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Function of the α Subunit of Rice Heterotrimeric G Protein in Brassinosteroid Signaling

Katsuyuki Oki[1], Noriko Inaba[1], Kanako Kitagawa[1], Shozo Fujioka[2], Hidemi Kitano[3], Yukiko Fujisawa[1], Hisaharu Kato[1] and Yukimoto Iwasaki[1,*]

[1]Department of Bioscience, Fukui Prefectural University, 4-1-1 Matsuoka Kenjyojima, Eiheiji-cho, Yoshida-gun, Fukui, 910-1195 Japan
[2]RIKEN, Advanced Science Institute, Wako-shi, Saitama, 351-0198 Japan
[3]Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, 464-8604 Japan

Regular Paper

The α subunit of plant heterotrimeric G proteins (Gα) plays pivotal roles in multiple aspects of development and responses to plant hormones. Recently, several lines of evidence have shown that Gα participates in brassinosteroid (BR) responses in *Arabidopsis* and rice plants. In this study, we conducted a comprehensive analysis of the roles of the rice Gα in the responses to BR using a defective mutant of the Gα gene, T65d1. Decreased sensitivity to 24-epi-brassinolide (24-epiBL) in the T65d1 mutant was observed in many processes examined, e.g. in the inhibition of root growth and the promotion of coleoptile elongation. The T65d1 mutant also showed similar phenotypes to those of BR-deficient mutants, such as the specifically shortened second internode and the constitutive photomorphogenic growth phenotype under dark conditions. However, a negative feedback effect by 24-epiBL on the expression of BR biosynthetic genes was observed in the T65d1 mutant, and the levels of BR intermediates did not fluctuate in this mutant. To determine the epistatic relationship between the T65d1 mutant and *d61-7*, a weak allele of a rice BR receptor mutant, the two mutants were crossed. The T65d1/*d61-7* double mutant showed no epistasis in the elongation inhibition of the internodes, the internode elongation pattern, the leaf angle and the morphological abnormality of leaf, except for the vertical length of seed and the seed weight. Our results suggest that the rice Gα affects the BR signaling cascade but the Gα may not be a signaling molecule in BRI1-meditated perception/transduction.

Keywords: Brassinosteroid • Heterotrimeric G-protein α subunit • Rice (*Oryza sativa*) • Rice brassinosteroid insensitive1 (OsBRI1).

Abbreviations: BR, brassinosteroid; DMSO, dimethylsulfoxide; 24-epiBL, 24-epi-brassinolide; Gα, G protein α subunit; RT–PCR, reverse transcription–PCR; SE, standard error; WT, wild-type.

Introduction

In many eukaryotes, heterotrimeric G proteins play pivotal roles in a wide range of physiological responses by transducing extracellular information into intracellular signaling components. This complex is composed of three subunits, namely the G protein α subunit (Gα), β subunit (Gβ) and γ subunit (Gγ). In a quiescent state, these three subunits form a complex. Once a ligand is detected by the G-protein-coupled receptor (GPCR) which works as a guanine exchange factor (GEF) for Gα, Gα exchanges GDP for GTP and becomes active. Subsequently, this complex dissociates into a Gα monomer and Gβγ dimer to regulate downstream effector proteins. In plants, Gα, Gβ and Gγ are encoded by a small number of genes, unlike the case in animals. Although there is a restricted variety of signaling components in plants, their functions are diverse, especially in plant hormone responses (Jones and Assmann 2004, Temple and Jones 2007).

The rice genome harbors one gene for each of Gα (*RGA1*) and Gβ (*RGB1*) and two genes for Gγ (*RGG1* and *RGG2*) (Kato et al. 2004). The rice dwarf mutant, *d1*, was identified as an *RGA1* mutant (Ashikari et al. 1999). Disruption of the *RGA1* gene causes an abnormal morphology, with dwarfism, erect leaves and small round seeds (Fujisawa et al. 1999). *d1* was originally recognized as a gibberellic acid signaling mutant due to its reduced sensitivity to gibberellic acid in the induction of α-amylase activity and up-regulation of gibberellic acid-responsive gene expression in aleurone cells (Ueguchi-Tanaka et al. 2000). It was also reported that *d1* showed

*Corresponding author: E-mail, iwasaki@fpu.ac.jp; Fax, +81-766-61-6015.

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impairment in the control of transcript abundance of gibberellic acid-responsive genes in aleurone tissue (Bethke et al. 2006). In contrast, it has been shown that *d1* responds normally to exogenous gibberellic acid in second leaf sheath elongation (Ueguchi-Tanaka et al. 2000). Thus, the cause of the dwarf morphology of *d1* seems not to be gibberellic acid deficiency. Recently, Wang et al. (2006) showed that *d1* displayed a less responsive to 24-epi-brassinolide (24-epiBL) phenotype, and suggested that the rice Gα was involved in brassinosteroid (BR) signaling.

Arabidopsis Gα (GPA1) has been shown to be involved in many physiological responses, including those to ABA (Pandey et al. 2006), gibberellic acid (Chen et al. 2004), BR (Ullah et al. 2002), D-glucose (Huang et al. 2006), blue light (Warpeha et al. 2007), sphingosine-1-phosphate (Coursol et al. 2003) and ozone (Joo et al. 2005). Ullah et al. (2002) proposed that the ABA-hypersensitive phenotype during the seed germination process in the *gpa1* mutant, a GPA1-defective mutant, may be due to the disruption of its BR response. Recently, genetic analysis with a double mutant for GPA1 and BRI1 (a BR receptor) or for GPA1 and DET2 (a BR biosynthetic enzyme) in *Arabidopsis* has suggested that the G-protein- and BRI1-mediated signaling pathways seem to cooperate regarding the modulation of cell proliferation (Gao et al. 2008). Since studies of plant Gα in BR signaling have been mainly conducted with the dicot model plant, *Arabidopsis*, we think that it is important to understand the function of Gα in BR signaling in detail using rice plants as the monocot model plant. In this study, we paid attention to the genetic background of rice. All mutants used in this study were derived from the Taichung 65 (T65) rice variety. We have conducted comprehensive studies using the T65d1 mutant, such as the quantification of BR intermediates, the negative feedback effect on BR biosynthesis genes by 24-epiBL and the analysis of the double mutant of the T65d1 mutant and *d61-7*, to elucidate the role of Gα in BR responses.

Results

Analysis of the responses to 24-epiBL in the T65d1 mutant

We first compared the T65d1 mutant and the wild type (WT) with regard to their responses to 24-epiBL at seedling stages, in order to examine whether the rice Gα is truly involved in responses to BR. Both plants were grown on agar media containing various concentrations of 24-epiBL under continuous light conditions for 1 week (**Fig. 1A**), and the lengths of the seminal root, adventitious root, aerial parts, second leaf sheath and coleoptile were measured (**Fig. 1B**; left column). The lengths of the tissues measured are also shown in terms of the relative values, i.e. the lengths expressed as a percentage of the tissue length for a given concentration of 24-epiBL relative to that for 24-epiBL-free medium (0 M) (**Fig. 1B**; right column).

The growth of the seminal root, adventitious root and aerial parts in the WT was inhibited by 24-epiBL in a concentration-dependent manner. However, the T65d1 mutant showed reduced responses to 24-epiBL in the growth of the tissues. For instance, the growth of the seminal root was inhibited 60% in the WT but 30% in the T65d1 mutant in the presence of 10^{-6} M 24-epiBL. The growth of the adventitious root was inhibited 80% in the WT but 30% in the T65d1 mutant in the presence of 10^{-6} M 24-epiBL. The growth of the aerial parts was inhibited 40% in the WT but not in the T65d1 mutant in the presence of 10^{-6} M 24-epiBL. The growth of the second leaf sheath and coleoptile of the WT was enhanced by 24-epiBL, but that of the T65d1 mutant was enhanced only by certain concentrations of 24-epiBL. The length of the second leaf sheath of the WT in the presence of 10^{-7} M 24-epiBL was 20% longer than that in 24-epiBL-free medium, but the second leaf sheath of the T65d1 mutant was barely elongated in 10^{-7} M 24-epiBL. The length of the coleoptile of the WT in the presence of 10^{-6} M 24-epiBL was 90% longer than that in 24-epiBL-free medium, but that of the T65d1 mutant was only 50% longer than that in 24-epiBL-free medium. On the whole, the T65d1 mutant showed hyposensitivity to 24-epiBL in the many tissues examined.

Effect of 24-epiBL on the degree of inclination of leaf lamina in the T65d1 mutant

We compared the effects of 24-epiBL on the size of cells in the lamina regions of the T65d1 mutant and WT. In the presence of 24-epiBL, especially at high concentrations, the bending angle of the T65d1 mutant was much smaller than that of the WT (**Fig. 2A**). Thus, the T65d1 mutant showed less sensitivity to 24-epiBL. By microscopic observation, the adaxial cells in this region of the WT were greatly expanded by 24-epiBL, while the cells in the T65d1 mutant was expanded only slightly (**Fig. 2B**). The cell number in the lamina region of the T65d1 mutant was almost the same as that in the WT. Thus, the cause of the reduction in the angle of lamina bending in the T65d1 mutant is reduced cell expansion.

The T65d1 mutant exhibits the features observed in BR-deficient mutants

We compared the phenotypes of the T65d1 mutant and typical BR-deficient mutants. First, the length of the second internode in the T65d1 mutant was dramatically reduced relative to the other parts (**Fig. 3**) and so the elongation pattern of internodes belongs to the dm type among six categories of elongation patterns (Takeda 1977). The elongation pattern of the internodes of the WT was named the N type in which the internodes shorten gradually as they lower. The d6 type is that in which all internodes barely elongate, except for the

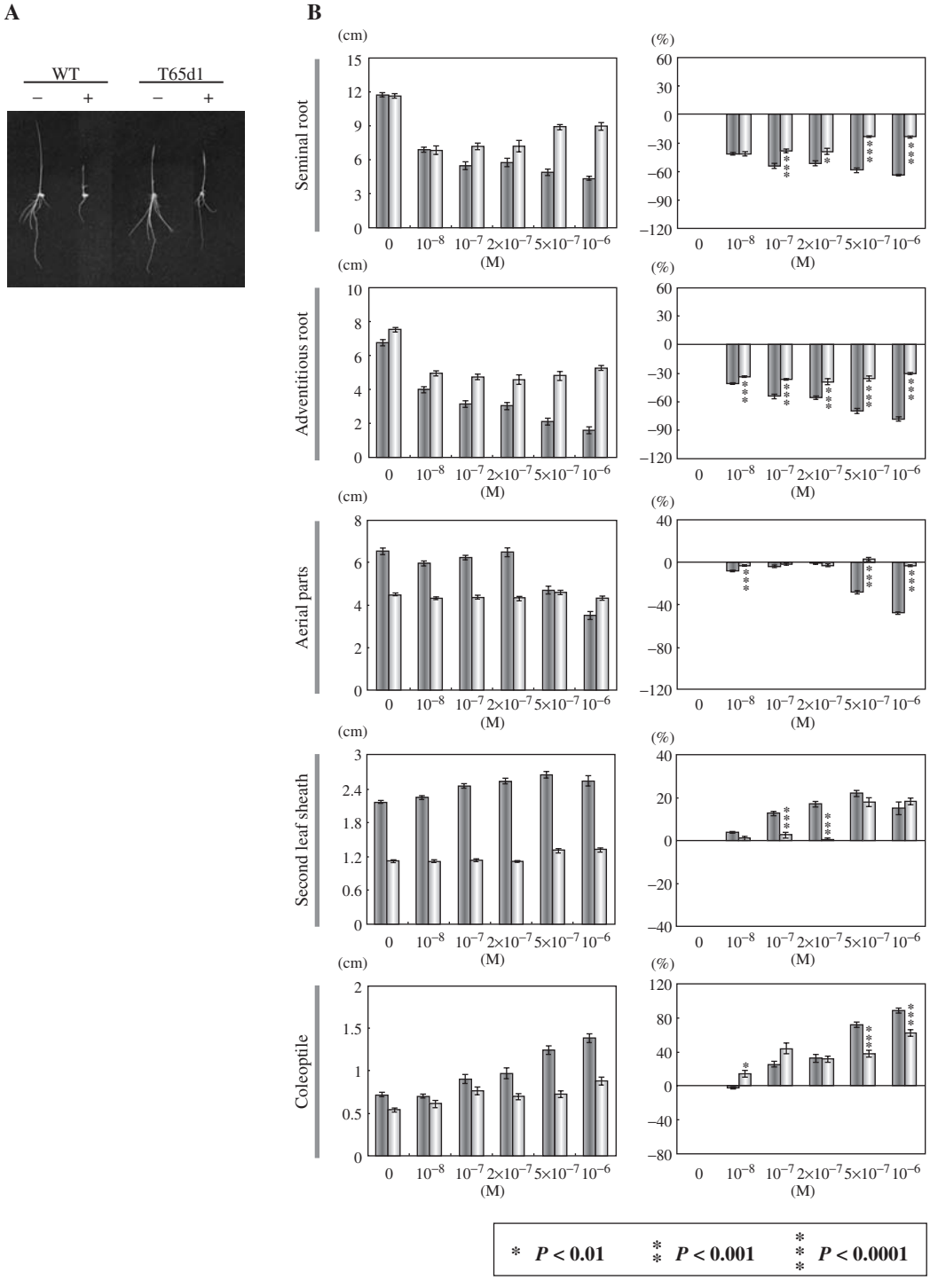

Fig. 1 Response to 24-epiBL in the T65d1 mutant. The responses of the WT and the T65d1 mutant to 24-epiBL were compared. The plants were grown for 1 week after germination on agar media with various concentrations of 24-epiBL. (A) Plants grown with (+) or without (–) 10^{-6} M 24-epiBL. (B) The lengths of the seminal root, adventitious root, aerial parts, second leaf sheath and coleoptile of these plants. Gray and white bars represent values for the WT and the T65d1 mutant, respectively. The left and right columns show the actual and relative values, respectively. The relative value is the percentage of the tissue length for a given concentration of 24-epiBL relative to that for 24-epiBL-free medium (0 M). Each value is the mean of 15 seedlings (error bars = SE).The experiment was repeated at least three times. Asterisks denote that the differences were statistically significant with the two-tailed *t*-test.

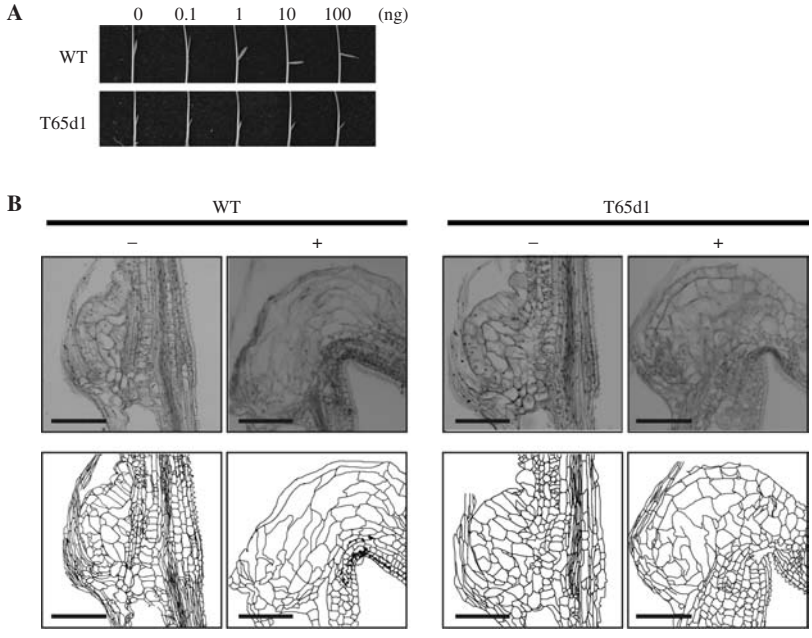

Fig. 2 Effect of 24-epiBL on the degree of inclination of leaf lamina in the T65d1 mutant. The effect of 24-epiBL on the promotion of the lamina joint inclination was investigated. (A) The WT and the T65d1 mutant were grown for 4 d under the same conditions as in **Fig. 1** without 24-epiBL. Then, various concentrations of 24-epiBL were applied at the second leaf lamina regions of these plants and subsequently grown for 3 d. (B) Longitudinal axis sections of the lamina regions of the WT and the T65d1 mutant treated with (+) or without (−) 1 μg of 24-epiBL (bar = 500 μm).

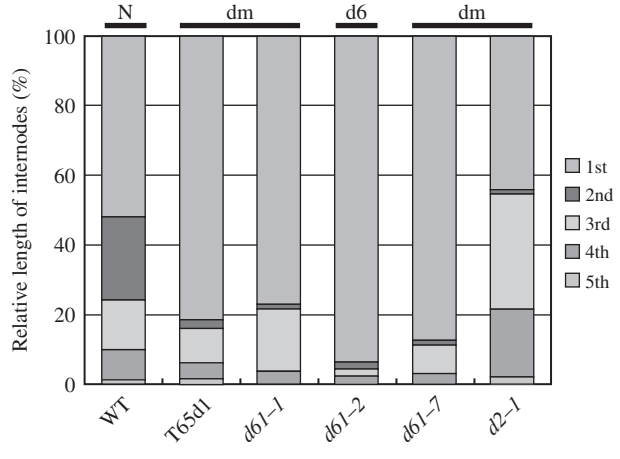

Fig. 3 Elongation patterns of internodes in the T65d1 mutant and BR-deficient mutants. The internodes of WT, the T65d1 mutant and BR-deficient mutants (*d61-1*, *d61-2*, *d61-7* and *d2-1*). The ordinate shows the length of each internode relative to that of the total culm. The T65d1 mutant is grouped into the dm type.

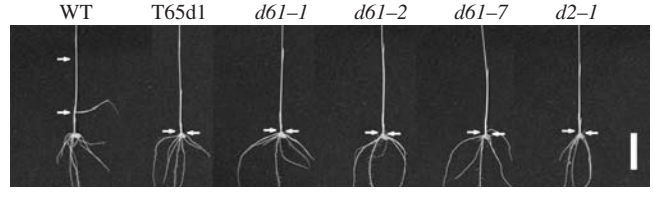

Fig. 4 Aberrant skotomorphogenesis of the T65d1 mutant. Gross morphology of the WT, T65d1 mutant and BR-deficient mutants (*d61-1*, *d61-2*, *d61-7* and *d2-1*) grown in darkness for 2 weeks on agar medium. The internodes (the part between the two arrows) of the WT elongated, indicating that the WT shows a skotomorphogenic growth phenotype. The internode of the T65d1 mutant did not elongate under the same conditions. Thus, the T65d1 mutant shows a de-etiolated phenotype similar to those in *d61-1*, *d61-2*, *d61-7* and *d2-1*. Arrows indicate the node positions (bar = 3 cm).

first one, and more severely abnormal BR-related mutants such as *d61-2* are grouped into this category. Secondly, the internode of the T65d1 mutant did not elongate in complete darkness, although that of the WT did (**Fig. 4**). Thus, the T65d1 mutant showed a constitutive photomorphogenic phenotype under dark conditions. Thirdly, the T65d1 mutant had erect leaves (**Fig. 5A**).

These three characters are also observed in *d11-1* which is a mutant of a BR biosynthetic enzyme, *CYP724B1* (Tanabe et al. 2005), in addition to *d2-1* which is a mutant of a BR biosynthetic enzyme, *CYP90D* (Hong et al. 2003), and *d61-2* (Yamamuro et al. 2000). In contrast, gibberellic acid biosynthesis mutants such as *d18* and *d35* can elongate the internodes when grown under dark conditions (Yamamuro et al. 2000). These results support the possibility that rice Gα may affect the BR signaling pathway which may concern the determination of the rice body plan during the developmental processes.

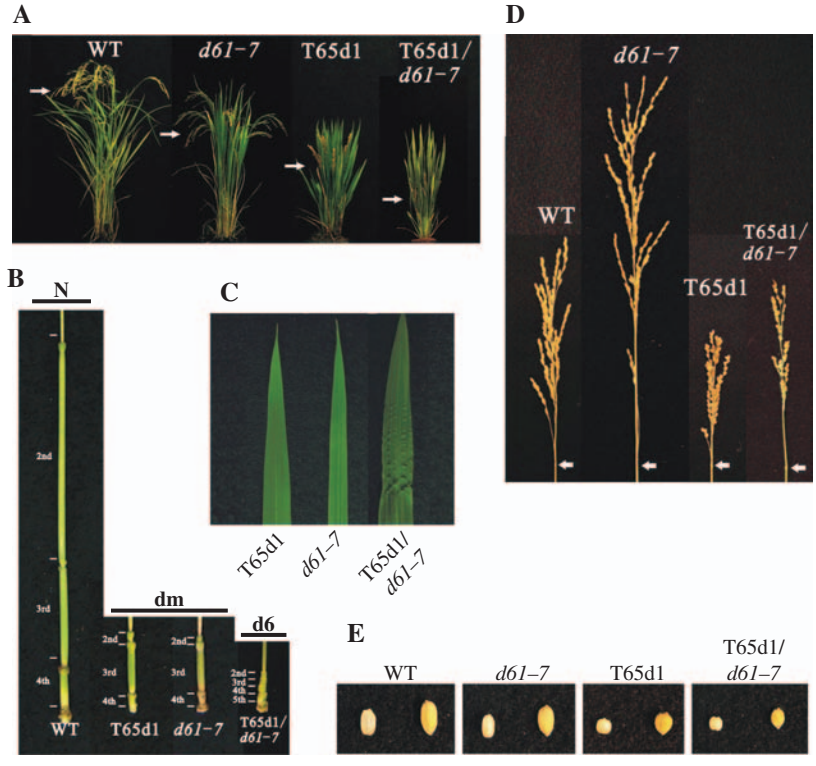

Fig. 5 Phenotype of the T65d1/*d61-7* double mutant. (A) Gross morphology of the WT, *d61-7*, the T65d1 mutant and the T65d1/*d61-7* double mutant at grain-filling stages. Arrows indicate the positions of panicles. (B) Internodes of the WT, *d61-7*, the T65d1 mutant and the T65d1/*d61-7* double mutant. The double mutant shows a d6-type internode elongation pattern which is found in more severe BR-deficient mutants. (C) Leaf blades of the T65d1 mutant, *d61-7* and the T65d1/*d61-7* double mutant. The leaf blade of the T65d1/*d61-7* double mutant is corrugated. (D) Panicles of the WT, *d61-7*, the T65d1 mutant and the T65d1/*d61-7* double mutant. The panicle in the double mutant is slightly longer than that in the T65d1 mutant. Arrows indicate the neck-ear nodes. (E) Seeds in the WT, *d61-7*, the T65d1 mutant and the T65d1/*d61-7* double mutant.

Analysis of the T65d1/*d61-7* double mutant

In order to investigate whether there is a link between the signaling pathways mediated by rice Gα and OsBRI1, we generated a T65d1/*d61-7* double mutant. The T65d1 mutant is a null allele of the *RGA1* gene (Fujisawa et al. 2001). *d61-7* is a leaky mutant with a single amino acid change in the extracellular leucine-rich repeat region of OsBRI1 and grouped into a mild allele (Nakamura et al. 2006).

At the grain-filling stage, the overall plant height of the T65d1/*d61-7* double mutant was shorter than that of each single mutant (**Fig. 5A**). The first, second and third internodes of the T65d1/*d61-7* double mutant were shorter than the corresponding internodes of the single mutants and the difference was statistically significant (**Fig. 5B** and **Table 1**). The elongation pattern of the internodes of the T65d1/*d61-7* double mutant were of the d6 type, which has been observed with more severe BR-deficient mutants such as *d61-2*, while the patterns of the T65d1 mutant and *d61-7* were observed to be grouped into the dm type (**Fig. 5B**). The angle of flag leaves of the T65d1/*d61-7* double mutant was much smaller than that of each single mutant and the difference was statistically significant (**Table 1**). The leaf blades of the double mutant at the adult plant stage were corrugated (**Fig. 5C**), and such corrugated leaves have also been observed in severe BR-deficient mutants (Hong et al. 2005). Thus, additive effects on the internode elongation pattern, the leaf angle and the morphological abnormality of leaf were observed in the T65d1/*d61-7* double mutant. Hence, the T65d1 mutant is not epistatic to *d61-7* in these morphological characters. The long panicle is a characteristic of BR-deficient mutants, e.g. *d2* (Hong et al. 2003), *d11* (Tanabe et al. 2005) and *d61* (Yamamuro et al. 2000). In contrast, the panicle of the T65d1 mutant is shorter than that of the WT. The panicle length of the T65d1/*d61-7* double mutant was slightly longer than that of the T65d1 mutant (**Fig. 5D** and **Table 1**). The panicle length in the T65d1/*d61-7* double mutant tended to be additively regulated, though a statistically significant difference was not clear. The T65d1 mutant set seeds with shorter lengths than did the WT, specifically in the longitudinal axis but not in the horizontal axis (**Fig. 5E** and **Table 1**). In contrast, the seeds of *d61-7* exhibited reduced lengths in both directions as compared with WT

Table 1. Morphological characters of the WT, T65d1, *d61-7* and T65d1/*d61-7*

	WT	T65d1	*d61-7*	T65d1/*d61-7*
Tissue length (mm)				
Panicle	207.5 ± 2 (100)	129.8 ± 11.2 (62.6)	345.5 ± 16.5 (166.5)	143.8 ± 6.9 (69.3)
Internodes				
First	398.3 ± 8.7 (100)	235.2 ± 3.7 (59.1)	257.5 ± 13.3 (64.6)	141.0 ± 7.7 (35.4)*
Second	173.0 ± 3.8 (100)	7.0 ± 0 (4.0)	5.8 ± 0.5 (3.4)	5.7 ± 0.4 (3.3)*
Third	94.2 ± 5.8 (100)	30.2 ± 6.3 (32.1)	25.2 ± 6.4 (26.8)	9.3 ± 0.7 (9.9)*
Fourth	63.2 ± 9.5 (100)	5.4 ± 1.3 (8.5)	9.5 ± 0.8 (15.0)	7.3 ± 1.1 (11.6)
Seed				
Vertical length	7.47 ± 0.13 (100)	5.19 ± 0.12 (69.5)	6.66 ± 0.07 (89.2)	5.25 ± 0.08 (70.3)
Horizontal length	3.78 ± 0.05 (100)	3.81 ± 0.04 (100.8)	3.58 ± 0.06 (94.7)	3.67 ± 0.05 (97.1)
Seed weight (mg)	31.5 ± 0.3 (100)	16.9 ± 0.4 (53.6)	24.7 ± 0.5 (78.4)	17.1 ± 0.6 (54.2)
Flag leaf angle (°)	35.7 ± 2.4 (100)	13.6 ± 2.0 (38.1)	15.0 ± 1.2 (42.0)	6.0 ± 0.4 (16.8)*

Data are averages of 10 tissue samples (±SE). The values in parentheses are the values of the indicated mutants as a percentage relative to the values of the WT. T65 used as the WT is a recurrent parent for the T65d1 mutant and *d61-7*. Asterisks denote statistically significant differences between the T65d1 mutant and the T65d1/*d61-7* double mutant with two-tailed *t*-test at a *P*-value <0.05.

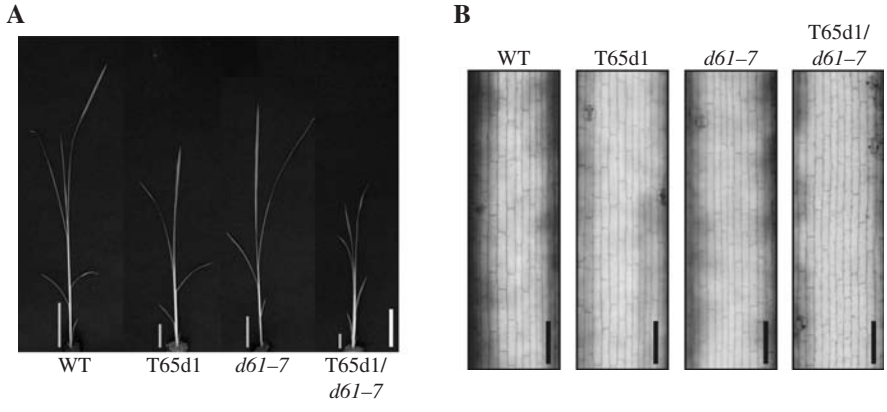

Fig. 6 Microscopic picture of the leaf sheath for the analysis of cell length and cell number of the T65d1/*d61-7* double mutant. (A) Gross morphology of the WT, the T65d1 mutant, *d61-7* and the T65d1/*d61-7* double mutant at seedling stages. Gray bars indicate the lengths of the third leaf sheaths (white bar = 7 cm). (B) Longitudinal axis sections of the third leaf sheaths of (A) (bars = 100 μm).

seeds. The seed length and seed weight of the T65d1/*d61-7* double mutant were almost the same as those of the T65d1 mutant. Thus, the synergistic effects on the vertical length of seed and seed weight raise the possibility that the T65d1 mutant may be epistatic to *d61-7* in the tissues.

Cell number and cell length in the T65d1/*d61-7* double mutant

To ascertain whether the enhanced dwarfism of the T65d1/*d61-7* double mutant is caused by reduction in cell number or cell length, we compared the leaf sheaths of the WT, the T65d1 mutant, *d61-7* and the T65d1/*d61-7* double mutant regarding the cell size using microscopic observations. The central part of the third leaf sheath at the sixth leaf stage of each plant was analyzed (**Fig. 6A, B**). The cell lengths of the T65d1 mutant and *d61-7* were shorter than those of the WT (**Table 2**). This result shows that rice Gα and OsBRI1 are positive regulators of cell elongation. The cell length of the leaf sheath in the T65d1/*d61-7* double mutant was not significantly different from that of *d61-7*. The synergistic effect on the cell length of the leaf sheath may suggest the possibility that *d61-7* is epistatic to the T65d1 mutant. The cell number decreased greatly in the T65d1/*d61-7* double mutant compared with that in each single mutant (**Table 2**). When the rates of reduction of the cell numbers in the T65d1 mutant (58.2%) and *d61-7* (72.9%) are multiplied together, the product is 31.1%. The actual rate of decrease in the cell number in the T65d1/*d61-7* double mutant was 27.9% (**Table 2**). Thus, the reduction in the cell number in the T65d1/*d61-7* double mutant may be caused by

Table 2. Cell length and cell number of third leaf sheaths of the WT, T65d1, *d61-7* and T65d1/*d61-7*

	WT	T65d1	*d61-7*	T65d1/*d61-7*
Third leaf sheath (mm)	84.0 (100)	37.0 (44.0)	54.0 (64.3)	20.0 (23.8)
Average cell length (μm)	154.0 ± 4.6 (100)	116.6 ± 2.1 (75.7)	135.8 ± 3.4 (88.2)	131.3 ± 2.1 (85.3)*
Deducible cell nuwmber	545.6 (100)	317.3 (58.2)	397.5 (72.9)	152.3 (27.9)

Average cell length is the average of the length of cells in a 5 mm parts of the central areas of the third leaf sheaths (±SE). The values in parentheses are the same as those in **Table 1**. The deducible cell numbers are the values from the average cell length. The asterisk denotes a statistically significant difference between the T65d1 mutant and the T65d1/*d61-7* double mutant with the two-tailed *t*-test at a *P*-value <0.05 regarding average cell length.

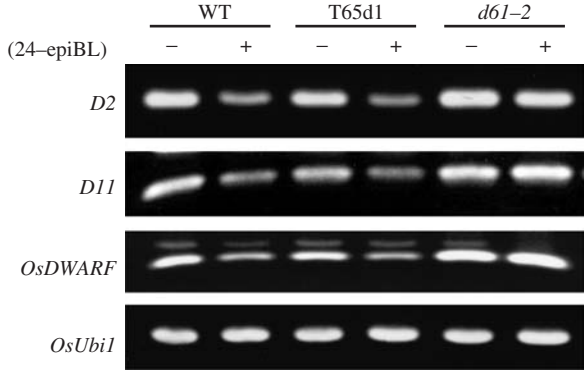

Fig. 7 Negative feedback effect of 24-epiBL on the expression of BR biosynthesis genes in the T65d1 mutant and BR-deficient mutants. RT–PCR analysis of the expression of *D2*, *D11* and *OsDWARF* genes in the WT, the T65d1 mutant and *d61-2*. Plants were treated with or without 1 μM 24-epiBL for 1 week. The levels of mRNAs of *D2*, *D11* and *OsDWARF* were reduced by application of 24-epiBL in the WT and the T65d1 mutant but not in *d61-2*. The negative feedback effect of 24-epiBL on the expression of the BR biosynthesis genes is normal in the T65d1 mutant. *OsUbi1* was used as an internal standard.

an additive effect between the T65d1 mutant and *d61-7*. The additive effect on the cell number of the leaf sheath may suggest that the T65d1 mutant is not epistatic to *d61-7*.

Negative feedback effect of 24-epiBL on the expression of BR biosynthesis genes is normal in the T65d1 mutant

It has been reported that mRNA expression of BR biosynthetic genes, *D2* (Hong et al. 2003), *D11* (Tanabe et al. 2005) and *OsDWARF* (Hong et al. 2002), undergoes negative feedback regulation by 24-epiBL. To examine whether rice Gα functions in the negative feedback regulation of BR biosynthesis genes, we compared the expression levels of BR biosynthesis genes in the T65d1 mutant and WT with or without 24-epiBL. The levels of mRNAs for *D2*, *D11* and *OsDWARF* were reduced by application of 24-epiBL in both the WT and the T65d1 mutant (**Fig. 7**). This demonstrates that the feedback regulation of the expression of *D2*, *D11* and *OsDWARF* genes by 24-epiBL occurs normally in the

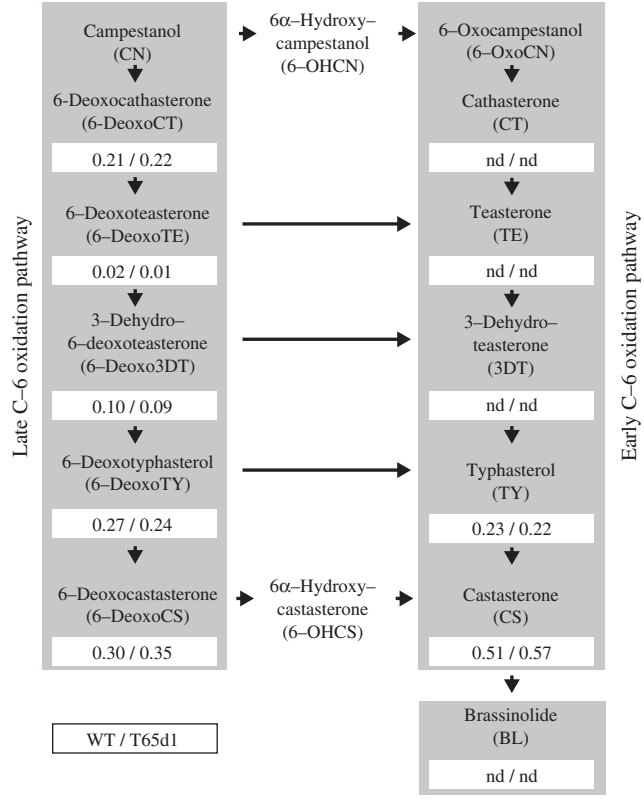

Fig. 8 The amounts of endogenous BR intermediates in the T65d1 mutant. The amounts of BL and its precursors (ng g^{-1} FW) in young panicles of the T65d1 mutant and the WT were quantified. The measurement was performed twice, and the average scores are presented here (ND = not detected). The intrinsic amounts of these compounds in theT65d1 mutant were similar to those in the WT.

T65d1 mutant as seen in the WT, while the regulation is not observed in *d61-2*.

The T65d1 mutant accumulates similar amounts of BR intermediates to the WT

Plant hormone levels must be strictly maintained for proper plant growth. The BR intermediates increase in *d61* in order to compensate for the impaired BR action in this mutant (Yamamuro et al. 2000). The measurement of the levels of BR intermediates would be helpful for characterization of

BR-related mutants. Considering that the T65d1 mutant shows reduced responses to 24-epiBL, the T65d1 mutant may be a BR signaling mutant. If so, the intrinsic amounts of precursors of BL in the T65d1 mutant would increase over those in the WT. To test this possibility, we quantified the amounts of BR intermediates. The amounts of BR intermediates in the T65d1 mutant were similar to those in the WT (**Fig. 8**). This suggests that the system sensing the amounts of BR intermediates is normal in the T65d1 mutant.

Discussion

In plants, heterotrimeric Gαs are well conserved among monocots and dicots. Many functional domains such as the myristylation site, effector-binding domain and receptor-binding region are commonly found in plant Gαs. The amino acid sequences of Gα in rice and *Arabidopsis* share 77% identity and the function of Gα seems to be evolutionarily conserved (Ishikawa et al. 1995). Previous studies with a dicot model plant, *Arabidopsis*, have suggested that Gα is involved in BR responses (Ullah et al. 2002, Gao et al. 2008). We have conducted a series of experiments to elucidate the functions of Gα in rice, a monocot model plant, and we examined whether rice Gα is really involved in BR responses using a rice Gα-defective mutant, T65d1, and rice BR-deficient mutants (three BR receptor mutants, *d61-1*, *d61-2* and *d61-7*, and one BR biosynthesis mutant, *d2-1*).

We observed that the T65d1 mutant exhibited decreased sensitivity to 24-epiBL in many of the aspects examined, such as in the growth inhibition by 24-epiBL of seminal root, adventitious root and aerial parts (**Fig. 1**), in the promotion of elongation of the coleoptile and second leaf sheath by 24-epiBL (**Fig. 1**), and in the enhancement of lamina joint inclination by 24-epiBL, accompanying an expansion of cell volume (**Fig. 2**). These results indicate that rice Gα is concerned with the BR responses in some way. The attenuated response of the seminal root, coleoptile and lamina joint to exogenous 24-epiBL has also been reported previously by Wang et al. (2006). Although some differences in the sensitivity to 24-epiBL were observed between the results of Wang et al. and ours, these may be due to a difference in the composition of the medium used for plant growth. Wang et al. used a 1/2 MS medium and we used an agar medium without MS. A marked contrast is not found between the results of Wang et al. and ours. Both studies found that the application of 24-epiBL to rice promoted growth of some tissues, such as the second leaf sheath and the coleoptile, and inhibited growth in other tissues, such as roots and aerial parts. 24-epiBL seems to cause an opposite function in a tissue-specific manner, but we are not able to explain its molecular mechanism. However, these results may indicate the presence of different BR sensing pathways in different tissues.

In addition, the morphology of the T65d1 mutant was observed to be similar to that of the already known BR-deficient mutants, such as specifically shortened second internodes (**Fig. 3**) and constitutive photomorphogenic growth phenotypes in darkness (**Fig. 4**). Furthermore, the T65d1 mutant bears erect leaves, as do other BR-deficient mutants (**Fig. 5**A). These characteristics have previously been reported with other BR-deficient mutants (Yamamuro et al. 2000, Hong et al. 2002, Tanabe et al. 2005). All the above results suggest that rice Gα may be concerned with BR signaling. The previous report by Wang et al. (2006) showed that the internode of the DK22 mutant, an allele of *d1*, elongated in darkness like the WT. On the other hand, the internode of the T65d1 mutant did not elongate in darkness in this study. These conflicting results may be due to a difference in the recurrent parents. Wang et al. (2006) used DK22 whose recurrent parent is Nipponbare and we used the T65d1 mutant whose recurrent parent is T65.

The present work suggests, however, that the Gα-mediated signaling seems not to be connected directly with the BR cascade via a rice BR receptor, OsBRI1. First, no apparent epistasis was observed in the internode length, the elongation pattern of the internode, the leaf angle, the leaf morphology and the cell number of the leaf sheath between the T65d1 mutant and *d61-7*, through analysis of the T65d1/*d61-7* double mutant, except for seed length and seed weight (**Figs. 5**, **6**, and **Tables 1**, **2**). A similar relationship has been observed between *gpa1* (an *Arabidopsis* Gα mutant) and *bri1* or *det2* (*Arabidopsis* BR-deficient mutants) (Gao et al. 2008). Secondly, we found that the feedback regulation of the expression of BR biosynthetic genes with 24-epiBL operated as it did in the WT (**Fig. 7**), although the regulation has been found to be impaired in a rice OsBRI1 mutant, *d61* (Yamamuro et al. 2000, Tanabe et al. 2005). The two results described above suggest that the Gα-mediated signaling is not directly connected with BR perception mediated by OsBRI1.

It has been demonstrated with *Arabidopsis* that BR signaling is mediated by the following components: the leucine-rich repeat (LRR)-receptor kinase BRI1 (Wang et al. 2001), receptor-like cytoplasmic kinase subfamily BSKs (Tang et al. 2008), serine/threonine protein phosphatase BSU1 (Mora-Garcia et al. 2004), protein kinase BIN2 (He et al. 2002) and nuclear/cytoplasmic shuttling transcription factors BZR1 and BES1 (Wang et al. 2002, Yin et al. 2002). Other candidates which may function downstream of BRI1 have also been reported (Nam and Li 2004, Ehsan et al. 2005). Recently, a rice homolog of *AtBZR1*, *OsBZR1*, was isolated (Bai et al. 2007), suggesting that this line of BR signaling machinery is conserved among monocot and dicot plants. Activated BZR1 is thought to bind to promoters of BR target genes to regulate their expression after perception of BR.

Since the feedback regulation with 24-epiBL in the T65d1 mutant is not impaired, the BR signaling cascade from OsBRI1 to OsBZR1 would function normally in this mutant.

In addition, we found that the amounts of BR intermediates in the T65d1 mutant were not different from those in the WT (**Fig. 8**). The amounts of BR intermediates in the OsBRI1 mutant, *d61* are higher than those in the WT (Yamamuro et al. 2000), probably due to compensating for the BR signaling power. From these results, a system sensing the amounts of BR intermediates is normal in the T65d1 mutant and Gα may not be an actual member of the BRI1-meditated signaling system.

Considering our results, we propose two models for the relationship between rice Gα- and OsBRI-mediated signaling pathways (**Fig. 9**). One model is that rice Gα affects the BR signaling cascade in a manner distinct from the BRI1-meditated perception/transduction (**Fig. 9**, model A). The possibility that rice Gα may amplify some BR responses that originate from OsBRI1 is not also ruled out (**Fig. 9**, model B).

Arabidopsis Gα is has been reported to be a positive regulator of cell proliferation (Ullah et al. 2001, Chen et al. 2006). This study shows that rice Gα is also a positive regulator of cell proliferation, because the cell number in the T65d1 mutant was found to decrease compared with that in the WT (**Table 2**). BRI1 has also been shown to be a positive regulator of cell proliferation in rice plants (Nakamura et al. 2006) and in *Arabidopsis* (Nakaya et al. 2002). Since an additive effect on cell proliferation was observed between Gα and BRI1 mutants in rice (this study) and *Arabidopsis* (Gao et al. 2008), the function of Gα in cell proliferation may be different from that of BRI1.

Rice Gα is a positive regulator of cell length, because the cell length in the T65d1 mutant is shorter than that in the WT (**Table 2**). The finding that the cell elongation induced by 24-epiBL was partially reduced in the T65d1 mutant (**Fig. 2**) supports the conclusion that rice Gα is a positive regulator of cell length. OsBRI1 is also a positive regulator of cell length, because the cell length in *d61-7* is shorter than that in the WT (**Table 2**). Thus, this study suggests that a synergistic effect on cell length between the T65d1 and *d61-7* mutants seems to occur. However, the finding that the T65d1 mutant may be epistatic to *d61-7* in terms of the seed length (**Table 1**) and *d61-7* may be epistatic to the T65d1 mutant in terms of the cell length of the leaf sheath (**Table 2**) are in contrast to each other. At present, we cannot explain the reason for this. In future, this contrast may be modified by analysis of double mutants between the T65d1 mutant and more severe BR-deficient mutants. The use of the BR biosynthesis inhibitor, brassinazole, may also be helpful to understand the synergistic effects. It may be expected that the phenotypes of the T65d1 mutant treated with some concentration of brassinazole may show a more severe abnormality than that of the T65d1/*d61-7* double mutant.

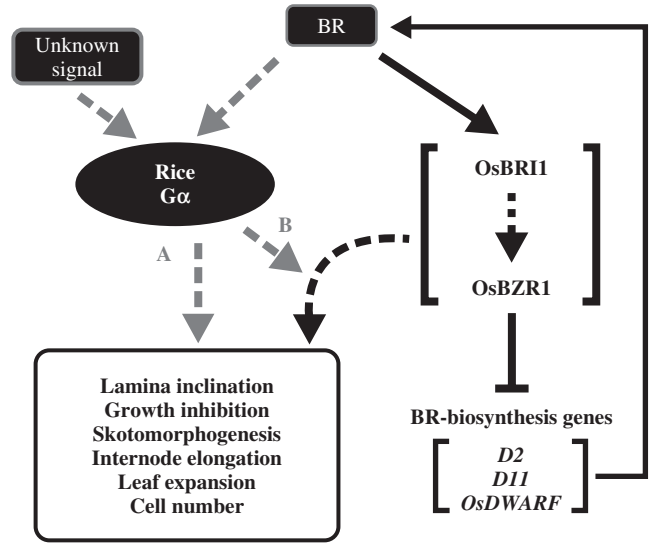

Fig. 9 Putative function of rice Gα in the BR signaling pathway. The T65d1 mutant showed phenotypes similar to BR-deficient mutants such as impaired responses to exogenous 24-epiBL (**Figs. 1**, **2**), the dm-type internode pattern (**Fig. 3**) and aberrant skotomorphogenesis (**Fig. 4**). However, the feedback regulation of BR biosynthesis genes (**Fig. 7**) and the control of intrinsic BR intermediates (**Fig. 8**) in the T65d1 mutant were normal. One model proposes that rice Gα affects the BR signaling cascade distinct from the BRI1-meditated perception/transduction (model A). The possibility that rice Gα potentiates in some aspects of the BRI1-mediated response is not ruled out (model B).

We had carried out our experiments on the basis of the supposition that the BRI1-mediated pathway was the major one in BR signaling in all cases and in every tissue. However, it would be better to postulate that the major pathways of BR signaling under different growth conditions, at different developmental stages or in different tissues are different from one another. Thus, we suppose that rice Gα may have the ability to transduce some BR signaling apart from OsBRI1-mediated signaling.

Materials and Methods

Plant materials and growth conditions

The following rice plants were used in this study: WT (*Oryza sativa* L. cv. Taichung 65); the T65d1 mutant, a null allele of the Gα subunit gene which has a two-base deletion at base positions 908 and 909 of the *RGA1* gene (Ueguchi-Tanaka et al. 2000; Fujisawa et al. 2001); *d2-1*, a BR-deficient mutant (Hong et al. 2003); and *d61-1*, *d61-2* and *d61-7*, BR-insensitive mutants (Yamamuro et al. 2000, Nakamura et al. 2006). The recurrent parents of the T65d1 mutant, *d2-1*, *d61-1*, *d61-2*

and *d61-7* are Taichung 65. Seeds were sterilized with 70% ethanol for 30 s and then with 1.25% (w/v) hypochlorous acid and 0.02% (w/v) Tween-20 for 15 min. After imbibition at 22°C for 3 d and subsequently at 30°C for 3 d, seeds were transferred to soil or agar medium. Plants in the paddy field or greenhouse were cultivated under sunlight. Plants at the seedling stage were grown in continuous light or in complete darkness at 30°C in an incubator room.

Analysis of responses to plant hormone

A brassinolide analog, 24-epiBL (635-00811), was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Imbibed seeds were sown on 0.6% agar media containing various concentrations of 24-epiBL. Plants were then grown in an incubator room in continuous light at 30°C for 1 week and the length of each tissue of the seedlings was measured. The 24-epiBL was dissolved in dimethylsulfoxide (DMSO). The same amount of DMSO was added to the 24-epiBL-free medium (0 M).

Statistical analysis

All data in graphs are presented with the standard error (SE). For the analysis of statistical significance between the WT and the T65d1 mutant, the *F*-test were performed and a *P*-value of <0.05 was considered to be an equal variance. In cases where an equal variance was observed, consequently, statistically significant *P*-values of differences were calculated with the paired two-tailed *t*-test; and in cases where a non-equal variance was observed, unpaired two-tailed *t*-tests were carried out.

Lamina joint inclination assay with 24-epiBL

Imbibed seeds were sown on 0.6% agar medium and grown in an incubator room in continuous light at 30°C until the third leaves began to emerge (4 d). Then, various amounts of 24-epiBL were applied to the lamina regions between the second leaf and leaf sheath. After 3 d, the bending angles of the lamina joint were measured.

Preparation of sections for histological analysis of the lamina joint region

Lamina joint regions of the WT and the T65d1 mutant treated with or without 10^{-6} M 24-epiBL for 3 d were fixed with formalin:glacial acetic acid:70% ethanol (1:1:18). Fixed tissues were embedded in PARAHISTO, melting point 56–58°C (Nacalai Tesque, Inc., Kyoto, Japan) and sectioned at about 10 μm thickness.

Quantification of endogenous BL and its precursors

Young florets of the WT and the T65d1 mutant were harvested just before the heading stage. Samples were immediately lyophilized in liquid nitrogen. To analyze endogenous BR compounds, lyophilized samples (equivalent to 20 g FW) were extracted twice with 250 ml of methanol:chloroform [4:1 (v/v)], and the BRs were purified and quantified according to previously described methods (Tanabe et al. 2005).

Semi-quantitative RT–PCR analysis

Imbibed seeds of the WT, the T65d1 mutant and *d61-2* were sown on half-strength MS medium (0.6% agar) without sucrose and supplemented with 10^{-6} M 24-epiBL. A 10^{-6} M 24-epiBL solution was also sprayed onto plants every 12 h for 1 week. Subsequently, total RNAs were extracted from aerial parts and roots of these plants using an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). cDNAs were synthesized with a SuperScript III system (Invitrogen, Japan K.K., Tokyo, Japan), using total RNAs. PCR was carried out with the KOD-Plus system (TOYOBO Ltd., Osaka, Japan). Primers used for PCR were as follows: *D2* (forward: 5′-agctgcctggcactaggctctacag-3′/reverse: 5′-atgttgtcggagatgagctcgtcgg-3′), *D11* (forward: 5′-tccttggtgagacgctgagg-3′/reverse: 5′-tggttcagctcctggtcacag-3′), *OsDWARF* (forward: 5′-tcgacatccaggccaagac-3′/reverse: 5′-tccaggaaggttgatgggc-3′) and *OsUbiquitin1* (forward: 5′-caccctgttctagggttcacaagtctgc-3′/reverse:5′-gcaaaatttggacacaatgattagggatc-3′),

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