the specific recognition of the pathogens and a rapid induction of localized host cell death (Ross, 1961). The secondary response is to develop an induced resistance to protect the plant’s body from further attacks by the pathogens (Kuc, 1982; McIntyre et al., 1981).

These responses are governed by hormonal regulation in which salicylic acid (SA), jasmonic acid (JA), or ethylene participates. Also, plants can activate distinct defense-signaling pathways depending on the type of pathogens (Dong, 1998; Glazebrook, 1999; Maleck and Dietrich, 1999; Pieterse and Van Loon, 1999). Systemic acquired resistance (SAR) is activated after infection by a necrotizing pathogen...
and confers resistance against a broad spectrum of plant pathogens in other uninfected parts (Chester, 1933; Durner et al., 1997). The existence of SAR has been demonstrated in many plant species, while SA was identified as a signaling molecule during the development of SAR in dicotyledonous plants such as tobacco and Arabidopsis (Delaney et al., 1994; Gaffney et al., 1993). However, the requirement of SA for the development of SAR in monocotyledonous plants is in dispute (Midoh and Iwata, 1997; Silverman et al., 1995). On the other hand, non-pathogenic rhizosphere-colonizing Pseudomonas bacteria triggers a similar systemic resistance called rhizobacteria-mediated induced systemic resistance (ISR) which requires JA-mediated but not SA-mediated signaling (Knoester et al., 1999; Pieterse et al., 2000; van Wees et al., 1999). Despite extensive studies on these signaling molecules, there is no conclusive evidence for the existence of a signaling molecule that is effective in both monocotyledonous and dicotyledonous species. However, SAR activators such as benzothiadiazole (BTH) (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996), N-cyanomethyl-2-chloroisouconitanamide (NCI) (Nakashita et al., 2002a), and probenazole (PBZ) (Midoh and Iwata, 1997; Nakashita et al., 2002b; Watanabe et al., 1979; Yoshioka et al., 2001) have been reported to induce disease resistance in monocotyledonous plants, suggesting that the defense mechanism between these two plant species are similar.

Brassinosteroids (BRs) have recently been recognized as a new class of phytohormones which play an important role in growth regulation, including the regulation of stem elongation, pollen tube growth, leaf bending, leaf unrolling, root inhibition, and cell elongation (Azpiroz et al., 1998; Clouse and Sasse, 1998; Mandava, 1988; Sakurai et al., 1999). Recent findings demonstrated that the shortage of BRs causes a dwarf phenotype in the light, and a seedling similar to the light-grown ones with developing leaves in the dark (Fujioka et al., 1997; Li et al., 1996; Schumacher and Chory, 2000; Szekeres et al., 1996). However, the importance of these molecules in living plant systems remains to be clarified. There have been several reports describing the relationship between BRs and plant stress responses such as activation of cold resistance (Hotta et al., 1998) and induction of ethylene biosynthesis (Yi et al., 1999), suggesting that BRs may play a role in stress-responding systems. Protective activities of BRs against plant diseases have been indicated based on evaluations from field trials (Khripach et al., 2000), but its mechanism at the molecular level remains to be clarified. Recent studies revealed the roles of some other phytohormones in disease resistance, as mentioned above, using model plant–microbe interaction systems. To investigate BR’s function in defense response against pathogens, we studied the potentiation capabilities and the mechanisms of action of brassinolide (BL), the most biologically active BRs to date, in model plants. We show that BL induces disease resistance both in tobacco and in rice, and that the resistance is systemically induced in tobacco with a mechanism different from known induced resistance.

Results

Brassinolide induces a broad range of disease resistance in tobacco

Tobacco is a good model for estimating the capability of chemicals to induce disease resistance (Friedrich et al., 1996; Ward et al., 1991), and is used to assess the ability of BL to enhance resistance to infection of various pathogens. Infection by tobacco mosaic virus (TMV) causes a necrotic lesion as the result of a defense response in Nicotiana tabacum cv. Xanthi nc, which possesses the N gene, a gene that confers resistance to TMV (Whitham et al., 1994). SA and SAR activators enhance this resistance and reduce the size of lesions (Durner et al., 1997; Friedrich et al., 1996; Ward et al., 1991). The average size of the lesions in BL-treated plants was about 50% smaller compared to water-treated control plants, indicating that BL enhanced the N gene-mediated resistance (Figure 1a). To ascertain whether the effect is limited to the treated leaves, two leaves of the same plants were treated and a challenge infection was performed on the distal upper leaves. The average size of the lesions in the upper leaves of BL-treated plants, as well as in the BL-treated leaves, was smaller than the control plants (Figure 1b). This indicates that the effect of BL was not only local but also systemic. Treatment of BL caused slight morphological changes in the treated leaves (Figure 1a) but not in the upper leaves. Thus, the resistance induction by BL is not correlated with the morphological changes. Generally, the development of SAR is in the order of 3–5 days when induced with PBZ. Surprisingly, a BL treatment of 24 h prior to TMV infection enhanced the resistance (Figure 1c). The effect of BL was also observed when the treatment was performed with the soil drench method (data not shown).

Next, the effect of BL on the interaction between tobacco plants and a virulent bacterial pathogen, Pseudomonas syringae pv. tabaci (Pst), was assessed. N. tabacum cv. Xanthi nc does not have a resistance gene specific to Pst and the relationship between the above plant and the pathogen is compatible. Susceptibility was estimated by measuring the growth of bacteria in leaf tissues after challenge infection. Treatment with 20 μM BL inhibited bacterial growth in the infected tissues, when compared to the water-treated plants (Figure 1d), although BL did not have any anti-microbial activity in liquid culture at tested concentrations (data not shown). This indicates that BL-induced resistance to Pst in tobacco plants. Treating
tobacco plants with 20 μM BL also reduced disease symptoms caused by infection with the virulent fungal pathogen *Oidium* sp. (powdery mildew). BL as well as benzisothiazole (BIT), an active metabolite of PBZ, and BTH induced resistance to the development of this disease (Figure 1e). As *Oidium* sp. is an obligate pathogen, the direct effect of BL on its growth could not be determined. Considering the above, we conclude that, in tobacco, BL induced a disease resistance to a broad spectrum of pathogens, which bears similarities to SAR.
Brassinolide activates a novel disease resistance mechanism in tobacco

In tobacco, the development of SAR is associated with the biosynthesis of SA and the expression of SAR marker genes, such as acidic PR-1, PR-2 and PR-5 genes. To test whether the BL-induced resistance requires SA biosynthesis, the levels of free and total SA (comprising free SA and salicylic acid glucoside, SAG) were measured in BL- and water-treated tobacco over a 7-day time course. The levels of both free and total SA detected in BL-treated plants were not significantly different from those observed in the water-treated controls at all times sampled, indicating that BL induces the resistance in tobacco without accumulation of SA (Figure 2a). This is also confirmed by the pathogen infection assays using NahG transgenic tobacco plants, which are unable to accumulate SA because of the expression of salicylate hydroxylase, a SA-degrading enzyme (Delaney et al., 1994; Gaffney et al., 1993). In TMV infection assay, the average size of lesions on the leaves of BL-treated NahG transgenic plants were smaller than for the water-treated controls (Table 1), indicating that the mechanism of resistance induction by BL does not require SA. In Pst infection assay, the bacterial growth in the infected tissues was inhibited in BL-treated plants compared to the water-treated control plants (Table 1). These data indicate that BL-induced resistance does not require SA biosynthesis for its development.

BTH can induce SAR by stimulating pathways that are downstream of SA accumulation, while BIT requires the biosynthesis of SA to induced SAR. To determine whether BL acts as a SAR activator in tobacco as BTH does, we analyzed its ability to induce PR gene expression in wild-type tobacco plants. Northern blot analysis indicates that transcripts for acidic PR genes accumulated neither in leaves treated with BL, nor in the water-treated leaves of control plants (Figure 2b). In contrast, the transcripts were detected in the leaves of BIT- or BTH-treated plants. Taken together, the BL-induced resistance is likely to be distinct from SAR.

On the other hand, in tobacco, abiotic stresses such as wounding induce systemic defense responses, which is induced via JA-mediated signaling pathway and associated with the systemic expression of basic PR genes. To determine whether BL activates this defense response in tobacco, we analyzed its ability to induce the basic PR-1 gene expression in wild-type tobacco plants. Northern blot analysis indicates that transcripts for basic PR-1 gene

Figure 2. Effect of BL on SA levels and PR gene expression.
(a) Accumulation of free and total salicylic acid in wild-type tobacco plants treated with BL. Leaves were harvested at the indicated times after treatment with 20 μM BL or water, and free and total SA (free SA and SAG) levels were quantified using HPLC. Open triangles, free SA in control plants; closed triangles, free SA in BL-treated plants; open circles, total SA in control plants; closed circles, total SA in BL-treated plants.
(b) Northern blot analysis of SAR marker gene expression in tobacco treated with BL. Plants were treated with 20 or 200 μM BL, 500 μM BIT, 100 μM BTH, or water by foliar spraying. BL-treated leaf samples were collected 1, 2 or 4 days after treatment and other samples were collected 2 days after treatment. Each lane was loaded with 5 μg total RNA. rRNA was used as an internal control for gel loading and transfer.
(c) Northern blot analysis of basic PR-1 gene expression in tobacco treated with BL. Plants were treated with 20 or 200 μM BL or water by foliar spraying or with 10 or 100 μg pot−1 BL by soil drenching. Leaves were collected 2 days after foliar treatment and 4 days after soil drench treatment. Wounded leaves (W) were collected 2 days after making cuts at the edges of leaves with scissors. L, BL-treated leaves; U, non-treated upper leaves.
accumulated neither in leaves treated with BL, nor in the water-treated leaves of control plants (Figure 2c). In contrast, the transcripts were detected in the wounded leaves. This result indicates that BL does not activate the wound-inducible signaling for defense response. Therefore, BL appears to induce resistance to a variety of pathogens by a mechanism different from those of known induced disease resistance in tobacco.

Requirement of brassinolide biosynthesis for disease resistance

During the development of known systemically induced resistances, the accumulation of related hormones in tissues is observed. For example, the infection of necrotizing bacteria induces SA accumulation and wounding induces JA accumulation. To determine whether BL biosynthesis is needed for plant defense response, we examined the effect of brassinazole 2001 (Brz) (Asami et al., 2001; Sekimata et al., 2001), an inhibitor for BL biosynthesis, on the N gene-mediated disease resistance of tobacco against TMV. The average lesion size on the leaves of Brz-treated wild-type plants was not distinct from that for the water-treated control plant (data not shown). However, the average lesion size on the leaves of Brz-treated NahG transgenic plants was larger than that for the water-treated control plants (Figure 3). Statistical analysis (ANOVA) indicated that lesion sizes of plants treated with 30, 150 µM Brz, and water were significantly different from each other (P < 0.01). Brz concentrations of more than 300 µM could not be tested because of severe affection on the plant growth as a result of the lack of BRs. Although Brz concentration slightly affected the plant growth, the suppressive effect of Brz on defense response was dose-dependent in the tested concentrations. In general, the larger TMV lesions appear on the larger leaves. However, the Brz-treated leaves were relatively smaller than the control leaves and formed larger TMV lesions. SA plays an important role in the defense response against TMV and removal of SA resulted in formation of enlarged lesions in NahG transgenic plants. Thus, in wild-type plants, it is speculated that more intense effect of SA on lesion formation covers the effect of Brz. We have not tested this effect on other pathogens, however, this result suggests that BR biosynthesis is involved in defense response at least against TMV. Conclusively, it is demonstrated that brassinosteroid-mediated disease resistance (BDR) takes part in plant defense responses independently from SA-mediated defense response.

As the experiment with Brz suggested that BL biosynthesis is required to some degree for the resistance against TMV, we then analyzed the levels of BL and the related compounds in tissues after TMV inoculation. In TMV-inoculated or mock-inoculated tobacco leaf tissue, the BL level was less than the detection limit but some biosynthetic precursors were detected (Figure 4). The levels of castasterone (CS) and 6-deoxocastasterone in TMV-inoculated leaves of wild-type plants were 0.27 and 2.88 ng g⁻¹ FW⁻¹, respectively, whereas those of mock-inoculated leaves were 0.16 and 1.52 ng g⁻¹ FW⁻¹, respectively (Figure 4), indicating that N gene-mediated defense response against TMV resulted in the increased levels of BRs in leaf tissues. These slight differences do not cause drastic morphological changes. This result also supports the theory that BDR functions in an innate immunity system in tobacco.

Simultaneous activation of systemic acquired resistance and brassinosteroid-mediated disease resistance results in an enhanced level of protection

It is reported that in Arabidopsis ISR and SAR are regulated by distinct pathways and exhibit additive effect against
pathogens (van Wees et al., 2000). BDR is likely developed through distinct signaling pathways from that of SAR because any characteristic phenomena of SAR were not observed in BDR development. The effect of simultaneous activation of SA-dependent SAR pathway and the BDR pathway on the level of systemically induced protection was examined in the wild-type tobacco plants. Lower leaves of the wild-type tobacco plants were treated with BIT or BTH to induce SAR and/or with BL to induce BDR, 5 days prior to TMV inoculation on upper leaves. The average size of lesions on leaves of SAR- or BDR-induced plants was significantly smaller than that for water-treated control plants. Plants induced by a combination of BTH and BL showed a statistically significant higher reduction of the average lesion size than plants treated with either inducer alone, indicating that SAR and BDR can exhibit additive effect on diseases (Figure 5a). This additive effect of SAR and BDR was not limited to TMV but was also observed against the bacterial pathogen *Pst* (Figure 5b), indicating that this additive effect likely depends on basic mechanisms of these resistances and is caused by a parallel activation of defense responses. Induction by a combination with BIT and BL showed the similar enhancement of resistance against TMV as the case with BTH and BL (Figure 5c), indicating that BDR induced by BL does not affect the BIT-induced SAR-signaling pathway including SA biosynthesis. This result also indicates that SAR development does not affect the BDR-signaling pathway downstream of BL.

**Brassinolide induces disease resistance in rice**

As opposed to dicotyledonous species, in monocotyledonous species, especially rice, the functions of SA and JA in defense response have not been clarified yet. To determine whether BL is able to induce resistance in monocotyledonous species, we tested the effect of BL on the disease resistance of rice plants. Rice blast disease, caused by *Magnaporthe grisea*, is one of the most economically important diseases that must be controlled in Asian countries. Several

**Figure 4.** Accumulation of BRs in TMV-inoculated tobacco leaf tissues. The wild-type tobacco plants were inoculated with TMV or water. The inoculated leaves with lesions and mock-inoculated leaves were harvested 5 days after inoculation. Levels of BRs in tissues were quantified using GC-MS: Biosynthetic pathway of BRs and levels of intermediates are shown.

**Figure 5.** Effect of simultaneous activation of BDR and SAR on pathogens. Lower leaves of tobacco plants (5-week-old) were treated by foliar spraying with either of 1 mM BIT, 200 μM BTH, 20 μM BL or water. For simultaneous activation, lower leaves of plants were treated with 20 μM BL 4 h prior to foliar treatment with BTH (a, b) or BIT (c). Challenge inoculation with TMV or *Pst* was performed 5 days later. Lesions were measured 5 days following the TMV inoculation (a, c). Growth of *Pst* in plant tissue was estimated by harvesting leaf disks 3 days post-inoculation (b). Values are shown as the mean ± SD. The experiments were repeated twice with similar results.
varieties of resistant cultivars have been created through classic breeding methods, which resulted in the appearance of many races classified by pathogenicity to cultivars. For example, Oryza sativa cv. Aichiasahi is compatible with M. grisea race 007 but incompatible with race 031. These interactions produce spreading necrotic lesions and very small, almost invisible lesions, respectively. We used the O. sativa cv. Aichiasahi–M. grisea race 007 model to assess the effects of BL. In contrast to many spreading lesions appearing on the leaves of water-treated control plants, sparse lesions were seen on the leaves of BL-treated (20 μg pot⁻¹) plants (Figure 6a). BIT, a SAR activator, reduced the size of lesions in this compatible interaction, as if it were incompatible (Figure 6a). The application of exogenous SA, however, did not have this effect on rice plants, despite its strong SAR-inducing activity in tobacco (data not shown). Treating with 100 μg pot⁻¹ of BL resulted in about 50–70% protection from pathogen infection without any visible morphological changes to leaf blades (Figure 6b). The resistance-inducing activity of BL was clearly observed starting from as low as 1 μg pot⁻¹ (data not shown), where the BL concentration in soil was estimated to be less than 200 nM. Thus, the concentrations tested are assumed to be physiologically appropriate. Although the BL-induced
resistance was not as strong as with BIT, and although the intensity of resistance varied between experiments (Figure 6b), this effect was reproducible in more than 15 independent experiments. Significant differences between the data in each experiment were statistically confirmed by ANOVA at \( P < 0.01 \) probability level.

The effect of BL treatment was also tested on rice bacterial blight caused by *Xanthomonas oryzae* pv. oryzae. Treating *O. sativa* cv. Aichiasahi plants with BL (100 or \( 10 \mu g \) pot\(^{-1} \)) reduced disease symptoms caused by infection with the virulent pathogen *X. oryzae* pv. oryzae race 003. By 14 days post-infection, the infected leaves of BL-treated plants showed slight bleaching whereas those of water-treated control plants exhibited severe bleaching (Figure 6c). Statistical analysis indicated that the lengths of infected plants treated with 100 or \( 10 \mu g \) pot\(^{-1} \) of BL, BIT or BTH were significantly shorter than those of water-treated control plants (\( P < 0.01 \)) (Figure 6d). Thus, the BL-induced resistance in rice plants is effective not only against fungal disease but also against bacterial disease, as was observed in tobacco.

The effect of Brz on defense response of rice against rice blast disease was examined, however, the result did not show the suppressive effect of Brz on defense response. This might be due to the high SA level in rice, because suppressive effect of Brz was observed in NahG transgenic tobacco but not in wild-type tobacco. However, the function of SA in defense response in rice is unclear and the rice plants without the influence of SA is not available. Thus, at the moment it cannot be determined whether BL biosynthesis is involved in defense response in rice.

To determine whether BL induces defense-related genes in rice, the expression of the *PBZ1* gene, which is induced by PBZ treatment, was examined. Northern blot analysis indicated that transcripts for *PBZ1* did not accumulate in leaves of BL-treated plants nor in water-treated control plants (Figure 6e), suggesting that the mechanism for inducing resistances is different between BL and PBZ. In conclusion, these data suggest that BDR functions also in rice plants, although the requirement of BR biosynthesis in this plant has not been determined.

**Discussion**

In this paper, we demonstrate that BL acts as an inducer of a broad range of disease resistance in tobacco as well as in rice. In tobacco, BL enhances resistance to the viral pathogen TMV, the bacterial pathogen *Pst*, and the fungal pathogen *Oidium* sp. In rice plants, BL enhances resistance to the fungal pathogen *M. grisea* and the bacterial pathogen *X. oryzae*. Measurement of SA accumulation and analysis using NahG transgenic tobacco revealed that the BL-mediated resistance enhancement does not require SA in tobacco. During induction of the resistance, BL itself does not induce the expression of the *PR* gene in either tobacco or rice. Analysis using Brz, an inhibitor for BR biosynthesis, in NahG transgenic tobacco demonstrated that BR functions in an innate immunity system in tobacco. Simultaneous activation of SAR and BDR in tobacco exhibited additive protective effect against TMV and *Pst*.

In field trials, 24-epibrassinolide was used to evaluate the effect on plant diseases (Khripach *et al.*, 2000). However, this molecule distributes in a limited number of plant species (three species) and the correlation between the activity and the natural occurrence remains to be determined. BL and its biosynthetic precursor CS distribute most widely in the plant kingdom, 25 and 37 species, respectively (Sakurai *et al.*, 1999). As it has been established recently that BL, CS and their related compounds are biosynthesized from campesterol (Fujiko *et al.*, 1997; Sakurai and Fujiko, 1997; Yokota *et al.*, 1997) and BL is the most biologically active BR, BL is considered to be the most important BR and to play pivotal roles in the hormonal regulation of plant growth and development. By using BL and model plant systems, we could show a physiologically important possibility that BRs function in defense response in higher plants, including dicotyledonous and monocotyledonous plant species.

In rice blast infection assay, the resistance induced by BL was not as intense as in an incompatible plant-pathogen interaction. The reason currently under speculation for the moderate level of induced resistance is the turnover of BL being used or the requirement of other factors. Further investigation is needed to clarify the detailed mechanism. JA was found to induce resistance to rice blast infection in the wounding model (Schweizer *et al.*, 1998), which was a moderate protection similar to the effect of BL. However, it was not effective when a whole plant was treated (Schweizer *et al.*, 1997). In rice plants only JA was suggested to be an endogenous compound that is effective against infection by pathogens. Our data demonstrate that BL is one of the most effective plant hormones to induce disease resistance in rice. In the rice blast infection assay, the intensity of BL-induced resistance varied between experiments. This may be a result of environmental factors, especially light, because the plants were cultivated in a temperature- and humidity-controlled greenhouse. As BRs play important roles in photo-morphogenesis (Fujiko *et al.*, 1997; Schumacher and Chory, 2000; Szekeres *et al.*, 1996), this observation suggests the possibility that light-signaling may be involved in the development of the BL-induced resistance.

A report that the complementation of a BL biosynthesis-deficient *Arabidopsis* mutant resulted in continuous *PR* gene expression (Szekeres *et al.*, 1996), suggests that BL induces *PR* gene expression. However, although the mechanism of SAR in *Arabidopsis* and tobacco is similar in many respects, our results show that treating wild-type
tobacco plants with BL does not lead to PR gene expression. An explanation for these different observations is that the BL used for the treatment causes a transient change in the in planta BL levels, while the complementation influences its constitutive change. Another possibility is that a cytochrome P450 enzyme, the product of the gene introduced, which is constitutively expressed under the control of the cauliflower mosaic virus 35S promoter, may have some physiological influences.

In Arabidopsis, NPR1 is a key regulator of both SAR and ISR (Cao et al., 1994; Delaney et al., 1995; Glazebrook, 1999; Shah et al., 1997). The development of ISR requires JA-dependent signaling and NPR1 but not SA (Pieterse et al., 1999). The BDR reported here shows characteristics that are similar to Arabidopsis ISR, such as independence from SA and PR gene expression and additive protective effect with SAR (van Wees et al., 1999). However, although very little is actually known about ISR in tobacco, it is proposed that SA is involved (De Meyer et al., 1999). Therefore, BDR shown here is likely to be different from ISR in this species, though more detailed studies using Arabidopsis are needed and are under investigation.

Clarke et al. (1998) demonstrated that the enhanced resistance against P. syringae pv. maculicola observed in the Arabidopsis mutant cpr6, which displays constitutive SAR, is blocked in the cpr6 npr1 double mutant, despite unaltered constitutive expression of the PR genes. This indicates that the induced resistance against P. syringae is independent of the PR proteins and that it must be accomplished through antibacterial factors, so far unidentified, that are regulated through NPR1. Our results are consistent with this observation, which indicates that the induced resistance against P. syringae is independent of PR protein, also in tobacco. However, whether the BDR is the same as ISR is currently unknown. Furthermore, although constitutive expression of PR-1 genes in tobacco is reported to confer enhanced resistance (Alexander et al., 1993), the enhanced resistance against TMV and Oidium sp., which does not involve PR gene expression, was also demonstrated. These suggest the existence of other unknown components functioning in the plant innate immune system.

Various types of cross-talks among phytohormone-mediated signalings in stress responses have been reported. BR induces the expression of 12-oxophytodienoate reductase, which is a JA biosynthetic enzyme (Muessig et al., 2000), and 1-aminocyclopropane-1-carboxylate synthase, which is an ethylene biosynthetic enzyme (Yi et al., 1999). However, whether JA or ethylene is required for BL-induced resistance in tobacco and rice is unknown so far. On the other hand, in Arabidopsis, SA is reported to inhibit JA-mediated signal transduction stimulated by abiotic stress (Niki et al., 1998; van Wees et al., 1999), although SA does not interfere with JA-mediated signaling for ISR induction (van Wees et al., 2000). SA is also reported to induce the expression of a steroid sulfotransferase which inactivates the effect of BRs in Brassica napus (Rouleau et al., 1999). Our results indicate that BL enhances resistance independently of SA and the combinations of BL and SAR inducers exhibit additive protective effect against pathogens. These mean that the SA-mediated signaling pathway for induced resistance is distinct from the JA/ethylene- and BL-mediated signaling pathways, however, cross-talks may exist between them. As the role of BL in defense response is moderately weak, the intense effect of SA may cover it as shown in the experiments with Brz. However, another possible explanation is that BL functions in the situation in which the induction of SA biosynthesis is not involved.

Similarities in the induced resistance between tobacco and rice are not yet defined. Application of exogenous SA, the most plausible signal compound for induced resistance in tobacco, is not effective in rice. Some SAR inducers such as BTH, NCI and PBZ/BIT, which stimulate SA-NPR1 signaling pathway in Arabidopsis, exert a similar effect in rice (Nakashita et al., 2002a,b). This indicates that similar signaling pathways or at least several common components for induced resistance exist in tobacco and rice, although the role of SA in the development of induced resistance of rice is unknown. Application of JA has some positive effects in rice as mentioned above, but has a suppressing effect on plant growth (data not shown). In contrast, BL at the concentration of up to 100 μg pot⁻¹ does not have such unwanted effects on the plants, whereas shortage of BL leads to dwarf plants (Choe et al., 1998; Fujio et al., 1997; Szekeres et al., 1996). As BL treatment enhanced resistance against various pathogens in both tobacco and rice, it is suggested that BL functions as one of the common signaling molecules in the innate immunity system of higher plants. This molecule is expected to give useful information to investigate the similarities and the differences in the mechanism of induced resistance between the dicotyledous and monocotyledous plant species. Our data indicate that the mechanism of BDR in tobacco is different from that of SAR and that BL induces resistance in rice in a different manner from SAR inducers. Thus, we speculate that the resistance mechanism mediated by BL is distinct from other identified ones and common to both tobacco and rice. Because BRs are essential phytohormone for the growth and development of plants, they may act in defense response by activating the fundamental biological systems which function not only in stress responses but also in other aspects of cellular regulation.

**Experimental procedures**

**Plant materials and growth conditions**

Nicotiana tabacum cv. Xanthi nc was grown in sterilized potting soil (Kureha, Japan) inside a growth chamber under a 16 h : 8 h...
light–dark regimen, at 22 °C, with 60% humidity. *Oryza sativa* was grown in sterilized potting soil in pots (5 cm × 5 cm × 5 cm) inside a greenhouse, at 25 °C during the day and at 19 °C during the night, with 50–60% humidity.

Tobacco pathogen infection assays

For the TMV infection assay, two leaves of 5-week-old plants were pre-treated by foliar spraying with various concentrations of BL, 30 or 150 μM Brz, or water. Unless otherwise mentioned, 5 days after pre-treatment, a challenge inoculation with TMV was performed on the pre-treated leaves and two non-pre-treated upper leaves, before incubation at 22 °C. Lesions were measured 5 days following the TMV inoculation.

For *Pst* and *Oidium* sp. infection assays, whole plants (5-week-old) were pre-treated with 20 μM BL, 500 μM BIT, 100 μM BTH, or water by foliar spraying. Five days after pre-treatment, a challenge inoculation was performed. *Pst* was cultured in nutrient broth at 28 °C for 2 days, and a bacterial suspension was prepared in 10 mM MgCl₂ (2 × 10⁸ colony-forming units (CFU) ml⁻¹). Challenge inoculation was performed by infiltration of the bacterial suspension using a 1-ml syringe without a needle. Leaf disks were taken from the infiltrated part of the leaves at 0, 1, 3 and 5 dpi and three disks from a plant were combined and homogenized in 10 mM MgCl₂. The number of CFU was estimated by growth on nutrient agarose plates after dilution. For each time point, three plants were used, and two samples were prepared from each plant.

*Oidium* sp. maintained on the tobacco leaves was transferred onto new tobacco leaves for inoculation and was incubated at 20–22 °C, 60–70% humidity, in 16 h: 8 h light–dark cycle. Sporulation was seen after 1 week, and the white sporophores were powdered onto the pre-treated plants placed together in a box (80 cm × 80 cm × 100 cm). Usually, about 25 cm² of fully sporulated leaf was used to infect 12 pre-treated plants. The plants were incubated as above and the degree of disease symptoms was evaluated (Nakashita et al., 2002a).

Rice pathogen infection assays

*Oryza sativa* cv. Aichiasahi was cultured in a pot (five or seven plants per pot) in a greenhouse and three-leaf stage plants were used for experiments. Pre-treatment with various concentrations of BL, BIT, BTH or water was performed by soil drench application. Five days after pre-treatment, challenge inoculation with *M. grisea* or *X. oryzae pv. oryzae* was performed on pre-treated leaves. In rice blast assay, plants were sprayed with *M. grisea* conidia suspension (10⁵ spores ml⁻¹), kept under dark condition with 100% humidity for 16 h, then incubated for 5 days in a greenhouse (25 °C). In rice bacterial blight assay, plants were cut at about 4 cm from the tip of leaf-4, sprayed with cell suspension (10⁶ CFU ml⁻¹) of *X. oryzae pv. oryzae*, and kept in a greenhouse under the following condition: 24 °C and 70% humidity for 10 h without light; 28 °C and 70% humidity for 14 h with light.

RNA analysis

Tobacco plants were treated with various concentrations of BL, 500 μM BIT, 100 μM BTH, or water by foliar spraying, and the leaves were harvested at various times after application. Rice plants were treated by soil drench method. Total RNA was extracted from frozen leaf samples of the plants using TRIzol reagent (Life Technologies, Rockville, MD, USA) following the manufacturer’s instructions. DNA fragment of the coding regions for tobacco acidic PR genes (Ward et al., 1991) and rice *PBZ1* (Midoh and Iwata, 1996) were amplified by polymerase chain reaction (PCR) from cDNA prepared from SA-treated tobacco and PBZ-treated rice, respectively. The PCR products were cloned into plasmid pCR2.1 (Invitrogen, Carlsbad, CA, USA) and the nucleotide sequences were confirmed. ³²P-labeled cDNA probes were synthesized by random priming of these fragments of PR-1, PR-2, PR-5 and *PBZ1* genes. Total RNA samples were subjected to 1.5% agarose, 1.1% formaldehyde gel electrophoresis and transferred to a nylon membrane (Hybond N⁺, Amersham, Buckinghamshire, UK). After the transfer, RNA was cross-linked to the membrane using an UV linker (GS GENEBINER, Bio-Rad, Hercules, CA, USA). Pre-hybridization was performed for 1 h or longer at 68 °C. Hybridization and washing were performed as described by Church and Gilbert (1984).

Extraction and analysis of brassinosteroids

TMV was inoculated on three to four leaves of 5-week-old tobacco plants and incubated for 5 days. Sterilized water was used for mock inoculation. The inoculated leaves with lesions and mock-inoculated leaves (40 g FW⁻¹ each) were harvested and stored at −80 °C for BR analysis. Extraction and gas chromatography–mass spectrometry analysis were performed as previously described (Noguchi et al., 1999).

Extraction and analysis of salicylic acid

Plants were treated by foliar spraying with 20 μM BL or water. At 2–7 dpi, leaves were harvested from the treated plants and SA and SAG levels were measured as previously described (Nakashita et al., 2002b; Yoshioka et al., 2001).

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