ORIGINAL ARTICLE

Heterotrimeric G protein α subunit is involved in rice brassinosteroid response

Lei Wang[1, 2], Yun-Yuan Xu[1], Qi-Bin Ma[1, 2], Dan Li[1, 2], Zhi-Hong Xu[1, 3], Kang Chong[1, 3]

[1]*Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Nanxincun 20, Xiangshan, Beijing 100093, China;* [2]*Graduate School of the Chinese Academy of Sciences, 100046 Beijing, China;* [3]*National Plant Gene Research Center (Beijing), Beijing 100093, China*

Heterotrimeric G proteins are known to function as messengers in numerous signal transduction pathways. The null mutation of *RGA* (rice heterotrimeric G protein α subunit), which encodes the α subunit of heterotrimeric G protein in rice, causes severe dwarfism and reduced responsiveness to gibberellic acid in rice. However, less is known about heterotrimeric G protein in brassinosteroid (BR) signaling, one of the well-understood phytohormone pathways. In the present study, we used root elongation inhibition assay, lamina inclination assay and coleoptile elongation analysis to demonstrated reduced sensitivity of *d1* mutant plants (caused by the null mutation of *RGA*) to 24-epibrassinolide (24-epiBL), which belongs to brassinosteroids and plays a wide variety of roles in plant growth and development. Moreover, *RGA* transcript level was decreased in 24-epiBL-treated seedlings in a dose-dependent manner. Our results show that RGA is involved in rice brassinosteroid response, which may be beneficial to elucidate the molecular mechanisms of G protein signaling and provide a novel perspective to understand BR signaling in higher plants.

Introduction

Heterotrimeric G proteins are known to function as messengers involved in numerous signal transduction pathways following perception by receptors on cell surfaces. Such proteins are composed of subunits, namely α, β and γ, respectively. The Gα subunit contains a Ras-like domain with a GDP/GTP nucleotide-binding site and GTP hydrolase activity [1]. The human genome contains about 23 Gα genes that belong to four subfamilies, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$, according to their functional and sequence attributes. In contrast, plant genomes contain a single copy of the Gα coding gene [2]. Nevertheless, recent studies in *Arabidopsis* have shown that plant G protein signaling is important to many fundamental processes, such as cell proliferation, hormone perception and ion-channel regulation [3, 4]. In rice, the G protein α subunit was first cloned and characterized in 1995 and designated *RGA1* (rice G protein α subunit 1) [5, 6]. The null mutant, Daikoku *d1*, of the G protein α subunit displays dwarfism and small-sized seeds [7]. Similar results were observed in transgenic antisense *RGA* plants that produce little or no mRNA of *RGA* [8]. Investigation of dose response to gibberellic acid (GA_3) revealed that the *d1* mutant showed defective induction of α-amylase activity in the aleurone layer as compared to wild-type plants. In addition, RNA blot analysis showed that GA-inducible genes, *OsMYB*, *Ca^{2+}-ATPase* and *Ramy1*, had a weaker response to GA in the *d1* mutant than that in control plants [9]. Epistatic analysis showed that *slr* (slender rice), caused by mutation in a crucial regulator in GA signaling, is epistatic to *d1* [9]. Undoubtedly, this evidence revealed that GA signaling is affected in the *d1* mutant. However, the precise underlying mechanism remains to be elucidated.

Recently, transgenic plants containing a constitutively active form of *RGA* were generated via site-directed mutagenesis at position 223, which caused the substitution of glutamine by leucine (Q223L), and showed GTP-binding activity but not GTPase activity. The transformants harboring *RGA* (Q223L) did not show a slender phenotype but, rather, were essentially normal cultivars, indicating that heterotrimeric G proteins do not directly regulate GA signaling in rice [10]. In contrast, there have been few reports on the function of the G protein in brassinosteroid (BR) signaling pathways. *OsBLE2* is a novel brassinolide-enhanced gene in rice. The transcript of *OsBLE2* was found to be slightly downregulated and less sensitive to BL treatment in the *d1* mutant as compared with control plants, and investigators speculated that heterotrimeric G protein might be involved in BL signaling [11]. In *Arabidopsis*, mutants of G protein α subunit were less sensitive to brassinolide with respect to stimulated germination and inhibition of vegetative growth [12]. However, little direct evidence exists about whether the G protein is involved in BR signaling in rice.

In BR signal transduction, *BRI1* encodes a plasma membrane-localized leucine-rich repeat receptor-like kinase [13]. BAK1, BRI1-associated receptor kinase, plays a critical role in BR signal perception via dimerization with BRI1 [14, 15]. In the present study, we used root elongation inhibition assay, lamina joint inclination assay and coleoptile elongation promotion assay to show that the *d1* mutant was less sensitive to 24-epibrassinosteroid (24-epiBL), a synthetic brassinosteroid that plays a wide variety of roles in plant growth and development. However, *d1* mutant plants did not display a canonical de-etiolated phenotype with no elongated mesocotyls, as did the *d61-1* mutant (a well-known BR-insensitive rice mutant caused by *OsBRI1* mutation) in the dark. In addition, 24-epiBL treatment also suppressed the expression of *RGA* in a dose-dependent manner. Our direct evidence supports that RGA is involved in affecting brassinosteroid response in rice. The result might be beneficial to elucidate the molecular mechanisms of G protein signaling and also provide a novel perspective to understand BR signaling in higher plants.

Materials and Methods

Plant materials and growth conditions

Seeds of wild-type rice (*Oryza sativa* ssp. *japonica* Nipponbare), *d1* mutant [7] and *d61-1* mutant [16] were used. The seeds were germinated at 30 °C in darkness for 2 days, and then transferred to agar plates containing half-strength Murashige and Skoog (MS) medium supplemented with different phytohormones. Seeds were cultured for 7 days at 25 ºC with a 16-h photoperiod for the physiological experiments.

Semi-quantitative RT-PCR

RNA was extracted for synthesis of first-strand cDNA as previously described [17]. Total RNA of 2 µg was reverse transcribed with Superscript II reverse transcriptase, according to the manufacturer's instructions (Gibco BRL life Technologies, UK). First-strand cDNA was generated with use of an oligo $(dT)_{18}$ primer. The first-strand cDNA of 5 µl was diluted to 20 µl. Diluted first-strand cDNA of 2 µl was used as a template in a subsequent PCR reaction. The abundance of *actin* gene was selected as an internal control. The specific primers (5'-TCA GAT GCC CAG TGA CAG GA-3', 5'-TTG GTG ATC TCG GCA ACA GA-3') were used to amplify *actin*. The primer for *RGA*-specific amplification was the same as that used by Ishikawa *et al.* [5]. PCR components consisted of 10 µl 2× GC buffer, 1 µl 10 mM dNTP, 0.5 µl *LA Taq* polymerase (Takara, Dalian, China), 1 µl of each primer (10 µM), and template first-strand cDNA in a 20 µl reaction volume. The PCR procedure consisted of the following steps: an initial denaturation (2 min, 94 °C), followed by 28 cycles of denaturation (45 s, 94 °C), annealing (45 s, 55 °C for *RGA* primers and 58 °C for *actin* primers) and primer extension (45 s, 72 °C). The final cycle was completed with a 10-min extension at 72 ºC, and then holding for 4 °C. The PCR products were run on 1.2% agarose gel. Images were analyzed by use of Bio-1D Microsoft (BIO-PROFIL, France, www.vilber.com).

Lamina inclination assay

Rice seeds were soaked in distilled water for 2 days in the dark at 30 °C after sterilization with 0.15% HgCl for 10 min, then transferred to half-strength MS medium and cultured at 30 °C for 7 days in dark. The second lamina joints were used for lamina inclination assay as described [18].

Results

Root elongation inhibition assay of d1 mutant

High concentrations of brassinolide can inhibit root growth in rice [16]. To test the response of the *d1* mutant to 24-epiBL, 2-day-old seedlings were treated with 0.1 nM to 10 µM 24-epiBL for 3 days to monitor the length of primary roots. Strikingly, the *d1* mutant seedling exhibited a less sensitive phenotype as compared with control plants (Figure 1). The primary root growth curves of the *d1* and *d61-1* mutants (another well-known BR-insensitive rice mutant, caused by mutation *OsBRI1*, encoding brassinosteroids receptor in rice) were compared. The results showed both mutants with a similar growth tendency until up to 1 µM 24-epiBL treatment, which is distinct from wild-type plants (Figure 1). Primary root length was not greatly inhibited at 1 µM 24-epiBL in the *d1* mutant as compared with the wild type. Increasing the concentration of 24-epiBL from 1 to 10 µM resulted in some inhibition of root length in *d1* plants while little inhibition was observed for *d61-1* (Figure 1), suggesting that heterotrimeric G protein α subunit functions in a different manner with OsBRI1 to affect BR response in rice.

Sensitivity of d1 mutant to 24-epiBL by lamina inclination assay
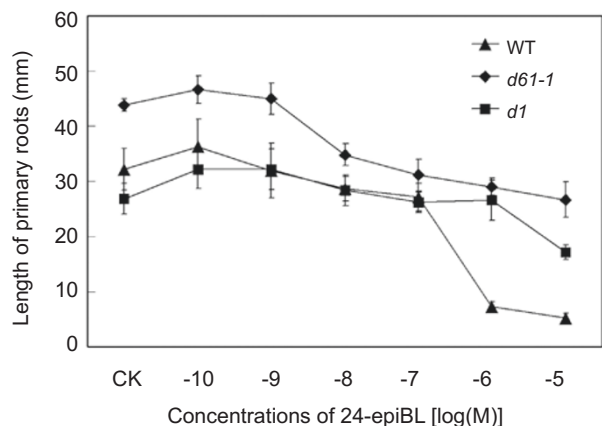
Lamina inclination assay is a classical and specific

Figure 1 Analysis of sensitivity of *d1* mutant to 24-epiBL by the root inhibition assay. Seedlings of the wild type, *d61-1* mutant and *d1* mutant were germinated on distilled water for 2 days and transferred to half-strength MS medium containing various concentrations of 24-epiBL. Roots were measured on day 5 after germination. Each data point represents the average root elongation of 15 seedlings. CK represents the mock-treated control.

method to determine the effect of BR in rice [18]. Both BR-insensitive and -deficient rice mutants display erect leaves [16, 19, 20]. BR-insensitive mutants *d61-1 and d61-2* were found to be less sensitive to BL than the wild type on lamina inclination assay [16]. To further identify the sensitivity of the *d1* mutant to 24-epiBL, lamina inclination assay was adopted. The results revealed that the degree of inclination between the lamina and leaf sheath in *d1* mutant plants was less than that of the control plants (Figure 2A). In *d1* mutant plants, the bending degree was increased with the increase in 24-epiBL concentration, but the extent of inclination was much less than that of control plants. The leaf angles were about 165° in the wild type but just 109° in the *d1* mutant at 100 nM 24-epiBL (Figure 2B), further confirming the lower sensitivity of the *d1* mutant to BR.

Coleoptile elongation response of d1 mutant to 24-epiBL

The rice coleoptile represents a feasible system to study hormonal signaling because its elongation is stopped at an early stage of germination [21]. In the presence of BR, the coleoptile of wild-type plants elongated more than that in the absence of BR; even the foliage leaves cannot break out of the coleoptile at high concentrations of BR in light [16]. These results suggested that BR is involved in the process of coleoptile elongation, and the extent of elongation can be considered reflect sensitivity to BR, since the length of elongation increased with increasing BR concentration. To further examine the sensitivity of the *d1* mutant to BR, we compared the effect of BR on coleoptile elongation in the
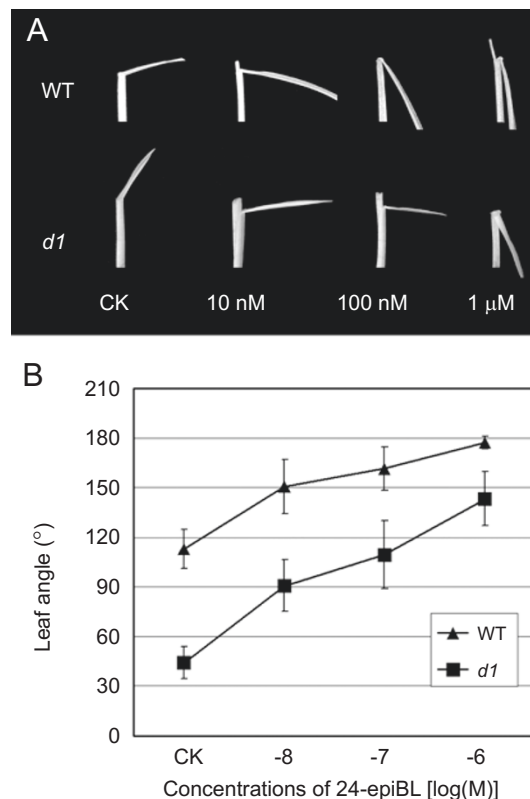

Figure 2 Comparison of 24-epiBL effect on etiolated lamina inclination in wild-type and *d1* plants. **(A)** Dose-response to 24-epiBL of the second lamina joint in wild-type (WT) and *d1* mutant seedlings. **(B)** Dose-response to 24-epiBL of the second lamina joint. Data represent the means of results from six seedlings. Error bars indicate SD, CK represents the mock-treated control.
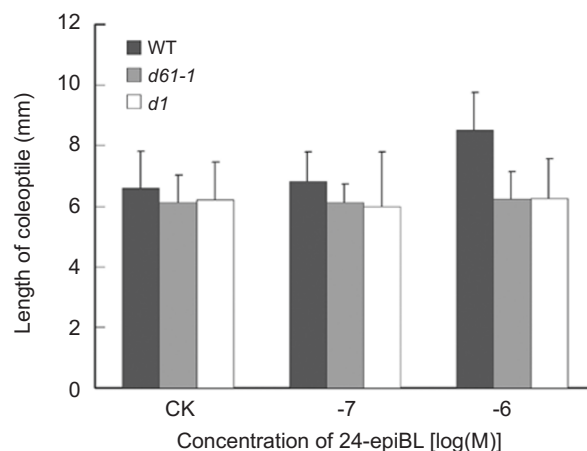

Figure 3 Sensitivity of coleoptile growth to 24-epiBL in *d1*. Seedlings of the wild type (WT) and *d61-1* and *d1* mutants were germinated on half-strength MS medium containing various concentrations of 24-epiBL. Coleoptiles were measured after germination for 7 days. CK represents the mock-treated control. Each data point represents the average coleoptile length of 10 seedlings.

d1, *d61-1* mutants and wild-type plants. The length of the coleoptile of control seedlings increased in the presence of 24-epiBL, whereas that of the *d1* and *d61-1* mutants was less sensitive to 24-epiBL (Figure 3).

Morphology of d1 mutant in the dark

To test whether the *d1* mutant is similar to other rice BR-related mutants that display canonical de-etiolated phenotypes such as unelongated mesocotyls and coleoptiles
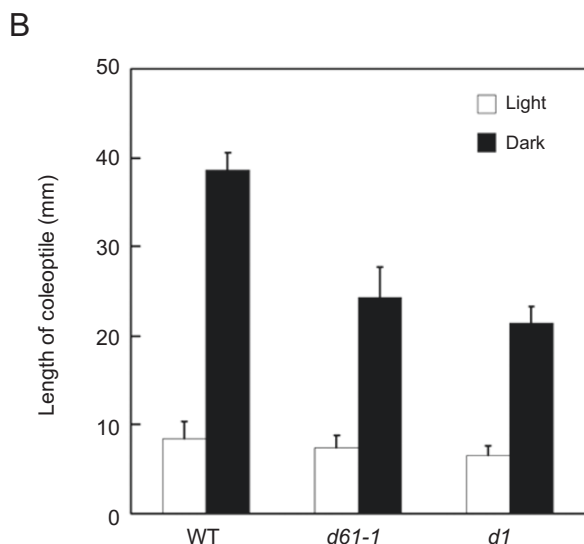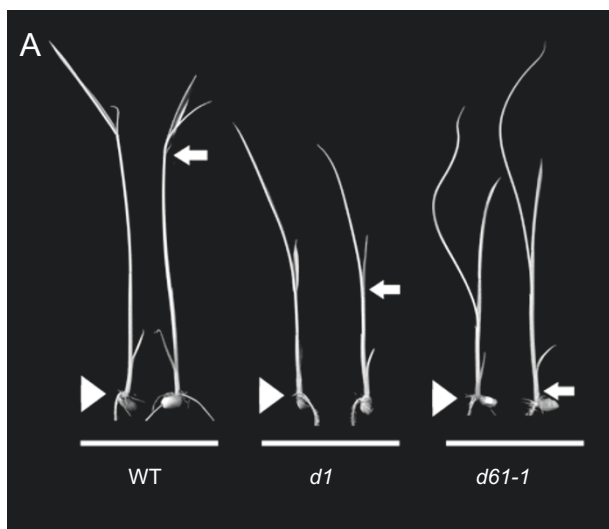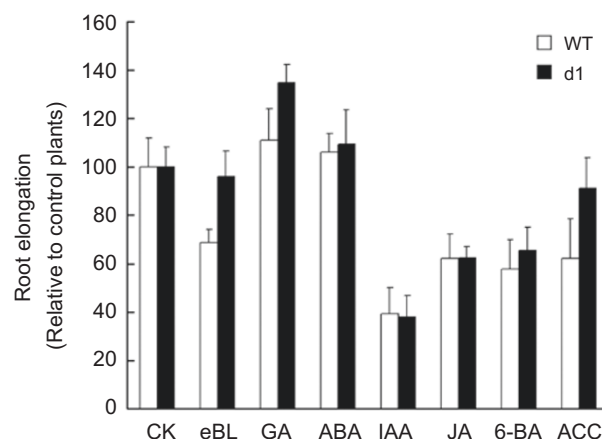

Figure 5 Inhibition of root elongation by plant hormone treatment in the *d1* mutant. Sterilized seeds were germinated on half-strength MS medium containing various plant hormones. The concentrations of plant hormones were 1 µM, except for GA_3, which was 50 µM. CK represents the mock-treated control. See text for details. Germination involved a 16 h photoperiod. Root lengths were measured on the 14th day after germination.

Figure 4 Morphology of *d1* mutant in darkness. **(A)** The *d1* mutant displays non-canonical de-etiolated phenotype as compared with *d61-1*, a mild *OsBRI1* mutation. Triangle points to the position of the mesocotyl, and arrowhead points to the position of the first collar. **(B)** Comparison of coleoptile length in wild type (WT), and *d1* and *d61-1* mutants in dark and light.

in the dark, we germinated *d1*, *d61-1* and wild-type seeds in half-strength MS medium without any plant hormones under darkness. The rice coleoptile has been regarded as an index of skotomorphogenesis. As shown in Figure 4B, the coleoptile elongation of the *d1* mutant in the dark was 3.25-fold of that in the light, while it was 3.28-fold for *d1* and 4.6-fold for wild type, respectively. However, compared with the *d61-1* mutant, the *d1* mutant has normal elongated mesocotyls as do wild-type plants, which is different from *d61-1* mutant with unelongated mesocotyls (Figure 4A). Taken together, we speculated that the *d1* mutant showed non-canonical de-etiolated phenotypes in darkness.

Response of d1 mutant to various plant hormones

In animals, heterotrimeric G protein mediates numerous extracellular environmental signals via coupled stimulus perception by G protein-coupled receptors (GPCRs) with numerous downstream effectors. Recent studies in *Arabidopsis* have shown that plant heterotrimeric G protein is involved in many fundamental processes such as cell proliferation, hormone perception and ion-channel regulation [1]. Many hormone-response mutants usually display changed sensitivity to multiple plant hormones [22]. We used root elongation assay to analyze whether other plant hormones can inhibit rice root elongation in the *d1* mutant. Germinated seeds were cultured on half-strength MS medium containing ABA (abscisic acid, 1 µM), known to be involved in stress response; ACC (1-aminocyclopropane-1-

carboxylate, 1 μM), a precursor for ethylene biosynthesis, JA (jasmonate, 1 μM), an important group of plant signaling molecules that play a pivotal role in the regulation of plant metabolism and defense against pathogens [23]; IAA (indoleacetic acid, 1 μM); 24-epiBL (1 μM), which belongs to brassinosteroids and plays a wide variety of roles in plant growth and development; 6-BA (6-benzylaminopurine, 1 μM), a kind of cytokinin with high activity to promote cell division; and GA_3 (50 μM), which play key roles in cell expansion. As shown in Figure 5, the *d1* mutant showed wild-type-like response to IAA, JA and 6-BA, but was less sensitive to 24-epiBL and ACC.

Response of RGA expression to 24-epiBL

To determine whether the expression of *RGA* was affected by BR, sterilized seeds were cultured on half-strength medium containing various concentrations of 24-epiBL and expression of *RGA* was determined by semi-quantitative RT-PCR at the transcript level. As shown in Figure 6A, the *RGA* transcript was downregulated by 24-epiBL in a dose-dependant manner (Figure 6A and 6B). Furthermore, downregulation of the *RGA* transcript was observed in the 24-epiBL-treated *d61-1* mutant (Figure 6A and 6B), suggesting that the hormone regulates the expression of RGA independently of OsBRI1.
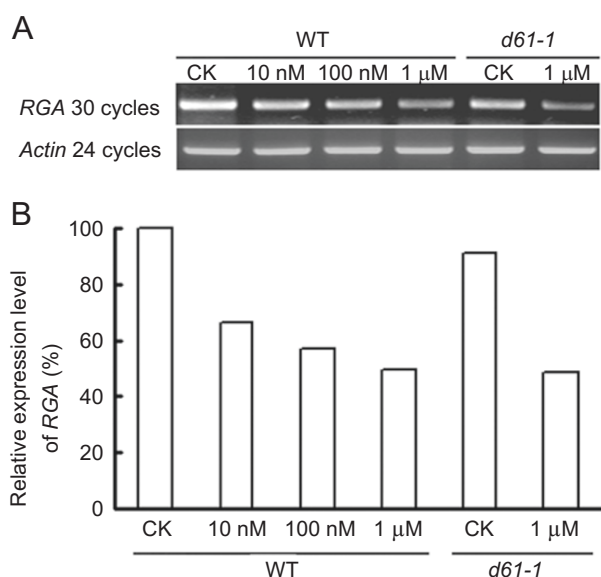

Figure 6 Effect of 24-epiBL on *RGA* expression at the steady-state level. 3-day-old seedlings of wild type (WT) and *d61-1* mutant were harvested after treatment with various concentrations of 24-epiBL. CK represents the mock-treated control. **(A)** Images of RT-PCR products run on 1.2% agarose gel. **(B)** Relative expression of *RGA* in response to different concentrations of 24-epiBL.

Discussion

In *Arabidopsis*, seeds of the *gpa1-1* mutant with a null mutation of heterotrimeric G protein α subunit displayed 100-fold less sensitivity to GA. Ullah *et al*. [12] further identified that *GPA1* potentiation action of GA rather than directly coupling GA to downstream effectors leading to germination, and the potentiality was further shown to couple with BR. Rice *d1* mutant also has been shown to have less sensitivity to GA as it is defective in GA_3-induced α-amylase activity in the aleurone layer [9]. Moreover, RNA blot analysis has shown three GA-inducible genes, *OsMYB*, Ca^{2+}-ATPase and *Ramy1*, with a weaker response to GA in *d1* mutant than in wild-type plant [9]. Thus, GA signaling is definitely altered in *d1* mutant. Transgenic plants containing a constitutively active form of *RGA* (Q223L) did not show a slender phenotype, but rather, were essentially normal cultivars, suggesting that heterotrimeric G proteins do not directly involve the GA signaling pathway in rice [10]. Recently, oligonucleotide microarray analysis demonstrated that genes upregulated by GA or ABA had a higher expression level in wild type than in the *d1* aleurone and genes downregulated by GA had a lower expression level in the wild type relative to the *d1* aleurone [24]. Further, Bethke *et al*. [24] identified that although changes in transcript abundance were smaller in the *d1* mutant than in wild type, the *d1* mutation did not result in a decrease in sensitivity to GA at the level of transcription.

In the present study, we used root elongation inhibition assay to test the response of *d1* mutant to 24-epiBL. Since *d61-1* mutant is well-documented as a rice BRs insensitive mutant by Yamamuro *et al*. [16], it was used as a positive control in root elongation inhibition assay. Our data clearly showed that *d1* mutant was less sensitive to 24-EBL compared to wild-type plants. At the same time, *d61-1* mutant, the positive control plants, also showed insensitivity to 24-EBL. It suggested that the physiological evidence is convincible. In addition, lamina joint bending assay and coleoptile elongation analysis both further proved that *d1* mutant is less sensitive to 24-EBL. Moreover, the *RGA* transcript was downregulated in 24-epiBL-treated seedlings in a dose-dependent manner. These results clearly show that the heterotrimeric G protein α subunit is involved in BR signal response in rice. The *Arabidopsis gpa1-1* mutant, caused by mutation in the *GPA1* gene (encoding the α subunit of heterotrimeric G protein), also showed reduced response to BL with respect to inhibition of vegetative growth and promotion of seed germination [12]. Overall, we speculate that the involvement of the α subunit of heterotrimeric G protein in BR signal response might be a common mechanism in plants. In addition, as was shown in the *det2-1* and *bri1-5* mutants in *Arabidopsis* [12], we have found that

the rice BR-related mutants *d2-1* and *d61-1* showed lower response to GA (our unpublished data) by the α-amylase activity analysis. Moreover, BR can rescue the germination phenotype of the severe GA-biosynthetic mutants *ga1-3*, *ga2-1*, *ga3-1* and the GA-insensitive mutant *sleepy1* in a concentration-dependent manner in *Arabidopsis* [25]. Thus, we speculate that the lower responsiveness of GA in the *d1* mutant might be due to cross-talk between BR and GA pathways.

In addition to our studies of altered BR responsiveness in the *d1* mutant, root elongation inhibition assay revealed changed ethylene sensitivity in the *d1* mutant. Actually, the transcription of ACC oxidase is increased in BR-treated lamina joints of rice [11]. The ACC synthase gene family was also regulated in mung bean [26]. Recently, both ethylene and BR were found to affect the elongation of cotton fibers. The inhibitory effect of BRZ (BR synthesis inhibitor) on fiber elongation can be rescued by ethylene, whereas the suppressed growth of fiber supplemented with 1-aminoethoxyvinylglycine, an ethylene synthesis inhibitor, cannot be reversed by BR, indicating that ethylene might act downstream of BR signaling [27]. Taken together, these results suggest cross-talk between ethylene and BR signaling. Therefore, the changed ethylene sensitivity in the *d1* mutant might be caused by altered BR responsiveness. However, whether BR is the singular plant hormone that couples with RGA and whether the reduced GA response in the *d1* mutant is influenced by the BR signal need to be further investigated.

The *d1* mutant is similar to the wild type in flowering time, whereas *d2-1* and *d61-1* (BR-deficient and -insensitive mutants, respectively) show delayed flowering time as compared with controls [16, 19]. Furthermore, in our studies, the *d1* mutant did not display a canonical de-etiolated phenotype, such as unelongated mesocotyls, as did *d61-1*. In addition, culture on media containing a high concentration of 24-epiBL (10 µM) resulted in inhibited growth of *d1* mutant primary roots (Figure 1), but *d61-1*, the allele weaker than *d61-2* and *d61-4* [16, 28], remained insensitive to 24-epiBL under the same condition. Moreover, the transcript level of RGA was downregulated by 24-epiBL in the *d61-1* mutant (Figure 5). Overall, we speculate that RGA cannot be a direct downstream effector of OsBRI1. Recent evidence strongly supports cross-talk between GPCRs and receptor kinases in animals [29-31]. Receptor kinases are also directly coupled by G proteins [32]. In higher plants, the *MLO* gene family has been demonstrated experimentally to have a 7-transmembrane domain topology similar to GPCRs in animals, with an estimated 35 *MLO* genes in *Arabidopsis*, one might be a G protein coupled receptor [33]. It would be interesting to investigate whether the action of a GPCR could functionally link the heterotrimeric G protein with BR signal response.

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