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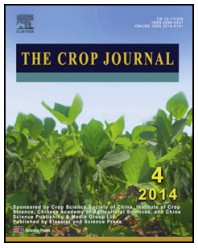

Phenotypic analysis and molecular characterization of an allelic mutant of the *D61* gene in rice

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ABSTRACT

Brassinosteroids (BRs) are a class of plant-specific steroidal hormones that play important roles in multiple biological processes. In this paper, a classic rice mutant *gsor300084*, showing erect leaves and semi-dwarf stature, was characterized. Morphological analysis in darkness showed that the mesocotyl of the *gsor300084* mutant did not elongate when grown in darkness. Coleoptile elongation and root growth were less affected by the exogenous application of brassinolide (BL), the most active form of BR, in *gsor300084* than in the wild-type rice variety Matsumae. Lamina joint bending analysis also showed that *gsor300084* was less sensitive to exogenous BL than Matsumae. These results suggested that the *gsor300084* mutant is defective in BR sensitivity. Map-based cloning indicated that *gsor300084* is a novel allelic mutant of the *DWARF61* (*D61*) gene, which encodes the putative BR receptor OsBRI1. A single-base mutation appears in the LRR domain of OsBRI1, changing the 444th amino acid from tryptophan (W) to arginine (R). Subcellular localization analysis suggested that both the wild-type and mutant OsBRI1 protein are localized at the cytoplasmic membrane. Structure modeling revealed that the W444R substitution may affect the perception of BRs by the LRR domain.

1. Introduction

Brassinosteroids (BRs) are a class of steroid compounds involved in diverse biological processes during plant growth and development, including seed size and germination, stem elongation, plant height regulation, vascular differentiation, reproductive development, flowering time, male fertility, photomorphogenesis, and stress response [1–9].

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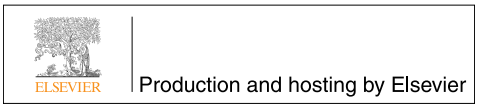

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A few BR-deficient or -insensitive mutants have been identified in *Arabidopsis* and rice, exhibiting pleiotropic phenotypes. BR-related mutants in *Arabidopsis* showed a distinctive dwarf phenotype with dark green leaves and exhibited defects in hypocotyl elongation and cotyledon closing when grown in darkness [10–13]. The rice BR-related mutants showed dwarf phenotype, erect leaves, and small and round seeds and exhibited defects in mesocotyl elongation in darkness and leaf angle enlargement in the lamina joint inclination assay [3,4,14].

In plants BRs are perceived at the cell surface by a member of the large family of leucine-rich repeat receptor-like kinases (LRR-RLKs), namely BRASSINOSTEROID INSENSITIVE 1 (BRI1) [15–17]. The *BRI1* gene was first cloned in *Arabidopsis* [18] and its ortholog *D61* was also identified in rice [4]. The BRI1 protein contains a hydrophobic signal peptide at the N-terminus, an extracellular leucine-rich repeat (LRRs) domain interrupted by a non-repetitive island domain, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain [18,19]. The kinase activity of BRI1 is essential for BR regulation of plant growth and development in rice [20]. The N-terminal signal peptide is likely to be required for translocation of the nascent protein across a membrane, while the transmembrane domain is required to anchor the protein in the plasma membrane [21]. The island domain and the adjacent C-terminal LRR repeat of the extracellular domain are responsible for perceiving BRs [22–24]. The LRR domain may be involved in facilitating protein–protein interactions between individual BRI1 molecules or with other proteins such as BAK1 [25].

BR binding can enhance BRI1 heteromerization with BAK1 (BRI1-associated kinase 1), another LRR-RLK that is localized to the plasma membrane [25]. In *Arabidopsis*, BAK1 and BRI1 share similar gene expression and subcellular localization patterns and physically associate with each other. BAK1/BRI1 interaction activates their kinase activities through transphosphorylation [26]. Structure analysis reveals that BAK1 acts as a co-receptor to recognize the BRI1-bound brassinolide and the extracellular domains of BRI1 and BAK1 interact with each other in a BL- and pH-dependent manner [27]. According to the solved crystal structure of the BRI1LRR-BL-BAK1LRR complex, the C-terminal two LRRs of BRI1LRR make extensive and direct contact with BAK1LRR [27]. Thus the structural stability of the BRI1 LRR domain is very important for both BR perception and association with the co-receptor BAK1.

In the present study we characterized a classic semi-dwarf mutant with erect leaves in rice, designated as *gsor300084*. *gsor300084* was insensitive to BRs and shown to be an allelic mutant of *D61* (*OsBRI1*). A point mutation in the LRR domain was found in the *gsor300084* mutant. The potential effect of this mutation on BRI1 protein structure and function is discussed.

2. Materials and methods

2.1. Plant materials and growth conditions

The *gsor300084* mutant and the wild-type variety Matsumae (*Oryza sativa* ssp. *japonica*, cv. Matsumae) were kindly provided by the USDA-ARS Dale Bumpers National Rice Research Center. The rice plants were grown in a paddy field at the experimental station of the Shandong Rice Research Institute, Shandong, China.

2.2. Skotomorphological analysis

Rice seeds were soaked in water for 24 h and then sprouted at 37 °C. Well-germinated seeds were transferred into 96-well plates supplemented with water and grown in the dark at 28 °C for 20 days.

2.3. Response of rice seedling to BL

Seeds of the *gsor300084* mutant and Matsumae were grown in half-strength MS solid medium with 0 or 1 μmol L^{-1} BL in a dark growth chamber at 28 °C for 4 days. Coleoptile and root elongation analysis was performed by measuring the length of coleoptile and root treated with or without BL.

2.4. Lamina joint bending assay

Rice seeds were grown in half-strength MS solid medium with 0 or 1 μmol L^{-1} BL at 28 °C under continuous light for 15 days. The angle between the second lamina and sheath was measured.

2.5. Map-based cloning

An F_2 mapping population from a cross between *gsor300084* and the *indica* variety Dular was generated. InDel markers (170 pairs) and 20 F_2 individuals showing mutant phenotypes were used in primary mapping. Seven InDel markers were developed and 358 F_2 individuals were used for fine mapping. Genomic DNA fragments of the *D61* gene from Matsumae and *gsor300084* were amplified and sequenced.

2.6. Subcellular localization

The coding sequence of the *D61* gene from the wild type and the *gsor300084* mutant was fused in-frame to the 3′ end of the sGFP gene in the transient expression vector pCAMBIA1205-GFP. The 1205-GFP-$d61^{300084}$ and 1205-GFP-D61 fusion constructs were transformed into protoplasts prepared from wild-type rice seedlings by polyethylene glycol treatment. The transformed protoplasts were incubated at 28 °C for 16 h. Green fluorescence of the GFP fusion protein was observed under a Zeiss LSM 510 META confocal microscope.

3. Results

3.1. Pleiotropic phenotypes of the gsor300084 mutant

The phenotype of the *gsor300084* mutant was different from that of the wild type variety Matsumae in many respects. The plant height of *gsor300084* was less than that of Matsumae from the seedling stage (Fig. 1-A). At maturity, the plant height of *gsor300084* was only about one half that of the wild type (Fig. 1-B and Table 1). In wild-type plants, the leaf blade bent away from the vertical axis of the leaf sheath toward the

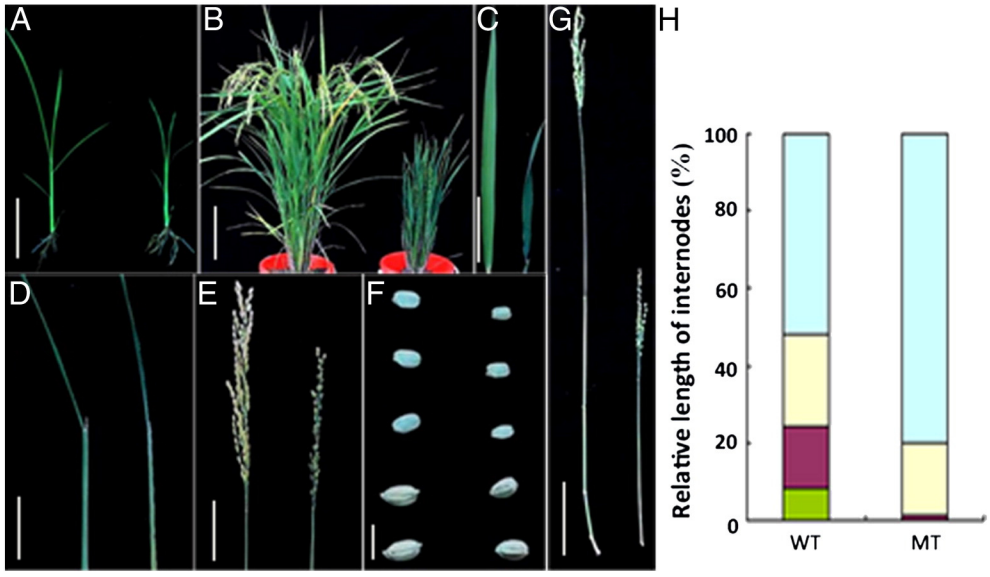

Fig. 1 – Morphological phenotypes of the *gsor300084* mutant. (A) Gross morphology of the wild type Matsumae (left) and mutant *gsor300084* (right) at the seedling stage. Bar = 5 cm. (B) Gross morphology at the maturity stage. Bar = 20 cm. (C) Flag leaf morphology of wild type (left) and mutant (right). Bar = 5 cm. (D) Leaf angle of wild type (left) and mutant (right). Bar = 5 cm. (E) Panicle structure of wild type (right) and mutant (left). Bar = 5 cm. (F) Seed morphology of wild type (left) and mutant (right). The upper three rows: dehusked seeds; the lower two rows: seeds with husk. Bar = 5 mm. (G) Phenotypic exhibition of internodes of wild type (left) and mutant (right). Bar = 10 cm. (H) Relative length of internode. From the top to the bottom are the first to the fourth internode, respectively.

abaxial side, but in *gsor300084* most of the leaves were erect (Fig. 1-D). The panicle of *gsor300084* was smaller than that of the wild type (Fig. 1-E). Moreover, the grains of *gsor300084* were smaller and rounder (Fig. 1-F and Table 1) and the grain weight was significantly reduced (Table 1). Internode elongation was severely inhibited (Fig. 1-G) except for the uppermost internode (Fig. 1-H), indicating that *gsor300084* is a d6-type dwarf mutant [28].

3.2. Skotomorphogenic phenotype

In rice mutants with defects in BR biosynthesis or sensitivity, elongation of the mesocotyl is inhibited when plants are grown in complete darkness [2]. To determine whether *gsor300084* is a BR-related mutant, the mesocotyl internode elongation pattern in the darkness was observed. After growth in complete darkness for two weeks, the wild type plants exhibited mesocotyl elongation, whereas no such elongation occurred in *gsor300084* (Fig. 2). The failure of mesocotyl elongation in *gsor300084* is similar to the phenotype of other rice BR-related mutants grown in darkness [4,29].

Table 1 – Comparison of morphological traits between gsor300084 and wild type.

Trait	Wild type	*gsor300084*
Plant height (cm)	93.66 ± 2.48	49.02 ± 2.80**
Panicle length (cm)	18.82 ± 1.81	13.43 ± 3.27**
Grain number per panicle	113.60 ± 8.01	37.22 ± 5.76**
Grain length (mm)	7.48 ± 0.32	5.37 ± 0.22**
Grain width (mm)	3.50 ± 0.18	3.43 ± 0.11
100-grain weight (g)	2.77 ± 0.06	1.95 ± 0.13**

Value = mean ± *SE*, $n = 15$. ** significantly different at $P < 0.01$ level.

3.3. The gsor300084 seedlings were less sensitive to BL than wild type

Based on the abnormal phenotypes of *gsor300084*, we suspected that the *gsor300084* mutant was defective in BR biosynthesis or sensitivity. To identify the type of BR mutant to which *gsor300084* belongs, we evaluated the coleoptile elongation and root length of wild type and *300084* seedlings in response to BL. Rice seeds were germinated in half-strength MS medium with 0 or 1 μmol L^{-1} BL in complete darkness. In wild-type plants, coleoptile elongation was promoted,

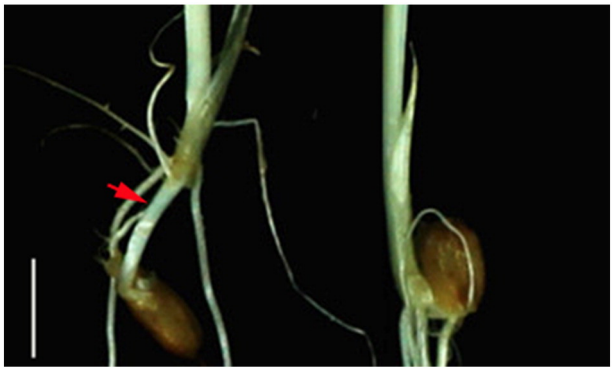

Fig. 2 – Skotomorphogenic phenotype of a wild type (left) and the *gsor300084* mutant (right). Arrowhead indicates elongated mesocotyl. Bar = 5 mm.

whereas root length was significantly inhibited by the presence of 1 μmol L^{-1} BL. However, coleoptile and root length did not vary significantly after BR treatment (Fig. 3-A, B and C).

3.4. *Lamina joint inclination assay confirmed the insensitivity of gsor300084 to BL*

A more sensitive method, lamina joint inclination assay [30], was used to examine sensitivity of *gsor300084* to BL. In the absence of BL, the bending angle of the leaf blade in *gsor300084* was smaller than that in wild-type plants (Fig. 4-A, B). In the presence of 1 μmol L^{-1} BL, the leaf angle increased dramatically in the wild type but remained almost unchanged in the *gsor300084* mutant (Fig. 4-A, B). This result further confirmed that *gsor300084* mutant seedlings were less sensitive to exogenous BL than wild-type seedlings.

3.5. *gsor300084 is a novel allelic mutant of the D61 gene*

Primary mapping using 20 F_2 mutant individuals derived from a cross between *gsor300084* and the *indica* variety Dular revealed that the mutation resided on the long arm of chromosome 1 between the InDel markers R1–12 and R1–13 (Fig. 5-A and Table 2). Fine mapping using 7 new inDel markers and 358 F_2 mutant individuals narrowed the location to a 107 kb region. According to the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), the *D61* gene (LOC_Os01g52050) encoding the putative BR receptor OsBRI1 is located within this region. Accordingly, the genomic DNA fragment of the *D61* gene from both *gsor300084* and Matsumae was amplified and sequenced. Sequence analysis revealed that only the 1330th base in the *D61* coding region was changed from T to A, causing the 444th amino acid tryptophan (W) to be substituted by arginine (R). This mutation was located in the LRR region of OsBRI1 and adjacent to the island domain (Fig. 5-A). Sequence alignment among BRI1 orthologs from different plant species revealed that this mutation site is highly conserved (Fig. 5-B), indicating that this residue is important for BRI1 protein function.

3.6. *Subcellular localization of OsBRI1 was not altered*

Although leucine-rich repeats (LRRs) are frequently involved in protein–protein interactions, a previous study had shown that mutations in the LRR domain led to changed protein subcellular localization [31]. We accordingly wondered whether the W444R substitution has any effects on the subcellular localization of OsBRI1. The wild-type and *gsor300084* mutant allele of the *D61* gene fused in-frame with the *sGFP* gene was transformed into rice protoplasts. Fig. 6 presents a confocal microscopy analysis of OsBRI1 expression, showing that OsBRI1::GFP fluorescence is localized to the cell surface. Thus the OsBRI1-directed GFP fluorescence is at the cell wall or plasma membrane but not in the cytoplasm. Since the cell wall has been removed during the preparation of protoplasts from rice seedlings, OsBRI1 should be localized at the plasma membrane. This result is consistent with the subcellular localization analysis of the *Arabidopsis* BRI1 protein, in which a plasmolysis experiment confirmed that BRI1 was localized at the plasma membrane rather than at the cell wall [24,26]. Interestingly, the green fluorescence of both the wild type and mutant sGFP-OsBRI1 was detected at the plasma membrane (Fig. 6). This result indicates that the W444R substitution has no effect on the OsBRI1 subcellular localization.

4. Discussion

Brassinosteroids (BRs) are a class of steroid compounds involved in diverse biological processes during plant growth and development. Here we have reported a classic semi-dwarf and erect-leaf rice mutant *gsor300084*. It belongs to the d6-type dwarf mutants, in which internode elongation was severely inhibited except for the uppermost internode. The *gsor300084* mutant was shown to be related to BR and was less sensitive to BRs by assays of coleoptile elongation, root growth inhibition, and lamina joint inclination in the presence of exogenous BL. All these results indicate that *gsor300084* is a BR-insensitive mutant. Map-based cloning showed that *gsor300084* is a novel allelic mutant of the *D61*/

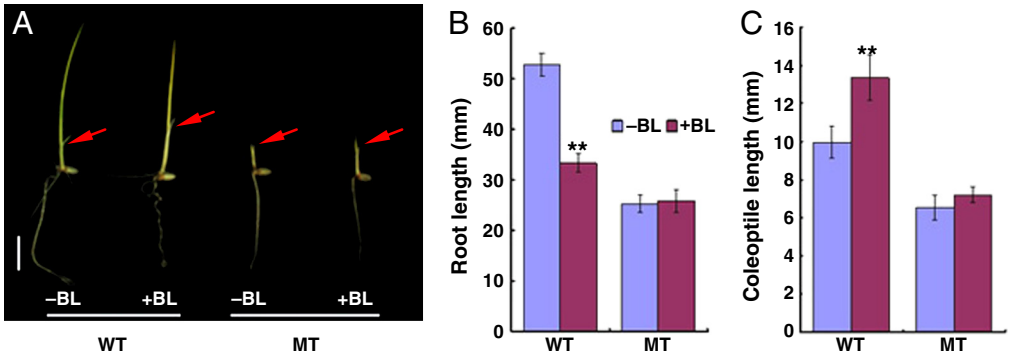

Fig. 3 – Response of rice seedlings to BL. (A) Effect of BL on coleoptile and root elongation in wild type (WT) and *gsor300084* mutant (MT). Arrow indicates the tip of coleoptile. Bar = 10 mm. (B) Root length of WT and MT seedling in absence or presence of 1 μmol L^{-1} BL. (C) Coleoptile length of WT and MT seedling in the absence or presence of 1 μmol L^{-1} BL. ** Significantly different at $P < 0.01$ level. Bar on the top of each column represents standard error, $n = 15$.

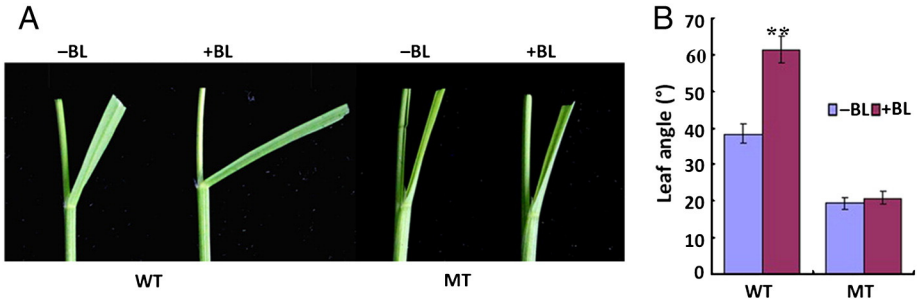

Fig. 4 – Lamina joint bending responses to BL in wild type (WT) and *gsor300084* (MT). (A) Effect of BL on lamina joint bending in WT and MT. (B) Change in leaf angle after BR treatment. ** Significantly different at $P < 0.01$ level. $n = 15$.

OsBRI1 gene. The 444th amino acid, tryptophan (W), located in the LRR domain, is substituted by arginine (R) and the mutation site is highly conserved among BRI1 orthologs from different plant species. These results suggest that this mutation site is important for BRI1 protein function and BRI1-mediated plant growth and development.

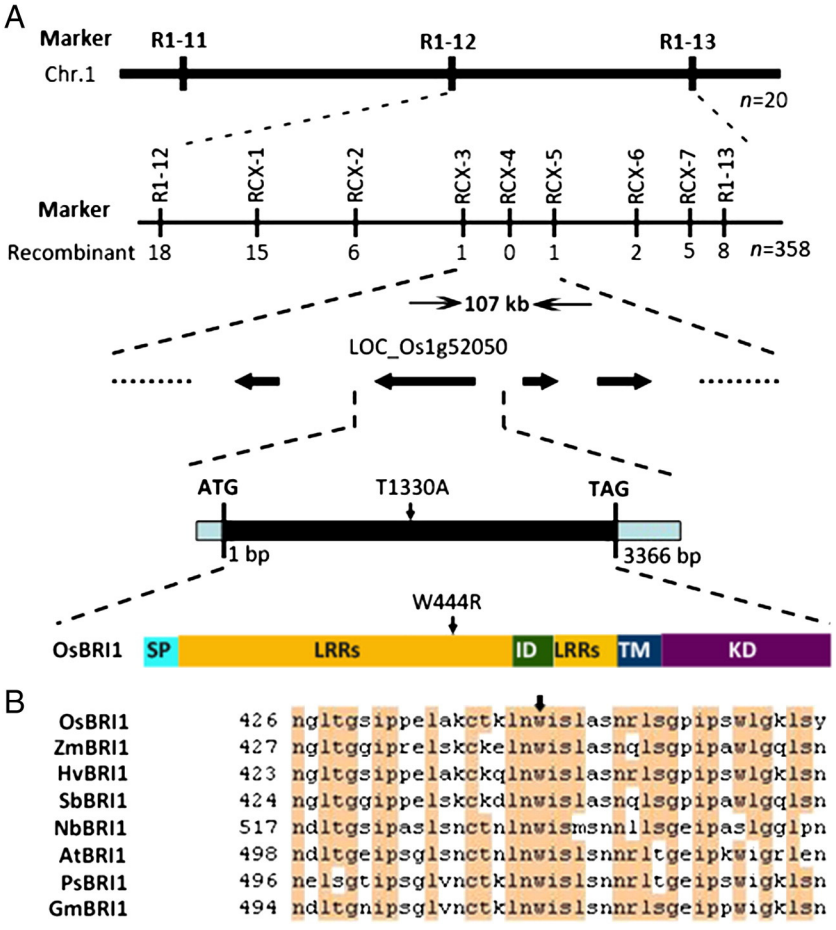

Fig. 5 – Map-based cloning of the target gene underlying the *gsor300084* phenotype and structure of the OsBRI1 protein. (A) Map-based cloning of the causative gene for the *gsor300084* mutant. The upper three rows show the map-based cloning process with molecular markers and recombinants indicated above and below the line, respectively. The lower two rows show the gene structure and protein domains of OsBRI1 with a signal peptide (SP) domain, leucine-rich repeat (LRR) domain, island domain (ID), transmembrane (TM) domain and kinase domain (KD). The change of T1330A in *gsor300084* results in the conversion of tryptophan (W) to arginine (R) at the 444th residue in the LRR domain. (B) Alignment of partial sequence surrounding the mutation site (indicated by arrow) in BRI1 orthologs from different plant species including *Oryza sativa* OsBRI1 (BAB68053), *Zea mays* ZmBRI1 (AFW83751), *Hordeum vulgare* HvBRI1 (BAD01654), *Sorghum bicolor* SbBRI1 (XP_002458412), *Nicotiana benthamiana* NbBRI1 (ABO27628), *Arabidopsis thaliana* AtBRI1 (NP_195650), *Pisum sativum* PsBRI1 (BAC99050), and *Glycine max* GmBRI1 (NP_001237411).

Table 2 – Primer sequences used for map-based cloning.

Name	Forward (5′–3′)	Reverse (5′–3′)
R1-11	GGATGATTTTGAGGAACATGG	ATCTTTGCCTGCAGAGTGCT
R1-12	CCACAAGCTCAAGCTTCAAA	TCGAATTACTCGCATTGATCC
R1-13	CGGTTGGTCATGAACTTGC	CAGCTACAAGCCCACCGTAT
RCX-1	TTCTGAGAAGCAGCTGGTTG	CACATTATATGCTGCATTTTC
RCX-2	GCCACCATAGCTCACTTCAA	TCCCATTGGTCATTGACCAC
RCX-3	ACGAGAAACAAATGGTAGGA	ATCGCATCCAAAGTTAGGAG
RCX-4	GTGCAAACAGAAGTGCAACG	ACCCATCATCAAACATCGGC
RCX-5	CGTGGATGAGGAAGTGGAAG	GCGAACTATATCTAGGGATG
RCX-6	TTGGAATAAGAGCATCACTC	CGTATATGGACTTTGGACTC
RCX-7	AGCTCCATCGGTATCGCCAA	GAGTGTACAAGGCTAAGGT
CXD61	GTACAAATGATCCCAGCAAC	CCTCACATGGAAACAGGAGC

More than ten allelic mutants of *D61* have been reported in rice [4,20,32,33]. Only four (*d61-2*, *d61-3*, *d61-5*, and *d61-7*) have distinctive mutations in the LRR domain and show various degrees of phenotypic severity. In *d61-2* the 491st amino acid, valine, is substituted by methionine, producing an intermediate phenotype [32]. *d61-3* and *d61-5* are two severe mutants that harbor the H420P and N426Y substitution, respectively. The phenotype of *d61-7*, in which the 467th amino acid is changed from alanine to valine, is milder [33]. The *gsor300084* mutant described in this study, harboring the W444R substitution, most resembles *d61-2*, showing an intermediate phenotype. Interestingly, the five mutation sites (H420P, N426Y, W444R, A467V, and V491M) are clustered together in a small portion of the LRR domain, which may be a potential essential motif for BRI1 function. However, the manner in which these mutations affect the OsBRI1 function remains unclear. Our protein localization analysis revealed that defects other than subcellular localization account for the OsBRI1 dysfunction.

The extracellular domain of *Arabidopsis* BRI1 contains 25 LRR repeats and a 70-amino acid island domain between the 21st and 22nd LRR [18]. The crystal structure of the extracellular domain of AtBRI1 has been resolved. The AtBRI1 LRR comprises a helical solenoid structure, while the separate island domain anchors onto the inner surface of the solenoid and spans six LRRs (LRR 17–22) [22,23]. The brassinolide molecule binds to a hydrophobic groove between the island domain and the inner surface of the LRRs. Thus both the island domain and the adjacent C-terminal LRR repeat (LRR 17–22) contribute to the formation of the hormone binding site [22,23]. Extensive non-covalent interactions occur between the island domain and the solenoid structure. For example, W516, I540, W564, and F658 in LRRs establish close contacts with the island domain [23]. Several *Arabidopsis* mutants in the island domain and adjacent LRRs exhibit a BR-insensitive phenotype. For example, *bri1-6*, carrying the G644D mutation in the island domain, shows a loss-of-function phenotype [34]; $bri1_{sud1}$, carrying the G643E mutation in the island domain, stabilizes the island domain and shows a gain-of-function phenotype [35]. The loss-of-function allele *bri1-9* (S662F in the 22nd LRR) has been mapped to the island domain—LRR interface and probably interferes with folding of the island domain [34].

The W444R mutation in the rice *gsor300084* mutant is equivalent to the W516 in the 19th LRR of the *Arabidopsis* BRI1 protein [18], which is involved in the formation of the brassinolide binding site as described above. Thus, although the W444R mutation occurs outside of the island domain (from L508 to F577), it still likely adversely affects the perception of BL. Compared with the *Arabidopsis* BRI1 (AtBRI1) protein, the rice BRI1 (OsBRI1) protein lacks three LRR domains, corresponding to the third to fifth LRR repeats of AtBRI1 [4]. Thus, the LRRs that contribute to the formation of the hormone binding site are expected to be LRR14-19 in OsBRI1. We performed *in silico* structure modeling of the extracellular domain of the wild-type and *gsor300084* mutant

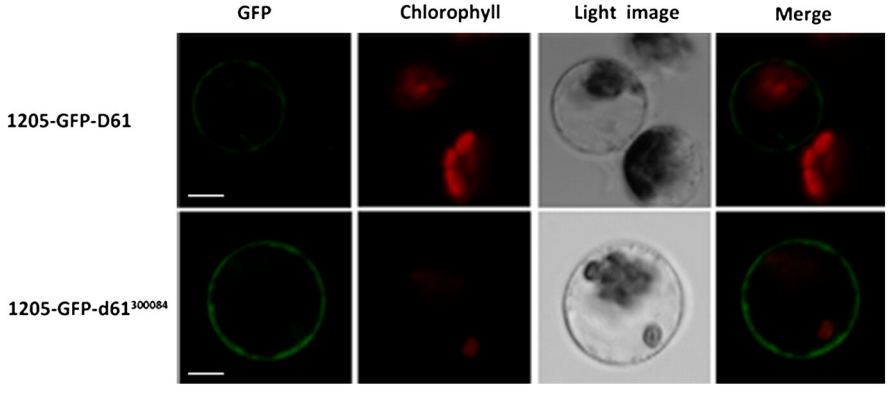

Fig. 6 – Subcellular localization of the wild type and mutant OsBRI1 protein. The 1205-GFP-D61 and 1205-GFP-d61^{300084} vector was transformed into protoplasts prepared from rice seedlings. Green fluorescence of the GFP fusion protein was viewed under a confocal microscope. Red fluorescence of chlorophyll and light-field images are also shown. Bar = 10 μm.

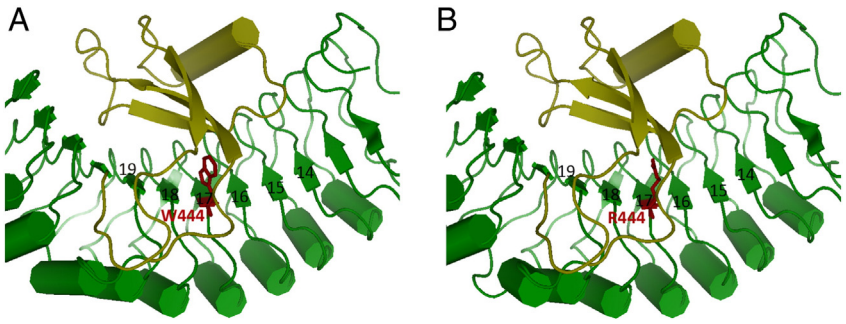

Fig. 7 – *In silico* structure modeling of the extracellular domains of the wild type and *gsor300084* mutant OsBRI1. The structure is predicted by CBS Prediction Servers (http://www.cbs.dtu.dk/services/), viewed and compared by PyMOL. (A) *In silico* structure modeling of the extracellular domain of the wild-type OsBRI1. (B) *In silico* structure modeling of the extracellular domain of the *gsor300084* mutant OsBRI1. The LRR and island domain are shown in green and yellow cartoons, respectively. The position of the W444R mutation is shown as a red stick.

OsBRI1. There was no dramatic change in the BR binding groove formed between the island domain and LRR14-19 (Fig. 7). However, the change from the neutral hydrophobic tryptophan to the basic hydrophilic arginine may exert a subtle effect on the hydrophobic environment of the binding groove (Fig. 7). So the W444R mutation can perturb local conformations and consequently hinder BRI1 recognition of brassinosteroids. The rice *gsor300084* mutant, together with other missense mutations, will play useful roles in assigning functions to specific domains or motifs and allow us to validate the structural model of the BRI1 protein.

Acknowledgments

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