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# Involvement of C-22-Hydroxylated Brassinosteroids in Auxin-Induced Lamina Joint Bending in Rice

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**The rice lamina joint is ideal material for investigating the activity of brassinosteroids (BRs) and auxin because of its high sensitivity to these compounds. Using a series of rice BR biosynthetic and receptor mutants, we conducted lamina joint tests to elucidate the mechanism of cross-talk between BR and auxin signaling in lamina joint bending. In BR biosynthetic mutants *d2* and *brd1*, which are defective in C-23 hydroxylase and C-6 oxidase, respectively, the lamina joint response to auxin was significantly higher than that of wild-type plants. The other BR-biosynthetic mutants, *brd2*, *osdwarf4* and *d11*, which are defective in C-22-hydroxylated BRs, showed less or no response to auxin. These results suggest that C-22-hydroxylated BRs are involved in auxin-induced lamina joint bending. The results were supported by the observation that inhibition of the hyper-response to auxin in *d2* was reduced by treatment with brassinazole, which inhibits the function of DWARF4, the C-22 hydroxylase. In *d61*, which is defective in *OsBRI1*, a possible BR receptor in rice, the bending angle of the lamina joint in response to auxin and C-22-hydroxylated 6-deoxoBRs was nearly the same as that in wild-type plants. This implies that C-22-hydroxylated BRs function in auxin signaling independently of OsBRI1. From these observations, we propose that C-22-hydroxylated BRs participate in auxin signaling via a novel OsBRI1-independent signaling pathway.**

**Keywords:** Auxin • Brassinosteroid • Lamina joint • Rice.

**Abbreviations:** BL, brassinolide; BR, brassinosteroid; Brz, brassinazole; CS, castasterone; CT, cathasterone; 3DT, 3-dehydroteasterone; 22-OHCR, (22S)-22-hydroxycampesterol; TE, teasterone; TY, typhasterol.

## Introduction

The lamina joint is a tissue between the leaf blade (leaf lamina) and leaf sheath that acts like a hinge to bend the leaf blade. The bending angle of the lamina joint is regulated by various factors, such as plant hormones, herbicides and fertilizers (Maeda 1965, Takeno and Pharis 1982, Cao and Chen 1995). The lamina joint bending assay is established as an indicator to evaluate the effects of plant hormones, herbicides and light on rice leaf bending, which is thought to affect crop yields (Maeda 1960, Maeda 1961, Maeda 1962, Maeda 1965). Isolation of the most active brassinosteroid (BR), brassinolide (BL), in the late 1970s (Grove et al. 1979) led to the discovery of strong lamina joint bending activity mediated through BRs (Wada et al. 1981). At the same time, synergistic effects of BRs and auxin were elucidated (Mandava 1988). The lamina joint inclination assay was then recognized as an extremely sensitive system for determination of biological activity of natural or synthetic BRs and auxin (Wada and Marumo 1981, Takeno and Pharis 1982, Kim et al. 1990, Fujioka et al. 1998).

Binding experiments with BRI1, the BR receptor in Arabidopsis, demonstrated that BL and castasterone (CS) are the active BRs (Wang et al. 2001). Recently, BR biosynthetic pathways deduced from in vivo metabolite conversion analysis were reconfirmed or redrawn using biochemical assays with heterologously expressed enzymes in Arabidopsis,

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tomato and rice. According to these studies, CYP724B2 (DWARF11) in rice and tomato, CYP90B1 (OsDWARF4) in rice and CYP90B3 in tomato were identified as C-22 hydroxylases, which convert campesterol to (22*S*)-22-hydroxycampesterol (22-OHCR), (24*R*)-ergost-4-en-3-one to (22*S*,24*R*)-22-hydroxyeragost-4-en-3-one, (24*R*)-5α-ergostan-3-one to (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one, campestanol to 6-deoxocathasterone (6-deoxoCT) and 6-oxocampestanol to cathasterone (CT) (Ohnishi et al. 2006b, Sakamoto et al. 2006). In Arabidopsis, CYP90C1 and CYP90D1 were identified as C-23 hydroxylases that convert 22-OHCR to (22*R*,23*R*)-22,23-dihydroxycampesterol, (22*S*, 24*R*)-22-hydroxyergost-4-en-3-one to (22*R*,23*R*)-22,23-dihydroxy-campest-4-en-3-one, (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one to 3-dehydro-6-deoxoteasterone (6-deoxo 3DT) and 6-deoxotyphasterol (6-deoxoTY) via 3-epi-6-deoxoCT and 6-deoxoCT to 6-deoxoteasteone (6-deoxoTE); and CT to TE, respectively (Ohnishi et al. 2006a). The activity of CYP85A1 (OsDWARF) of rice confirmed it was a C-6 oxidase that converts 6-deoxoTE to TE, 6-deoxo3DT to 3DT, 6-deoxoTY to TY and 6-deoxoCS to CS (Hong et al. 2002).

Since an auxin response element containing the sequence TGTCTC was demonstrated as a cross-talk point for BR and auxin signaling (Nakamura et al. 2003, Nemhauser et al. 2004), studies on cross-talk between these two plant hormones have become one of the most active research areas in plant science. Because the lamina joint inclination assay is extremely sensitive to BRs and auxin, we considered it an appropriate system for studying cross-talk between BRs and auxin. To date, several BR-related rice mutants have already been isolated. Erect leaves observed in mild BR-related mutants suggest that regulation of lamina joint bending is related to the endogenous BR level and BR signaling (Yamamuro et al. 2000, Hong et al. 2003, Nakamoto 2004, Sakamoto et al. 2006). In this study, we conducted a series of lamina joint inclination assays using various BR-related mutants to investigate cross-talk between BRs and auxin in rice. The mutants used were the relatively weak *osbri1* mutants, *d61-1* and *d61-2* (Yamamuro et al., 2000), *brd2*, which is defective in the conversion of 24-methylenecholesterol to campesterol (Hong et al. 2005), *d11-3* and *osdwarf4-1* (*osdwarf4*), which are defective in the C-22 hydroxylation step (Tanabe et al. 2005, Sakamoto et al. 2006), *brd1-3*, which is the weakest allele of the rice C-6 oxidase mutant (Hong et al. 2002), and *d2-2* and *d2-4* (Hong et al. 2003). The *d2* mutants that lack CYP90D2 were first reported as possibly defective in the conversion steps from 6-deoxoTE to 3-dehydro-6-deoxoTE and from TE to 3-dehydroTE (Hong et al. 2003). However, later in vitro experiments demonstrated that the ortholog for CYP90D2, CYP90D1 in Arabidopsis, catalyzes C-23 hydroxylation steps, as described above (Ohnishi et al. 2006a). Thus, according to the homology to CYP90D1, it is natural to consider that CYP90D2 also catalyzes the C-23 hydroxylation step and that the *d2* mutant is defective in C-23-hydroxylated BRs in rice. We discuss the results based on information from redrawn BR biosynthetic pathways. The results suggest that C-22-hydroxylated BR is involved in auxin-induced lamina joint bending without conversion into CS or BL, and imply that the response is OsBRI1 independent.

## Results

### Age-dependent response of lamina joint bending to brassinosteroids and auxin

First, we investigated the age-dependent sensitivity of the lamina joint to BL, the most active BR, and IAA. Responses to BL and BL with IAA were highest in plants 2.5–3 d after germination, when the lamina joint of the second leaf had appeared and the second leaf blade was just about to open, after which the response decreased (**Fig. 1**). The response to IAA was observed in plants 3 d after germination and the bending angle was approximately the same in 3- and 4-day-old plants (**Fig. 1**). These results suggest that age-dependent response patterns of the lamina joint in response to IAA differ from those to BL.

### Differences in auxin response between biosynthetic and receptor mutants of brassinosteroids

To investigate cross-talk between BRs and auxin in rice, we analyzed the lamina joint response to auxin in a BR biosynthetic mutant, *d2* (Hong et al. 2003), and in the BR-insensitive mutant *d61*, which is defective in the BR receptor, OsBRI1

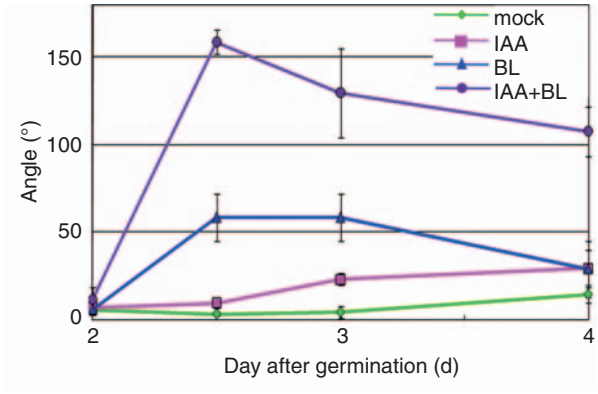


**Fig. 1** Age-dependent lamina joint response to brassinolide (BL) and IAA in wild-type plants. The age-dependent lamina joint response to BL and IAA was observed in wild-type plants. A 10 ng aliquot of BL and/or 5 μg of IAA dissolved in 1 μl ethanol was dropped onto the tip of the second leaf blade on the indicated day after germination and the bending angle measured after 2 d. Data represent the means ± SD of at least eight plants. The results were reproduced in two independent experiments.

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(Yamamuro et al. 2000). In *d61*, the response to auxin was relatively weaker than that in the wild type. In contrast, in *d2* the bending angle induced by auxin was much higher than that in the wild type (**Fig. 2A**). This contradiction suggests that the lamina joint response induced by auxin is affected by endogenous BR levels and BR signaling; however, the cross-talk is not straightforward. Thus we conducted several lamina joint inclination assays using various BR biosynthetic mutants: *brd2* (Hong et al. 2005), *d11-3* (Tanabe et al. 2005), *osdwarf4-1* (Sakamoto et al. 2006), *d2-2* and *d2-4* (Hong et al. 2003), *brd1-3* (Hong et al. 2002) and alleles of *d61* mutants, *d61-1* and *d61-2* (Yamamuro et al. 2000), as described in detail in the Introduction. The hyper-response to auxin was observed only in alleles of *d2* and *brd1-3*, which are defective in later BR biosynthetic pathways, and less or no response to auxin was observed in the biosynthetic mutants, *brd2*, *d11* and *osdwarf4*, which are defective in the earlier BR biosynthetic pathways (**Fig. 2B**). In *d61* mutants, the auxin response was similar to or relatively weaker than that in the wild type, as described above (**Fig. 2B**).

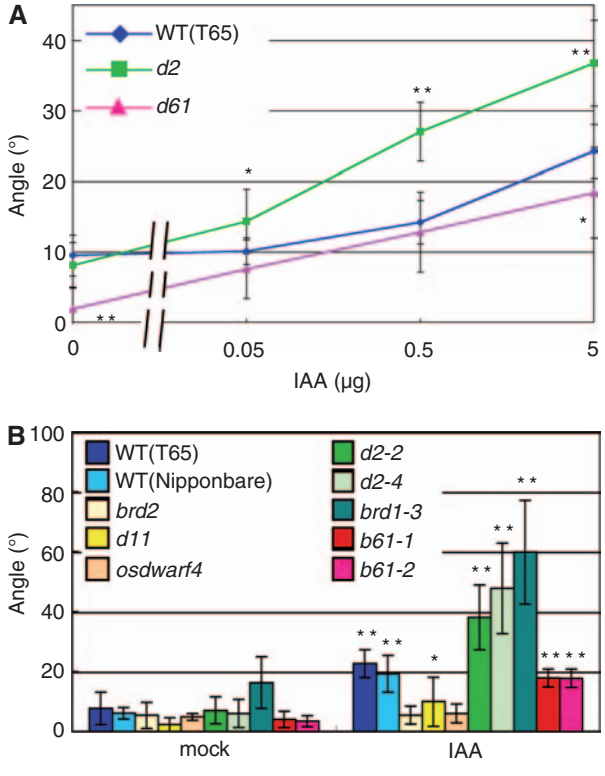


**Fig. 2** Auxin-induced lamina joint bending in BR-related mutants. (A) The bending angle of the lamina joint in the BR-deficient mutant *d2*, and in the OsBRI1-deficient mutant *d61* in response to 5 μg of IAA. (B) The bending angle of the lamina joint in BR-deficient mutants, *brd2*, *d11*, *osdwarf4-1*, *d2-2*, *d2-4* and *brd1-3*, and alleles of the OsBRI1-deficient mutants, *d61-1* and *d61-2*, in response to 5 μg of IAA. The mutants *d11*, *d2-2*, *d61-1* and *d61-2* are of the T65 ecotype, and *brd2*, *osdwarf4-1*, *d2-4* and *brd1-3* are of the Nipponbare ecotype. Data represent the means ± SD of at least eight plants (**P* < 0.05, ***P* < 0.005, Student's *t*-test). The results were reproduced in two independent experiments. The mutants *brd2*, *d11* and *osdwarf4* showed less or no response to auxin, while *d2* and *brd1* showed hyper-sensitivity to auxin. The auxin response in *d61* mutants was relatively smaller than that in wild-type plants.

## The lamina joint response to 6-deoxobrassinosteroids with auxin in osdwarf4

One of the differences between hyper-auxin-sensitive BR mutants and the less auxin-sensitive BR mutants described above is likely to be the kinds of BR accumulated in the mutants. We used 6-deoxoBRs as representatives of C-22-hydroxylated BRs (described as C-22-hydroxylated 6-deoxoBRs in this manuscript). Thus, we treated plants of *osdwarf4* with C22-hydroxylated 6-deoxoBRs (e.g. 6-deoxoCT, 6-deoxoTE, 6-deoxo3DT, 6-deoxoTY and 6-deoxoCS) and IAA and measured the resultant angle of the lamina joint. As expected, C22-hydoxylated 6-deoxoBRs themselves show little activity in *osdwarf4* (**Fig. 3**). However, the angle of the *osdwarf4*

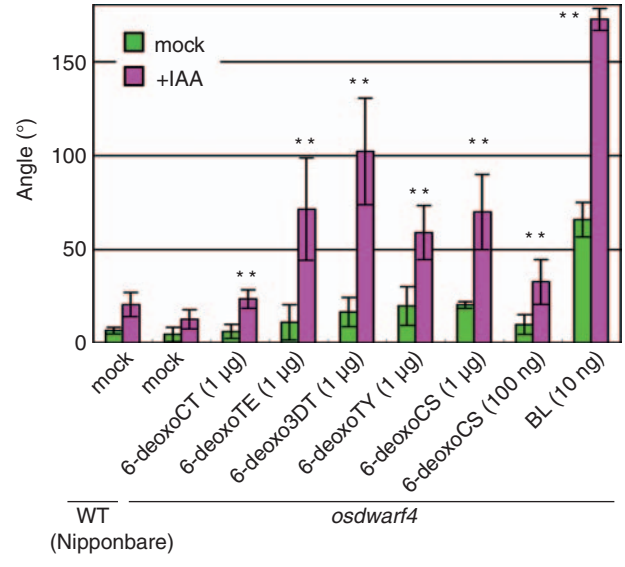


**Fig. 3** Effect of C-22-hydroxylated 6-deoxoBRs on auxin-induced lamina joint bending in *osdwarf4* plants. The effect of C-22-hydroxylated 6-deoxoBRs on auxin-induced lamina joint bending in the *osdwarf4* mutant was examined. A 1 μg aliquot of C-22-hydroxylated 6-deoxoBRs, 1 μg or 100 ng of 6-deoxoCS or 10 ng of BL with or without 5 μg of IAA was given to 3-day-old *osdwarf4* mutants for 2 d and the resultant bending angle of the lamina joint measured. Data represent the means ± SD of at least seven plants (**P* < 0.05, ***P* < 0.005, Student's *t*-test). The bending was synergistically induced by C-22-hydroxylated 6-deoxoBRs and IAA, and by BL and IAA. 6-deoxoCT, 6-deoxocathasterone; 6-deoxoTE, 6-deoxoteasterone; 6-deoxo3DT, 3-dehydro-6-deoxoteasterone; 6-deoxoTY, 6-deoxotyphasterol; 6-deoxoCS, 6-deoxocastasterone.

lamina joint in response to auxin with the C-22-hydroxylated 6-deoxoBRs was synergistically increased, despite the lower or lack of activity of IAA or the C-22-hydroxylated 6-deoxoBRs alone (**Fig. 3**). We also found that the auxin response was significantly higher in *osdwarf4* mutants treated with 6-deoxo3DT and IAA than when treated with 6-deoxoTY and IAA (Student's *t*-test, $P < 0.05$). The result is noteworthy because if these C22-hydroxylated 6-deoxoBRs are converted into the active BRs, CS or BL, and show activity as BRs, 6-deoxoTY must induce stronger lamina joint bending than 6-deoxo3DT because 6-deoxoTY is a product of 6-deoxo3DT.

## The lamina joint response to auxin in brassinazole-treated d61 and d2

To analyze further whether C-22-hydroxylated BRs are required for the auxin-induced lamina joint response, we investigated the bending angle of the lamina joint in *d61* when treated with a series of concentrations of brassinazole (Brz), a specific inhibitor of the BR biosynthetic enzyme, DWARF4 (Asami et al. 2001). Because the angle of the lamina joint in *d61* is very small without any hormone treatment, a response to Brz alone was not observed (**Fig. 4A**, blue line); with IAA treatment, the angle increased to 20°. When plants were pre-treated with Brz for 12 h, the angle induced by IAA treatment decreased in a dose-dependent manner as the concentration of Brz increased (**Fig. 4A**, pink line). This suggests that the auxin-induced lamina joint response depends on the amount of endogenous BRs, especially BRs downstream of the C-22 hydroxylation catalyzed by DWARF4. Thus, we investigated whether the hyper-response observed in *d2* was canceled out by Brz treatment. We analyzed the bending angle of *d2* pre-treated with 1, 10 and 100 µM Brz followed by IAA treatment. The bending angle induced by IAA was decreased by Brz treatment in *d2*, as observed in *d61*, in a Brz concentration-dependent manner (**Fig. 4B**, green line). This result strongly supports the above hypothesis that C-22-hydroxylated BRs participate in the lamina joint response to auxin.

## The synergistic response of brassinosteroids and auxin in d61

We investigated whether the interaction with IAA of C-22 hydroxylated BRs is activated via the OsBRI1-mediated signaling pathway. Wild-type and *d61* plants were treated with the C-22-hydroxylated 6-deoxoBRs, CS and BL with or without IAA. The lamina joint response induced with C-22-hydroxylated 6-deoxoBRs alone was small, in both wild-type and *d61* plants (**Fig. 5A**, dark blue bars and red bars, respectively). As expected, both CS and BL clearly induced lamina joint bending in wild-type plants after application of IAA, and the induction was inhibited in *d61* (**Fig. 5A**). When

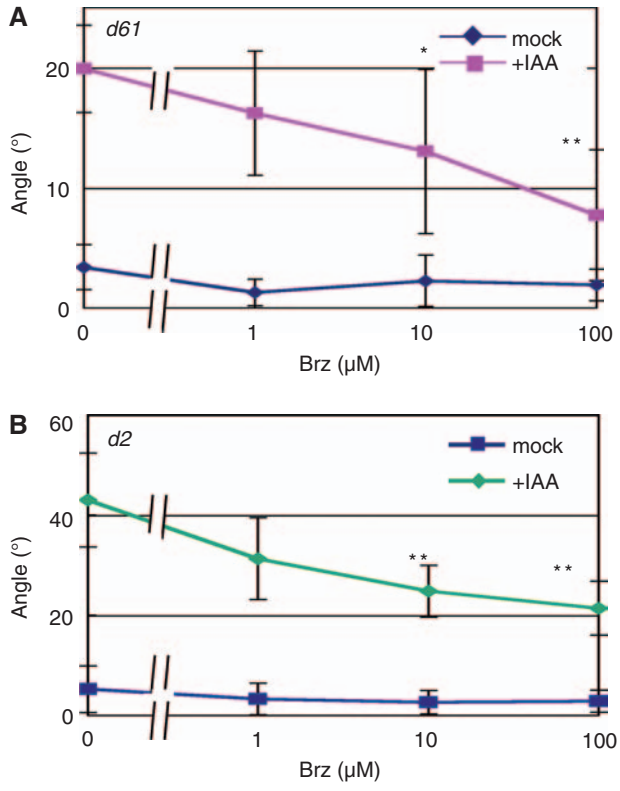


**Fig. 4** Effect of brassinazole (Brz) on auxin-induced lamina joint bending in *d61* and *d2* plants. The effect of Brz on auxin-induced lamina joint bending in (A) *d61* and (B) *d2* was investigated. Plants were treated with the indicated concentrations of Brz on the tip of the second leaf 12 h before IAA treatment. A 5 µg aliquot of IAA was given to 3-day-old plants for 2 d and the bending angle of the lamina joint measured. Data represent the means ± SD of at least eight plants (*$P < 0.05$, **$P < 0.005$, Student's *t*-test). The results were reproduced in two independent experiments. In both *d61* and *d2*, the auxin response was significantly inhibited by Brz treatment.

C-22-hydroxylated 6-deoxoBRs were applied to wild-type plants with IAA, the lamina joint response was significantly higher than with IAA alone (**Fig. 5A**, light blue bars). Furthermore, the lamina joint response to C-22-hydroxylated 6-deoxoBRs with IAA was not affected by the *d61* mutation, while that to CS or BL with IAA was inhibited by the mutation (**Fig. 5A**, compare light blue bars with orange bars). This implies that the synergistic response to treatment with IAA and C-22-hydroxylated 6-deoxoBRs is not mediated by OsBRI1. We also analyzed the bending angle of wild-type and *d61* plants in response to IAA with a series of BL concentrations. The response to IAA and BL is apparently larger in the wild type than in *d61*, especially when the amount of applied BL was <1 ng. In contrast to the response to C-22-hydroxylated BRs with IAA, these results suggest that the synergistic response to IAA and BL is also mediated by the OsBRI1-dependent pathway (**Fig. 5B**).

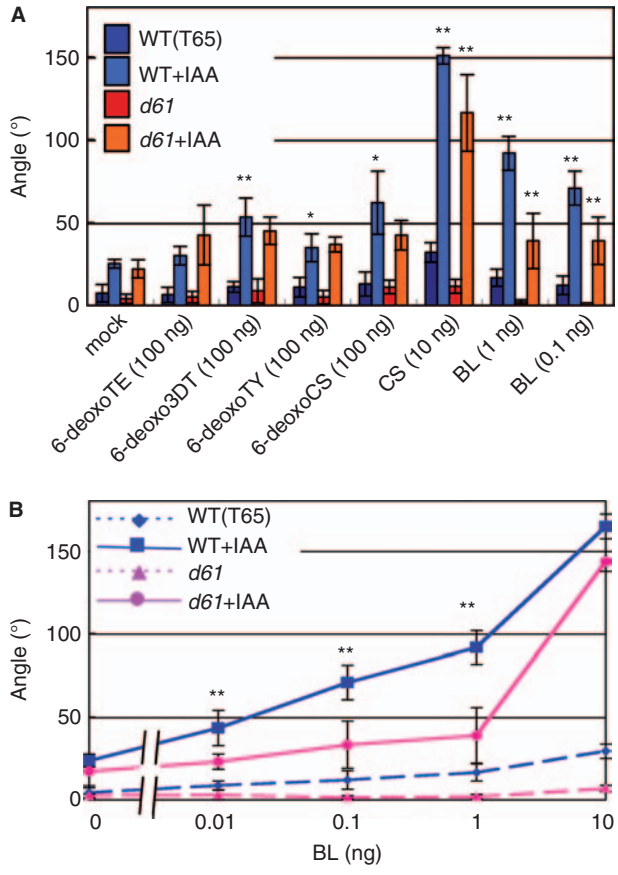


**Fig. 5** Auxin- and BR-induced lamina joint response in *d61* plants. (A) The effect of the *d61* mutation on lamina joint bending induced by IAA and BRs was examined. Three-day-old wild-type and *d61* plants were treated with 100 ng of C-22-hydroxylated 6-deoxoBRs, 10 ng of CS, or 1 or 0.1 ng of BL, with or without 5 μg of IAA, for 2 d and the bending angle of the lamina joint measured. Data represent the means ± SD of at least seven plants. (*$P < 0.05$, **$P < 0.005$, Student's *t*-test. The *P*-values of wild-type plants treated with IAA were calculated against those of corresponding mock-treated wild-type plants, and the *P*-values of *d61* plants treated with IAA were calculated against those of corresponding IAA-treated wild-type plants.) All CS, BL and C-22-hydroxylated 6-deoxoBRs significantly increased the bending angle in response to IAA in the wild type, and the increase induced by C-22-hydroxylated 6-deoxoBRs, but not by CS or BL, was not affected by the *d61* mutation. (B) The dose-dependent response to BL with or without IAA was analyzed. Three-day-old wild-type or *d61* plants were treated with the indicated amount of BL with or without 5 μg of IAA for 2 d and the bending angle of the lamina joint measured. Data represent the means ± SD of at least seven plants. (**$P < 0.005$, Student's *t*-test. *P*-values were calculated against the value of corresponding IAA-treated *d61* plants.) The results were reproduced in two independent experiments. The response to IAA with BL was considerably greater in wild-type than in *d61* plants.

## Brassinolide regulation of the hyper-response to auxin in d2

We investigated whether the hyper-response to auxin observed in *d2* was regulated by exogenous BL application. The hyper-response to IAA observed in *d2* was dose-dependently inhibited by application of 0.01–0.1 pg of BL (**Fig. 6**), while with ≥1.0 pg of BL, a synergistic response was observed, as in the wild type. This suggests that the auxin-induced hyper-response in *d2* was negatively regulated by BL.

## Cell elongation in auxin-treated brassinosteroid mutants

Because lamina joint bending occurs exclusively through cell elongation (Maeda 1965), we compared the length along the longitudinal axis of epidermal cells of the lamina joint in IAA-treated wild type, *d2*, *d2* treated with 0.1 pg of BL, *osdwarf4* and *d61*. In the wild type, the length of cells increased after IAA treatment (compare **Fig. 7A, F, K**). The length of lamina joint cells of *d2*, *d2* treated with 0.1 pg of BL and *osdwarf4* was approximately the same as that of the wild type (**Fig. 7A–D, K**), while that of *d61* was decreased (**Fig. 7E, K**). In IAA-treated *d2*, which shows a hyper-response to IAA, the length of lamina joint cells was apparently longer than that of IAA-treated wild-type plants (**Fig. 7F, G, K**), while elongation was restricted by application of 0.1 pg of BL (**Fig. 7H, K**). As observed in the bending angle of the lamina joint, cell elongation was also not induced by IAA treatment in *osdwarf4* (**Fig. 7D, I, K**). In *d61*, because the original cells were much shorter than those of the wild type (**Fig 7A, E, K**), the resultant cell length in IAA-treated *d61* was approximately the same as in mock-treated wild type (**Fig. 7A, J, K**); however, we confirmed that cell elongation was significantly induced by IAA treatment (**Fig. 7E, J, K**). Thus, the length of lamina joint cells was well correlated to the bending angle of the lamina joint. Our results also suggest that even though the bending angle of the lamina joint was relatively smaller than that of the wild type, auxin definitely induced cell elongation in *d61*.

## Discussion

In this study, we investigated cross-talk between BR and auxin in rice using the lamina joint test as an indicator. The elongation of epidermal cells of the lamina joint was well correlated with the bending angle of the joint (**Fig. 7**), demonstrating that the resultant bending angle of the lamina joint reflects regulation at the cellular level.

Lamina joint tests using a series of BR-related mutants demonstrated that C-22-hydroxylated BRs are required for auxin-induced lamina joint bending. This finding is supported by the results described below. First, sensitivity to auxin was enhanced in *d2* and *brd1*, but not in *brd2*, *osdwarf4* or *d11*, suggesting that differences between mutants

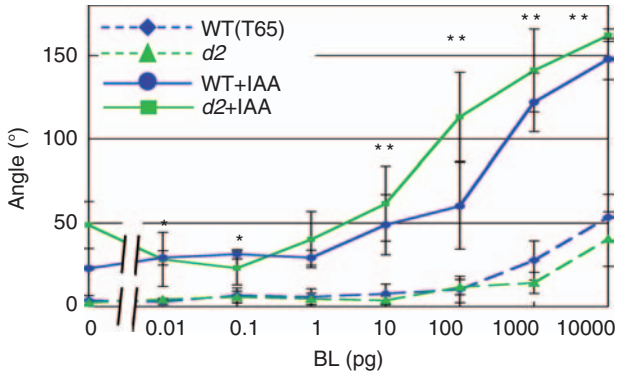


**Fig. 6** Effect of BL on IAA-treated *d2* plants. The dose-dependent response to BL of IAA-treated *d2* plants was examined. Wild-type and *d2* plants 3 d after germination were treated with the indicated amount of BL with or without 5 μg of IAA for 2 d, and then the angle of the lamina joint was measured. Data represent the means ± SD of at least eight plants. In the data for IAA-treated *d2*, *P*-values were calculated against the value of 0 M BL-treated plants (**P* < 0.05, ***P* < 0.005, Student's *t*-test).The hyper-response observed in *d2* was cancelled by application of 0.01–0.1 pg of BL. The results were reproduced in at least two independent experiments.

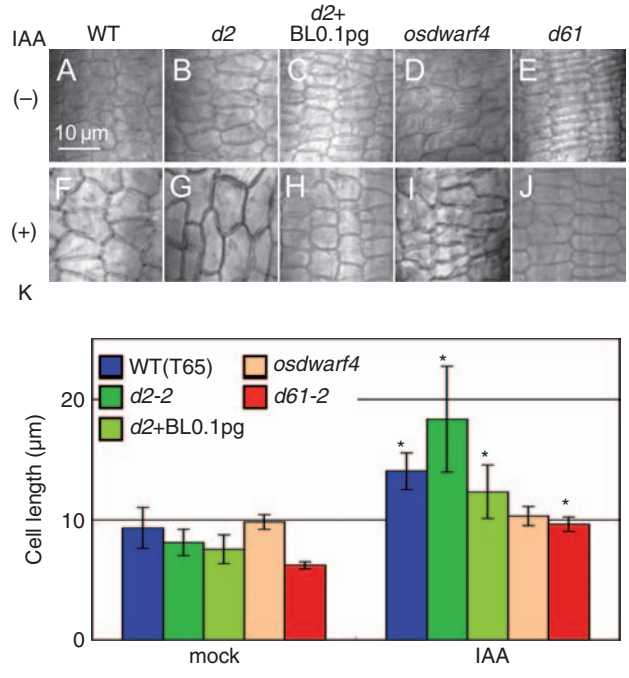


**Fig. 7** Length of epidermal cells of the lamina joint. (A–J) The morphology of epidermal cells of the lamina joint was examined. The lamina joints of wild-type (A, F), *d2* (B, G), *d2* given 0.1 pg of BL (C, H), *d61* (D, I) and *osdwarf4* (E, J) plants were given 1 μl of ethanol (mock; A–E) or 1 μl of ethanol containing 5 μg of IAA (F–J). Bar = 10 μm. (K) The lengths of epidermal cells of the lamina joint in wild-type, *d2*, *d2* with 0.1 pg of BL, *d61* and *osdwarf4* plants with or without 5 μg of IAA are presented in A–J. Data represent the means ± SD of at least four independent plants (**P* < 0.05, Student's *t*-test).

affect auxin signaling. The evident difference between these mutants was in accumulation of BRs through a defective step in the BR biosynthetic pathway. Namely, the latter three mutants contain much less C-22-hydroxylated BR than the former two mutants, suggesting that the amount of C-22-hydroxylated BRs affects auxin signaling (**Fig. 8A**; Hong et al. 2002, Hong et al. 2003, Hong et al. 2005, Sakamoto et al. 2006). Secondly, C-22-hydroxylated 6-deoxoBRs greatly and synergistically enhanced the response to IAA in *osdwarf4* (**Fig. 3**), even though the responses to auxin or C-22-hydroxylated 6-deoxoBRs alone were restricted, suggesting that the activity of auxin is enhanced by C-22-hydroxylated 6-deoxoBRs or vice versa. Thirdly, the auxin-induced lamina joint responses in both *d2* and *d61* were inhibited by Brz treatment (**Fig. 4A, B**; Asami et al. 2001). In *d61*, in which endogenous BRs accumulate (Yamamuro et al. 2000), the total amount of C-22-hydroxylated BRs may decrease after Brz treatment, while in *d2*, in which endogenous BRs are basically lower than in the wild type (Hong et al. 2003), endogenous BRs may be further decreased by Brz treatment. These results suggest that the critical component for auxin-induced lamina joint bending is the amount of C-22-hydroxylated BRs, and not the total amount of BRs or the activity of OsBRI1. It is possible that the bending angle induced by auxin was decreased by Brz treatment because of the loss of the resultant level of active BRs, such as CS or BL. However, this is unlikely, because the bending angle induced by auxin was increased in *d2* and *brd1* mutants, in which the amount of CS was decreased (Hong et al. 2002, Hong et al. 2003) compared with that of the wild type (**Fig. 2**), and because the hyper-response to auxin in *d2* was decreased by application of 0.01–0.1 pg of BL (**Fig. 6**). These results suggest that C-22-hydroxylated BRs themselves are critical for the auxin-induced lamina joint response.

The result in **Fig. 5** suggests that the C-22-hydroxylated BR-mediated auxin response is rather OsBRI1 independent because the response to auxin with C-22-hydroxylated 6-deoxoBRs was not inhibited by the *d61* mutation, while that with CS and BL was inhibited (**Fig. 5A**). This implies that auxin induces lamina joint bending via at least two different BR signal pathways in rice; one is the well-known OsBRI1-mediated signal pathway and the other is an OsBRI1-independent C-22-hydroxylated BR-mediated pathway. Very recently, it was demonstrated that a moss (*Physcomitrella patens*) has BRs, but not CS and BL, while no orthologous genes for BRI1 were found in the moss genome (Yokota et al. personal communication, Rensing et al. 2008, PHYSCObase: http://moss.nibb.ac.jp/). We confirmed that none of the genome sequences of moss was homologous to the full-length BRI1 amino acid sequence. However, genes homologous to those that participate in the well-characterized auxin signaling, such as *Aux/IAA* or *TIR1*, were present in moss (Imaizumi et al. 2002,Hayashi et al. 2008), suggesting that

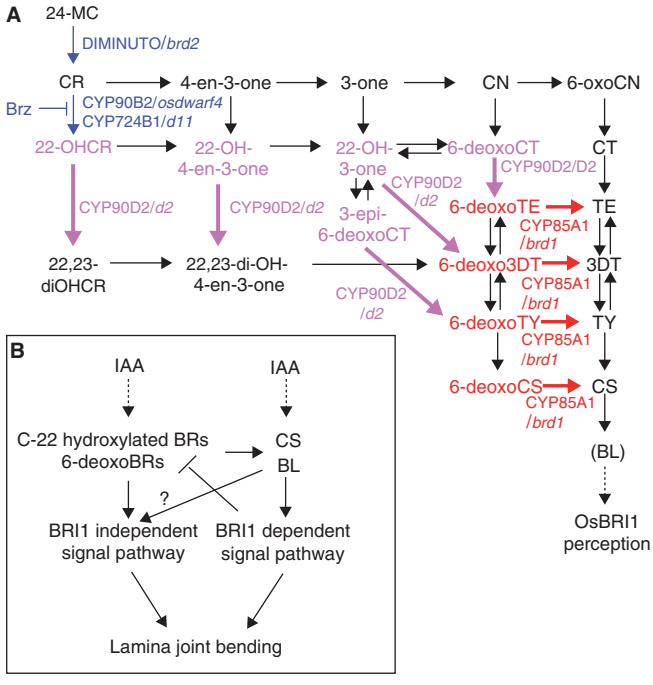


**Fig. 8** Possible BRs involved in auxin signaling and a model of cross-talk between BR and auxin in lamina joint bending. (A) The recently revised BR biosynthetic pathway and possible BRs involved in auxin signaling. BRs that can accumulate in *d2* and *brd1* mutants are indicated in pink and red, respectively. Defective pathways in *brd1* (red) and *d2* (pink) mutants are indicated by bold arrows. The catalytic pathway for CYP90D2 is deduced from the catalytic pathway of CYP90D1 in Arabidopsis (Ohnishi et al. 2006a). Defective pathways in *brd2*, *d11* and *osdwarf4* mutants are indicated by blue arrows. (B) The proposed model of cross-talk between BR and auxin in lamina joint bending. Auxin induces lamina joint bending through at least two signal pathways. One is a C22-hydroxylated-BR-dependent pathway and the other is the well-known CS- and BL-dependent pathway. The C22-hydroxylated-BR-dependent pathway is negatively regulated by the BL-mediated signaling pathway. 24-MC, 24-methylenecholesterol; 22-OHCR, (22*S*)-22-hydroxycampesterol; 22-OH-4-en-3-one, (22*S*,24*R*)-22-hydroxyergost-4-en-3-one; 22-OH-3-one, (22*S*,24*R*)-22-hydroxy-5α-ergostan-3-one; 22,23-diOHCR, (22*R*,23*R*)-22,23-dihydroxycampesterol; 22,23-di-OH-4-en-3-one, (22*R*,23*R*)-22,23-dihydroxy-campest-4-en-3-one.

auxin signaling is conserved from mosses to higher plants. Considering these results, C-22-hydroxylated BRs might participate in auxin signaling as components of the signaling in ancient plant species, while the well-identified BRI1-mediated BR signaling is a newly constructed BR-specific pathway that functions in higher plants.

The dose-dependent response to BL in IAA-treated *d2* revealed that small amounts of BL (0.01–0.1 pg per plant) reduce the hyper-response to IAA in *d2* (**Fig. 6**), suggesting that the hyper-response to auxin is negatively regulated by BL (**Fig. 6**). In Arabidopsis, a transcription regulation factor, BZR1, is involved in negative feedback regulation of BRI1-mediated BR signaling by binding the promoter region of the *DWARF4* gene (He et al. 2005). Thus the negative regulation observed here may also be caused by a decrease of *OsDWARF4* expression regulated by OsBZR1. Namely, in the *d2* or *brd1* mutant, the response to auxin is enhanced by the accumulated C-22-hydroxylated BRs without BL treatment; however, with treatment with 0.01–0.1 pg of BL, the OsBRI1-mediated signaling is activated and the expression of *OsDWARF4* is repressed. Then the amounts of C-22-hydroxylated BRs are decreased, and the auxin-induced lamina joint response is decreased.

The lamina joint response to BL with IAA in the wild type was obviously higher than that in *d61* (**Fig. 5B**). Thus, it is likely that the synergistic response to auxin and BRs is mostly mediated by OsBRI1-dependent, well-characterized BR signaling. The regulation of the bending angle of the lamina joint is critical to the viability of plants. Plants may strictly regulate the lamina joint bending by using various BRs and auxin via at least two signaling pathways. The model described herein is summarized in **Fig. 8B**. There are at least two auxin signal pathways for lamina joint bending. One is the C-22-hydroxylated BR-mediated pathway, which might be OsBRI1 independent. The other is mediated by the well-characterized BR signaling, which starts with the binding of CS or BL to OsBRI1, and through the OsBRI1-mediated pathway, both of which are negatively regulated. Which C-22-hydroxylated BRs are the specific BRs that participate in auxin signaling, and how these BRs are involved in auxin signaling, remains to be clarified.

## Materials and Methods

### Plant materials and growth conditions

Wild-type rice plants (*Oryza sativa* L. cvs Taichung 65 and Nipponbare) and BR-deficient or BR-insensitive mutants *brd2* (Hong et al. 2005), *d2-2* and *d2-4* (Hong et al. 2003), *d11* (Tanabe et al. 2005), *osdwarf4-1* (Sakamoto et al. 2006), *brd1-3* (Hong et al. 2002), and *d61-1* and *d61-2* (Yamamuro et al. 2000) were grown in a plant box containing Murashige and Skoog medium with 3% (w/v) sucrose under continuous light at 30°C. The mutants *d11*, *d2-2*, *d61-1* and *d61-2* are of the T65 ecotype, and *brd2*, *osdwarf4-1*, *d2-4* and *brd1-3* are of the Nipponbare ecotype.

### Hormone treatment and measurements

Plants were grown for 3 d after germination unless otherwise indicated, and well-grown uniform seedlings were selected and used for the experiments. The indicated amounts of plant hormones or inhibitors for bioassay were dissolved in 1 µl of ethanol and applied to the inside of the second leaf blade. After incubation for the indicated number of days, the angle of the leaf blade and sheath (A) and the cell size were measured. The value (180° – A) was used as the angle

required in the results. The lengths of the cells at the center of the adaxial side of the lamina joint were measured using microscopy (IX70, Olympus, Japan, equipped with an Olympus DP50-C).

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