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A Novel Gene Involved in Brassinosteroid Signaling in Rice

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**BRASSINOSTEROID UPREGULATED 1**, Encoding a Helix-Loop-Helix Protein, is a Novel Gene Involved in Brassinosteroid Signaling and Controls Bending of the Lamina Joint in Rice

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Abstract

Brassinosteroids (BRs) are involved in many developmental processes and regulate many subsets of downstream genes throughout the plant kingdom. However, little is known about the BR signal transduction and response network in monocots. To identify novel BR-related genes in rice, we monitored the transcriptomic response of the brassinosteroid deficient 1 (brd1) mutant, with a defective BR biosynthetic gene, to brassinolide (BL) treatment. Here, we describe a novel BR-induced rice gene BRASSINOSTEROID UPREGULATED 1 (BU1) encoding a Helix-Loop-Helix (HLH) protein. Rice plants overexpressing BU1 (BU1:OX) showed enhanced bending of the lamina joint, increased grain size and resistance to brassinazole, an inhibitor of BR biosynthesis. In contrast to BU1:OX, RNAi plants designed to repress both BU1 and its homologues displayed erect leaves. In addition, compared to the wild-type (WT), the induction of BU1 by exogenous BL did not require de novo protein synthesis and it was weaker in a BR receptor mutant OsbriI (Oryza sativa brassinosteroid insensitive 1, d61) and a Rice G protein alpha subunit (RGA1) mutant d1. These results indicate that BU1 protein is a positive regulator of BR response: it controls bending of the lamina joint in rice and it is a novel primary response gene that participates in two BR signaling pathways through OsBRI1 and RGA1. Furthermore, expression analyses showed that BU1 is expressed in several organs including lamina joint, phloem and epithelial cells in embryos. These results indicate that BU1 may participate in some other unknown processes modulated by BR in rice.
Introduction

Brassinosteroids (BRs) are essential growth regulators, involved in many physiological processes, e.g. cell expansion and division, vascular bundle differentiation, skotomorphogenesis, flowering, senescence, abiotic and biotic stresses (Szekeres et al., 1996; Clouse and Sasse 1998; Nakashita et al., 2003; Yu et al., 2008). The main components of BR signaling have been mostly identified in Arabidopsis (Belkhadid and Chory, 2006). BRs are perceived by a leucine-rich repeat receptor kinase known as BRASSINOSTEROID INSENSITIVE 1 (BRI1; Li and Chory 1997; Kinoshita et al., 2005). BRI1 heteromerizes with the BRI1 ASSOCIATED KINASE 1 (BAK1), and this protein complex phosphorylates BRASSINOSTEROID SIGNALING KINASES (BSKs; Nam and Li 2002; Tang et al., 2008). BSKs inhibit the kinase activity of BRASSINOSTEROID INSENSITIVE 2 (BIN2), which encodes a GLYCOGEN SYNTHASE KINASE -3 (GSK3)-like kinase. In the absence of BR, BIN2 phosphorylates two transcription factors, BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), and abolishes their functions and BR response (He et al., 2002; Yin et al., 2002). On other hand, BRs induce dephosphorylation of BZR1 and BES1 which are transcriptional regulators of target genes in the nucleus (He et al., 2002; Yin et al., 2002).

BZR1 and BES1 have been identified by genetic screens based on skotomorphogenesis (Wang et al., 2002; Yin et al., 2002). In Arabidopsis, it has been reported that hypocotyls of BR deficient and insensitive mutants, such as constitutive photomorphogenic dwarf (cpd) and bri1, are shortened and de-etiolated (Szekeres et al., 1996; Li and Chory, 1997). In contrast, hypocotyls of constitutive BR-response mutants
are not shortened and de-etiolated by a BR deficiency that was artificially induced by brassinazole (BRZ, Asami et al., 2000). The \textit{bzl1-D} mutant was screened as a BRZ-resistance gain of function mutant (Wang et al., 2002). A suppressor of a \textit{bzl1} mutant, \textit{besl-D}, also showed phenotypes similar to \textit{bzl1-1D} (Yin et al., 2002).

We have previously isolated the \textit{brd1} mutant that had a defective BR biosynthetic gene, \textit{BRD1 (OsBR6ox/OsDWARF)} in rice (Mori et al., 2002). Analyses of \textit{brd1} mutants clarified many BR effects in rice. The \textit{brd1} mutant showed severe dwarfism, sterility, small seeds, abnormal vascular bundles, shortened internodes and inhibition of elongation of the coleoptile and mesocotyl in the dark (Mori et al., 2002; Hong et al., 2002). The lamina joint, which is a border region between the leaf blade and sheath, is an especially sensitive organ to BR and it is severely bent after exposure to active BRs (Wada et al., 1981). Loss of function mutants of BR biosynthesis, \textit{d2} and \textit{d11}, have erect leaves because of BR deficiency (Hong et al., 2003; Tanabe et al., 2005). In addition, BR-deficient rice mutants have much shorter grains than wild-type (WT) (Mori et al., 2002; Hong et al., 2003; Tanabe et al., 2005). On the other hand, hyperproduction of BR by overexpression of \textit{DWARF4}, which encodes a BR biosynthetic gene, increased seed size and yield in Arabidopsis (Choe et al., 2001). In rice, overexpression of \textit{DWARF4} increased grain filling, but there was no mention of its effect on grain size (Wu et al., 2008).

Some components of BR signaling have been also identified in rice. OsBRI1 and OsBZR1 have been respectively identified as counterparts of BRI1 and BZR1 in Arabidopsis and their loss of function mutants display BR deficient mutant -like phenotypes such as erect leaves (Yamamuro et al., 2000; Bai et al., 2007). OsBZR1 directly binds to the promoter of \textit{DWARF AND LOW TILLERING (DLT)} gene, whose
product is needed for the full BR response in rice (Tong et al., 2009). Recently, rice heterotrimeric G protein alpha 1 (RGA1), known to be involved in gibberellic acid signaling and disease resistance (Ueguchi-Tanaka et al., 2000; Suharsono et al., 2002), was also implicated as a BR signaling component (Wang et al., 2006; Oki et al., 2009). Loss of function mutant of RGA1, \(d1\), displayed less sensitivity to BL in lamina joints and primary roots. Since expression patterns of OsBZR1-regulated genes were not altered by BL in \(d1\) mutants, RGA1 may be involved in a BR signaling pathway distinct from that of OsBZR1 (Oki et al., 2009).

Some transcriptional regulators identified in BR signaling encode basic/helix-loop-helix (bHLH) proteins in Arabidopsis. BES1 interacts with another transcription factor BES1-INTERACTING MYC-like 1 (BIM1), which encodes a MYC-like bHLH protein, and both proteins together bind to a promoter region of the target gene to regulate expression (Yin et al., 2005). In addition, three redundant bHLH proteins, BR ENHANCED EXPRESSION 1-3 (BEE1-3), are products of early BR response genes and they are required for the full BR response (Friedrichsen et al., 2002). Although some bHLH proteins play a critical role in BR signaling in Arabidopsis as described above, little is known in rice. Here, we report the identification and characterization of a novel rice gene BRASSINOSTEROID UPREGULATED 1 (\(BU1\)), which encodes a bHLH protein but presumably lacks a basic region, by using rice plants overexpressing and repressing \(BU1\).
RESULTS

Isolation of BR-induced Genes

To identify novel BR-regulated genes, RNA were obtained from young shoots of 50-day-old brd1 mutants, with or without 40 nM of BL treatment for 24 hrs and analyzed using a 22K rice microarray. 96 of 21,938 genes were up-regulated by BL application, as defined by a more than 2-fold difference in the fold change values that represent ratios of hybridization signals between BL-treated and control brd1 plants. Several Arabidopsis genes reportedly involved in BR signaling, such as BEE1-3 and BIM1-3, contain basic/helix-loop-helix (bHLH) motifs. Therefore, we searched the bHLH motif among the 96 upregulated genes and identified three genes. In this paper, we report one of the three genes with the bHLH motif that we have designated as BU1 (Os06g0226500) (Fig.1A), whose induction by exogenous BL has been confirmed by real-time PCR analysis both in brd1 and WT plants (Supplemental Fig. S1 and Fig. 4A). We will provide details of our microarray results and other up-regulated genes in future publications.

In silico Analysis of BU1 Protein

Transcription factors with the bHLH motif regulate a large array of target genes and play a pivotal role in cellular signaling in living organisms (Ledent and Vervoort, 2001; Toledo-Ortiz et al., 2003; Li et al., 2006). A bHLH protein has two functional domains, the basic region and the HLH region. The former is required for binding to DNA to regulate the expression of genes, while the latter is required for interaction with other bHLH proteins for hetero- or homodimerization (Murre et al., 1989). In rice, more than 160 bHLH genes have been identified (Li et al., 2006). Although the BU1 protein is not
described by Li et al. (2006), it has an HLH motif and is included in the bHLH protein family (Fig. 1A). Since BU1 lacks the basic region needed for binding to DNA, it is classified into Group D of bHLH proteins, the non-DNA binding protein family (Li et al., 2006). In rice, there are three proteins which have high identity with BU1. However, Os02g0747900 may not be a functional protein, since Os02g0747900 lacks one helix motif of the HLH domain (Fig. 1A). BU1 and these proteins have a high similarity with PACLOBUTRAZOL RESISTANCE 1 (PRE1) of Arabidopsis, an HLH protein which also lacks the basic region. Since PRE1 functions in gibberellin (GA) signaling (Lee et al., 2006), we expect that BU1 may also play an important role in plant hormone signaling, especially BR. Therefore, we used phylogenetic analysis to determine the similarity of the HLH domain of BU1 to those of BEE1 and BIM1, which are bHLH proteins related to BR signaling in Arabidopsis (Fig. 1B). Since the NJ tree indicates that the similarity between BU1 and BEE1 or BIM1 is very low, BU1 and its homologs may be a novel protein family involved in BR signaling. In contrast to BU1, because the induction of the three homologs by exogenous BL was not detected in the WT shoot (data not shown), we preferentially analyzed BU1 in this paper.

**BR Action Phenocopies Rice Plants Overexpressing BU1**

To characterize BU1, we made transgenic rice plants overexpressing BU1 (BU1:OX) under the control of a maize Ubiquitin promoter. Many T0 plants showed enhanced bending of the lamina joint, but most of them were sterile. As a result, we obtained seeds from only two overexpressed lines, BU1:OX-3 and 4 (Fig. 2A). The expression level of BU1 is higher in OX-3 and much higher in OX-4 than in WT plants (Fig. 2B). BU1:OX showed increased bending of the lamina joint toward the abaxial
side depending on the expression levels of *BU1* (Fig. 2C) and had larger grains than WT (Fig. 2D and E). Since, it is known that bending of the lamina joint and grain size are regulated by BR (Yamamuro et al., 2000; Mori et al., 2002; Hong et al., 2003), these results suggest that *BU1* may be involved in BR signaling or biosynthesis. In addition, spikelet numbers were drastically reduced in OX-4, although they were similar in WT and OX-3 (data not shown). This observation may be the result of poor growth due to high transcript levels of *BU1* in OX-4.

*BU1*:OX also has some characteristic abnormalities in the pattern of internode elongation (Fig. 2F), progression of tillers in nodes of aerial part (Fig. 2G), and lignification and development of crown roots in these nodes (Supplemental Fig. S2). The first internode was severely shortened, but the 5th and 6th internodes were slightly elongated. Panicles did not emerge perfectly from the flag leaf sheath as a result of the shortened first internode in OX-4.

**BU1 Is Involved in BR Signaling, Not BR Biosynthesis**

In Arabidopsis, a BR signal component, BZR1, was isolated by genetic screens using elongation of hypocotyls with or without BRZ (Wang et al., 2002). Similar to hypocotyls in Arabidopsis, elongation of the mesocotyls and 2nd internodes in rice grown in complete darkness was severely dependent on BR (Yamamuro et al., 2000). To ascertain whether *BU1* functions in BR signaling or biosynthesis, we used the inhibition of elongation of the mesocotyl and 2nd internode in dark with or without BRZ, similar to the test performed in Arabidopsis hypocotyls. Mesocotyls and 2nd internodes in WT were elongated in half-strength MS medium without BRZ, but shortened in half-strength MS medium containing BRZ (Fig. 3A and B). On the other
hand, in BU1:OX-4, the length of the 2nd internodes was not affected by BRZ. Mesocotyls of OX-4 were greatly elongated compared to WT and the elongation was not much affected by BRZ. These results indicate that BU1:OX-4 is less sensitive to BRZ than wild-type.

Bending of the lamina joint is very sensitive to BR. BU1:OX shows enhanced bending of the lamina joint as described above. In contrast, the loss of function mutant of OsBRI1, d61, has erect leaves and it is less sensitive to BL. We generated d61 plants overexpressing BU1 and observed the resulting phenotypes, especially their lamina joints. BU1 overexpressor in the d61 background showed increased bending of the lamina joint (Fig. 3C) and a pattern of internode elongation similar to BU1:OX-4 (Fig. 3D), indicating that overexpression of BU1 complemented the BR-deficient phenotype of d61.

In addition, the WT and OX-4 did not differ markedly in their level of expression of BRD1, a gene that encodes a P450 that catalyzes the conversion of 6-deoxocastasterone (6-Deoxo CS) to castasterone (CS) (Supplemental Fig. S3A). We also measured endogenous BR contents in BU1:OX-4 and WT. There were no obvious differences between OX-4 and WT in the amounts of both CS (presumably the most active endogenous BR in rice) and 6-Deoxo CS, the main precursor of CS (Supplemental Fig. S3B). These results indicate that the biosynthesis of BRs is not activated in BU1:OX. Therefore, BU1 is a signaling component of BR in rice.

BU1 Is an Early Response Gene by BL through Dual Pathways, OsBRI1 and RGA1
To test the response to other plant hormones, we performed real-time PCR analysis using RNA samples of seedlings subjected to a variety of hormones and chemical treatments. Previous research showed that genes induced by auxin overlapped with those induced by BR, and both hormones synergistically modulate plant architecture (Yin et al., 2002; Nemhauser et al., 2004). However, expression of \textit{BUI} was not induced by IAA or by GA$_3$. On the other hand, ABA, a known antagonist to BR response, repressed expression of \textit{BUI} (Fig.4A). The mechanism whereby ABA represses the primary signaling outputs of BR in Arabidopsis (Zhang et al., 2009) may be conserved in rice as well.

\textit{BUI} was greatly upregulated by exogenous BL in WT plants. In addition, \textit{BUI}:OX showed many BR-related phenotypes. These results suggest that \textit{BUI} may act as an early response gene in BR signaling. We tested the response of \textit{BUI} expression to exogenous BL treatment with cycloheximide (CHX), an inhibitor of \textit{de novo} protein synthesis. A previous report in Arabidopsis indicated that induction of primary response genes by BR signaling, e.g., \textit{BEE1-3}, did not need \textit{de novo} protein synthesis (Friedrichsen et al., 2002). Induction of \textit{BUI} by exogenous BL was not affected by CHX (Fig. 4B). This result indicates that induction of \textit{BUI} does not need \textit{de novo} protein synthesis and \textit{BUI} is a primary response gene to BR signaling.

To determine whether the induction of \textit{BUI} by endogenous BRs is through OsBRI1, a transmembrane BR receptor, we compared the response of \textit{BUI} to exogenous BL between the \textit{d61-2} (\textit{Osbril}) mutant and its WT, T65 (Fig. 4C). In T65, induction of \textit{BUI} by exogenous BL was 8.7 times more than the control, and in the \textit{d61-2} mutant, 6.6 times greater than the control. The expression level of \textit{BUI} in \textit{d61-2} after BL treatment was about one-half compared to that in T65. These results indicate that \textit{BUI} is less
sensitive to exogenous BL in *d61-2* compared to T65 and it is induced through OsBRI1 by BRs. On the other hand, the sensitivity of *BU1* to exogenous BL still remained in *d61-2*. This result suggests that the induction of *BU1* by BRs may be also through another pathway distinct from the one associated with OsBRI1. Recent research showed that RGA1, encoding the rice heterotrimeric G protein alpha subunit, was also involved in BR signaling in rice (Wang et al., 2006; Oki et al., 2008). To determine whether the induction of *BU1* by BRs is not only through OsBRI1 but also through RGA1, we compared the response of *BU1* to exogenous BL between T65 and the *dl* mutant, a loss of function mutant of RGA1 in the T65 background (Fig. 4D). In the WT (T65), induction of *BU1* by BL was 5.9-fold to control but only 2.7-fold in the *dl* mutant. The expression level of *BU1* in *dl* after BL treatment was about one-third compared to that in T65. These results indicate that *BU1* is less sensitive to exogenous BL in *dl* and induced through both OsBRI1 and RGA1 pathway by BRs.

**Expression Analysis of *BU1***

We compared the expression level of *BU1* in various organs by real-time PCR analysis (Fig. 5A). The expression level of *BU1* is higher in the lamina joint before bending than the lamina joint after bending. Aside from the lamina joint, *BU1* is highly expressed in the leaf blade, leaf sheath and the panicle at heading stage. To analyze the expression pattern of *BU1* in detail, we inserted a 2 kb segment of the promoter region of *BU1* to drive the *GUS* gene, then transformed rice plants with this gene cassette. We performed the histochemical GUS assay using these transgenic plants (Fig. 5B). GUS staining was observed in various organs. We examined the lamina joint region of the second and 8th leaf (Fig. 5B (1 and 2)), leaf blade and sheath near the lamina joint in
9-day-old seedlings (Fig. 5B (3)). The veins in the leaf blade and sheath were well
stained (Fig. 5B (4 and 5)). Using a light microscope, we observed staining in phloem in
transverse sections of leaf blades (Fig. 5B (6)). In the reproductive stage, staining was
observed in the ovule and filaments before anthesis (Fig. 5B (7)). In particular, staining
was observed in the vascular bundles of the ovule, lemma and palea (Fig. 5B (8, 9, 10)).
In the seed, epithelial cells were stained in the embryo 3 days after imbibition (Fig. 5B
(11, 12)). The expression of \textit{BU1} in the lamina joint, verified by two independent
experiments (Fig. 5A and B (2)), strongly suggests that \textit{BU1} is involved in bending of
the lamina joint in WT plants. In addition, the results of GUS staining suggest that
expression of \textit{BU1} that was detected by real-time PCR in the leaf blade and panicle may
be localized in the vascular bundles (phloem) in these organs.

Expression of \textit{BU1} is higher in the embryo than in the endosperm; however, \textit{BU1}
expression is not influenced by imbibition (Fig. 5C). Although elongation of the 2nd
internode and mesocotyl is very sensitive to BR and these organs were elongated in
\textit{BU1}:OX compared to WT, in the presence of BRZ in dark (Fig. 3A and B), GUS
staining was not observed in 10-day-old seedlings grown in the dark (data not shown).
This result suggests that \textit{BU1} may not be involved in BR response in the absence of
light in WT plants.

To determine the intracellular localization of \textit{BU1} protein, we constructed a plasmid
DNA for fusion protein in which \textit{BU1} was fused to the C-terminus of an enhanced
green fluorescent protein (eGFP), that we subsequently introduced into rice coleoptiles
via particle gun bombardment. Dispersed fluorescent signal of eGFP-\textit{BU1} fusion
protein throughout the coleoptile cells was observed (Supplemental Fig. S4).
Transcription factors are basically localized in nucleus. Therefore, BR signal transduction by BU1 may be mediated by a different mechanism.

**Rice Plants Suppressing Both \textit{BU1} and Its Homologues Show Erect Leaves**

We generated transgenic plants suppressing \textit{BU1} using the 3’-UTR region (340bp) as a trigger region (\textit{BU1}:RNAi), and investigated the effect of silencing by morphological evaluation. The angle of the lamina joint and other morphological characters in rice suppressing \textit{BU1} did not markedly change as compared to WT plants (Fig. 6A to C). As described in Fig. 1A, there are three proteins which have a high similarity in amino acid sequence to BU1. We supposed that these three proteins may have functions that overlapped with those of BU1, therefore we generated transgenic plants suppressing both \textit{BU1} and these homologs using the whole ORF region (270bp) of \textit{BU1} as a trigger region (\textit{BU1F}:RNAi). \textit{BU1F}:RNAi plants showed erect leaves in contrast to \textit{BU1}:OX (Fig. 6D to F). In addition, we compared the sensitivity between \textit{BU1F}:RNAi and WT to BL by using the lamina joint bending assay. In \textit{BU1F}:RNAi, the angles of the lamina joint were narrower than those of the WT at all concentrations of BL (Fig. 6G), indicating that the sensitivity of \textit{BU1F}:RNAi to BL is lower than that of the WT. Moreover, the expression levels of \textit{BU1} homologs were reduced in the lamina joint of BRZ-treated WT seedlings as \textit{BU1} (Supplemental Fig. S5). These results indicate that \textit{BU1} controls bending of the lamina joint and the three homologs have redundant function with \textit{BU1} in the lamina joint. However, we did not observe changes of phenotype in the vascular bundles of \textit{BU1F}:RNAi (data not shown).
DISCUSSION

Although some BR signaling components have been isolated in Arabidopsis, little is known about BR signaling components in rice, other than OsBZR1 and DLT functioning in the OsBRI1 pathway and RGA1. In this study, we demonstrate that BU1, a small helix-loop-helix protein, is a novel BR-signaling component. BU1:OX shows many characteristic phenotypes associated with BR effects, e.g. enhanced bending of the lamina joint, large grain and elongation in dark-grown seedlings. In addition, BU1:OX is resistant to BRZ and overexpression of BU1 in d61, a BR receptor mutant, complements the BR-insensitive erect leaf phenotype of d61. These results indicate that BU1 is involved in BR signaling, but not biosynthesis. Real-time PCR analysis showed that BU1 is a primary response gene of BR signaling and upregulated through both OsBRI1 and RGA1 pathways by BRs. The expression pattern of BU1 also provides information about the native function of BU1. From analysis of phenotypes of BU1:RNAi plants and the lamina joint bending assay, it is clear that BU1 controls the bending of the lamina joint.

BU1:OX shows abnormal phenotypes that includes previously known and also possibly new BR effects. The length of the first internode of culm is shortened but that of the 5th internode is elongated, and the 6th internode protrudes in BU1:OX (Fig. 2F and G). d61 has a relatively longer first internode and shorter lower internodes (Fig. 3D) in comparison to BU1:OX. This result suggests that alternation of length of internodes in BU1:OX may be a characteristic BR effect on growth and development of culm. Progression of tillers in lower aerial nodes (Fig. 2F) may also be a possible BR effect, since rice plants overexpressing DWARF4, a BR biosynthetic gene, have more tillers whereas dlt mutants have less tillers (Wu et al., 2008, Tong et al., 2009). Severe
lignification and abnormal development of crown roots in nodes was observed in 
*BU1:OX*. It is known that epidermal cell death in nodes correlates with root emergence 
in rice and it is mediated by ethylene (Steffens and Sauter, 2009). Since ethylene 
biosynthesis is activated by BR (Arteca et al., 1983; Yi et al., 1999), this observation 
may be a BR effect mediated via ethylene biosynthesis. Moreover, most of *BU1:OX* 
plants displayed severe sterility. Plants suppressing *OsSPY*, a negative regulator of GA 
signaling and BR biosynthesis, rarely developed fertile flowers (Shimada et al., 2006). 
Overexpression of genes related to plant hormones often causes severe sterility.

The expression pattern of *BU1* revealed by the histochemical GUS assay suggests 
that *BU1* may be involved not only in bending of the lamina joint but also in vascular 
differentiation in the leaf blade, leaf sheath and floral organs. The characterization of 
the loss of function mutant of BRI1 and its homologs, BRL1 and BRL3 in Arabidopsis 
and OsBRI1 in rice showed that BR plays an important role in vascular differentiation, 
enhances the differentiation of the xylem and inhibits the differentiation of the phloem 
(Caño-Delgado et al., 2004; Nakamura et al., 2006). However, we could not detect 
morphological changes in vascular bundles of *BU1:OX* and *BU1:F:RNAi*. According to 
the results of the GUS assay, *BU1* is mainly expressed in the phloem, a vital organ for 
long-distance transport of photosynthetic products, nutrients, ions, proteins and 
hormones. It is possible that BU1 may regulate gene expression related to transport or 
unknown function in the phloem. The epithelium is also an important organ for the 
transport of nutrients, ions and sugars from the endosperm, and gibberellins from the 
embryo. However, the expression level of *BU1* was scarcely affected by imbibition. 
This result suggests that BU1 in the epithelium may not be involved in expression of
growth-related genes but homeostatic-related genes. Therefore, genes regulated by \textit{BU1} in the epithelium may be different from those in the phloem.

In this study, we showed that \textit{BU1} is a novel primary response gene to BR signaling through both OsBRI1 and RGA1. Previous reports describe that OsBZR1 functions as a transcriptional repressor for downregulating \textit{DLT} and BR biosynthesis genes such as \textit{AtBZR1} (He et al., 2005; Bai et al., 2008; Tong et al., 2009). On the other hand, real-time PCR clearly showed that \textit{BU1} is a primary response gene upregulated by exogenous BL, suggesting that BU1 may be upregulated by a novel transcription factor (maybe an activator) distinct from OsBZR1.

In addition, we compared the expression of known BR-marker genes between WT and two independent \textit{BU1}:OX lines. However, BR-marker genes, \textit{OsXTR1}, \textit{OsXTR3}, \textit{OsBLE2} and \textit{OsBLE3} (Uozu et al., 2000; Yang et al., 2003, 2006), were not upregulated in \textit{BU1}:OX (data not shown). Therefore, downstream genes of BU1 may be different from known BR-related genes regulated by the OsBZR1 pathway in rice.

\textit{BU1} protein is categorized as a putative non-DNA binding bHLH protein, since it lacks a basic region (Fig. 1A). Therefore, downstream genes of BU1 may be regulated by more complex mechanisms, different from the general mechanism of transcriptional regulation of target genes by a transcription factor. Molecular mechanism of non-DNA binding HLH proteins is understood well in human Inhibitor of DNA binding (Id) proteins. Id proteins heterodimerize with other DNA binding bHLH protein partners via HLH motif and abolish their functions as transcription factors, binding to the cis-element of their target genes (Benezra et al., 1990; Sun et al., 1991). Consequently, expression of their target genes by partners of Id proteins is abolished by Id proteins. Likewise, BU1 may also interact or form a complex with putative BR-negative
regulators (maybe bHLH proteins), and inhibit their functions as transcription factors. Taking into account these studies and perspectives, we propose a model of BR signaling in rice (Fig. 7).

BU1 has high similarity with PRE1, encoding a HLH protein involved in GA signaling in Arabidopsis (Lee et al., 2006). In addition, elongation of internodes in darkness is affected not only by BRs but also by GAs, suggesting that BU1 may also be involved in GA signaling. To test whether BU1 is also a GA signal component, we performed two kinds of experiments, the 2nd leaf sheath elongation assay and the α-amylase induction assay. First, we compared the effect of paclobutrazol, an inhibitor of GA biosynthesis, against 2nd leaf sheath elongation in BU1:OX and its WT. BU1:OX showed shortened leaf sheaths and displayed sensitivity to paclobutrazol similar to the WT (Supplemental Fig. S6A and B). Second, we examined α-amylase induction by GA in BU1:OX-4 and BU1F:RNAi-6 seeds by using the starch plates. For example, slender rice, a constitutive GA response mutant, produces and secretes amylase from embryoless half seeds without GA addition and a plaque zone around half seeds is observe on agar plates after staining by iodine (Ikeda et al., 2001). Production of amylase from embryoless half seeds of OX-4 and RNAi was observed only on GA applied agar plates (clear zones), but not on control plates. There was no significant difference in sensitivity to GA relative to the WT (Supplemental Fig. S6C). These results indicate that BU1 is not directly involved in GA signaling. Our study suggests that many non-DNA binding bHLH proteins may play an important role not only in GA signaling but also in some plant hormone signaling similar to the function of human Id proteins, although little is known about functions of HLH proteins in plants.
BRs play an important role in plant morphogenesis. In this study, we show that *BU1* controls bending of the lamina joint via BR signaling. BR-defective and insensitive mutants, which have erect leaves, have higher biomass and grain yield than WT plants at high planting density, since they may increase light capture for photosynthesis by their erect leaves (Morinaka et al., 2006; Sakamoto et al., 2006). Therefore, control of bending of the lamina joint via regulating expression of *BU1* may lead to increased biomass and grain yield. In addition, *BU1*:OX displays a valuable phenotype, large grains. Further studies of downstream effects of BU1 may provide important insights for engineering the plant’s architecture and increasing grain yield.
MATERIALS AND METHODS

Plant Material and Growth Condition

All transgenic and WT plants were generated from *O. sativa* japonica cultivar Nipponbare, except for the *d61-2* mutant, *d1* mutant and their WT, which were obtained from japonica cultivar Taichung 65 (T65). All transgenic plants were grown in isolated green houses at 28°C.

For mRNA analysis of plant hormones including brassinolide or cycloheximide treatment, dehusked seeds of rice plants were surface-sterilized and sown on one-half-strength MS medium containing 3% (w/v) sucrose and 0.4% (w/v) Gelrite (Wako Pure Chemicals, Osaka, Japan). Seedlings were transplanted into Kimura’s B solution medium about 10 days after sowing (Sato et al., 1996). After 4 days, seedlings were incubated with 1 μM BL (Daiichi Fine Chemical, Toyama, Japan), 10 μM IAA (Wako Pure Chemicals), 100 μM ABA (Wako Pure Chemicals), 10 μM GA₃ (Sigma Aldrich, Saint Louis, MO) or an equal volume of the pertinent solvent (DMSO, NaOH, EtOH) for 24hr. For cycloheximide treatment, plants were pretreated with 100 μM cycloheximide (Nacalai tesque, Kyoto, Japan) for 3 hr before BL treatment. Plants were grown in a growth chamber at 25°C under long-day conditions (14hrs light [60-70 μmol m⁻² s⁻¹] / 10 hrs dark).

For etiolation analysis, transgenic plants and WT were germinated and grown on half-strength MS medium supplemented with 10 μM BRZ2001 (Sekimata et al., 2001) or its equal volume of solvent (DMSO) in a dark chamber at 25°C. The length of the mesocotyl and 2nd internode were measured after 2 weeks.

For the 2nd leaf sheath elongation assay, seeds of transgenic plants and WT were sterilized, sown on distilled water for 2 days at 28°C and germinated. Germinated seeds
were sown on half-strength MS medium supplemented with 2 μM paclobutrazol (Wako pure chemicals) or its equal volume of solvent (DMSO), and grown at 25°C under long-day conditions. After a week, the lengths of the 2nd leaf sheath were measured. The method described by Sekimata et al. (2001) was used as reference in this experiment.

**Oligo DNA Microarray Analysis**

We used a rice 22K oligo DNA microarray kit (G2554A; Agilent Technology, Santa Clara, CA) containing 21,938 oligonucleotides based on the sequence data of the rice full-length cDNA project (Kikuchi et al. 2003; Yazaki et al., 2004). Total RNA was extracted using Isogen (Wako Pure Chemicals) and further purified with an RNeasy Plant Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA amplification, labeling, hybridization, scanning and image analysis were performed according to the manufacturer’s instructions (Agilent Technology). The microarray results were filtered to select candidate clones with P-value log ratios of less than 0.01. A 2.0-fold expression cutoff was applied, and the cases in which dye swapped replications passed this cutoff were scored as differential expression.

**Vector Construction and Rice Transformation**

To generate overexpression lines, the full-length cDNA of *BU1* (AK071601), provided by the Rice Genome Resource Center in NIAS, was cloned into the *SfiI* site of the pBIGS RiceFox vector (Nakamura et al., 2007) between the maize *Ubiquitin* promoter and the nopaline synthase terminator. To generate RNAi lines, the PCR product that was designed to trigger RNAi was first subcloned into the pENTR entry
vector (Invitrogen, Carlsbad, CA). PCR products were amplified using the following primer pairs: \textit{BU1:RNAi} 5’-CACCGCTCGTGCCTGTAGCTA-3’ and 5’-GGACGACTCTACTGCATCAAGGA-3’; \textit{BU1F:RNAi} 5’-CACCGACGATGTGAGCCGGAG-3’ and 5’-GCGGAGGCTGCGGATGATCTC-3’. Using the Gateway system, the fragment was cloned into the pPANDA destination vector through a LR clonase reaction (Miki and Shimamoto, 2004; Miki et al., 2005). The rice plants were transformed according to the \textit{Agrobacterium}-mediated method of Toki et al., (2006).

**Quantitative Real-Time RT-PCR Analysis**

After RNA extraction, first-strand cDNAs were synthesized from equal amounts of total RNA (1 μg / reaction) with a PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Tokyo, Japan) in a total volume of 20 μl, as described by the manufacturer. Synthesized cDNAs were used for real-time PCR. Real-time PCR was performed with Thermal Cycler Dice (TaKaRa Bio). The following primer pairs were used for real-time PCR: \textit{BU1} 5’-GTAGCCAGCTTGATCTCATCTC-3’ and 5’-GGGACGACTCTACTGCATCA-3’. RUBQ2 5’-GGTCATCCCGAGCCTCTGTT-3’ and 5’-GCAAATGAGCAAATTGAGCA. 18S rRNA 5’- GGAAGGAGAAGTCGTAACAAGG-3’ and 5’-CAGGGTCACGACAATGATCC-3’. Os02g0747900 5’-TAGCACTAGGGGCCTAAGCA-3’ and 5’-AAGGGAGAGGGAAAAACCAA-3’. Os03g0171700 5’-GGTATGTACTGTACCAGGGCTAA-3’ and 5’-ATCTCTGAGCTTATCAAAGCAGCT-3’. Os10g0403800 5’-GGTCAAAACTCGAAGCCCTTT-3’ and 5’-TCCATTCTGATTGTCCGTAGA-3’.
**Histochemical GUS Activity Assay**

For analysis of *BUL* expression, a 2.0 kb promoter region was amplified by PCR with primer pairs 5’-GGGGTACCCTCGACAATTTGACATGCCGTGCAT-3’ and 5’-GGTCTAGAGAAGCAGAAAGGGAGAGGGGGCA-3’, and cloned into the pSMAHdN632L-M2GUS vector between the *Kpn*I and *Xba*I sites in front of the beta-glucuronidase (GUS) gene. T1 plants were selected with 50 μg ml⁻¹ hygromycin and used for GUS assay. GUS staining was performed according to the method of Kosugi et al. (1991). Transverse section and organs were observed under a LEITZ DMR light microscope (Leica, Wetzler, Germany) and a VHX-500 digital microscope (Keyence, Osaka, Japan).

**Lamina Joint Bending Assay**

Detailed method is described in Fujioka et al. (1998). Seeds of transgenic plants and WT were dehusked, sterilized and grown on half-strength MS medium at 25°C under long-day conditions. After a week (when 3rd leaf began to emerge), 1 μl of ethanol:DMSO (9:1, v/v) solution containing 0, 10, 100, 1000 ng BL was spotted on the lamina joint of the 2nd leaf of seedlings. After 3 days, the angles of the lamina joint were measured.

**Quantification of Endogenous BRs**

Mature shoots (*BUL*:OX, 50g fresh weight; Vector control, 50g fresh weight) grown in soil in an isolated green house for two months were used for the experiment. The detailed method is described in Mori et al. (2002).
Subcellular Localization of BU1 Protein

The *BU1* cDNA fragment was cloned into a multi-cloning site in frame at the C-terminus of EGFP in the pSAT6-EGFP-C1 vector (Tzifara et al., 2005). This construct was introduced into coleoptiles of 5-day old rice seedlings by particle bombardment method (Bio-Rad Biolistic PDS-1000/He Particle Delivery System, Bio-Rad, Hercules, CA; Heiser, 1992; Sanford et al., 1993). After incubation at 28°C for 24h, GFP fluorescence was observed using a fluorescent microscope.

α-amylase Induction Assay

Five embryoless half seeds per plate were dehusked and sterilized. These half seeds were placed on starch plates (0.2% (w/v) starch and 2% (w/v) agar) with or without 2 μM of GA₃. These plates were incubated for 5 days at 28°C in darkness. For detection of secreted amylase, iodine vapor was applied to plates (Ueguchi-Tanaka et al., 2000). Purple zones indicated unhydrolyzed starch, while clear zones (plaques, not turned to purple) indicated starch hydrolyzed by the amylase present in the half seeds.

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Result of real-time PCR analyses of *BU1*.

**Supplemental Figure S2.** Close-up view of third node of culm in wild-type, *BU1:OX*-3,4.
Supplemental Figure S3. Role of BU1 in BR biosynthesis.

Supplemental Figure S4. Subcellular localization of eGFP-BU1 fusion protein.

Supplemental Figure S5. Expression level of BU1 and its homologs analyzed by real-time PCR.

Supplemental Figure S6. BU1 is not involved in GA signaling.

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**Figure Legends**

**Figure 1.** Protein sequence analysis of BU1. A, Alignment (A) and phylogenic tree (B) of Helix-Loop-Helix (HLH) domains for BU1 (Os06g0226500), its three homologous proteins, PRE1, and two bHLH proteins associated with BR signaling, BEE1 and BIM1. Upper five protein sequences are full-length. HLH domains are indicated below the sequences. Black and gray backgrounds indicate identical and similar amino acids, respectively. The bar corresponds to 0.1 amino acid substitutions per site.

**Figure 2.** Phenotypes of rice plants overexpressing *BU1* (*BU1*:OX). A, Gross morphology of wild-type (WT, left), *BU1*:OX-3 (center), -4 (right) at the heading stage. Bar = 50 cm. B, Relative expression level of *BU1* in *BU1*:OX-3,4 to WT, analyzed by real-time PCR and normalized by *RUBQ2*. The error bars indicate SD. C, Lamina joint of second leaf from flag leaf in WT and *BU1*:OX-3,4. *BU1*:OX showed increased bending of the lamina joint. D and E, Grain morphology and size of WT and *BU1*:OX-3,4. *BU1*:OX-3,4 (center, right) have larger grains than that of WT (left). Bar = 2 mm. The error bars indicate SD (n=10). Photograph (F) and length as a percentage (G) of internodes in WT and *BU1*:OX-3,4 (n=8). Uppermost internode of *BU1*:OX-3,4 was shorter, but its two lower internodes were longer than WT. Bar = 50 cm.

**Figure 3.** Involvement of *BU1* in BR signaling. A and B, Effect of brassinazole (BRZ). Etiolated seedlings of WT and *BU1*:OX-4 were grown in complete darkness with or without 10 μM of BRZ2001 for 2 weeks prior to measurement of the length of the 2nd lower internodes and mesocotyls. Arrows indicate nodes. Organs between arrowheads
and seeds indicate mesocotyls. Bar = 5 cm. The error bars indicate SD (n = >9). C, Gross morphology of d61, a BR insensitive mutant which has an inactive BR receptor OsBRI1 (left), and d61 overexpressing BUI (right). The erect leaf phenotype of d61 was complemented by overexpression of BUI. Bar = 50 cm. D, Culm of d61 (left) and d61 overexpressing BUI (right). Arrows indicate nodes. Bar = 10 cm.

Figure 4. Expression pattern of BUI analyzed by real-time PCR. Total RNA used in these experiments were isolated from shoots of 2-week-old seedlings after a variety of chemical treatments for 24 h. Data is the average of three or four independent experiments and normalized by 18S rRNA. The error bars indicate SD. A, Response of BUI to various plant hormones. IAA 10 μM, ABA 100 μM, GA 10 μM, BL 1 μM. B, Effect of cycloheximide (CHX) on BUI induction. Wild-type (Nipponbare) plants were treated with mock, CHX, BL, and both CHX and BL. 2 week-old seedlings were pretreated with or without 100 μM CHX treatment for 3 h, before 1 μM BL or mock treatment for 24 h. C and D, Expression of BUI in response to BL treatment in either d61-2 and d1 or WT (T65). Plants were treated with 1 μM BL for 24 h.

Figure 5. Expression pattern of BUI. A and C, Expression analysis of BUI in various organs and seeds by real-time PCR. Data is the average of three or four independent experiments and normalized by RUBQ2. Total RNA was isolated from various organs (A) and embryo and endosperm of 10 dehusked seeds (C). LJ , lamina joint. YP, young panicle. DAH, day after heading. The error bars indicate SD. B, Histochemical GUS staining of rice plants harboring BUI::GUS. (1,2) Close-up view of second lamina joint region of 9-day-old seedling and 8th lamina joint region. (3) 9-day-old seedling. Leaf
blade was stained. Leaf blade (4) and sheath (5) of 9-day-old seedling. (6) Transverse section of leaf blade. (7) Flower before anthesis. (8) Close-up view of stigma and ovule. (9) Transverse section of ovule. (10) Palea and lemma of young panicle. Stained blue line is a vascular bundle. (11) Embryo and (12) cross section of embryo 3 days after imbibition. Epithelial cells were stained.

**Figure 6.** Phenotype of rice plants suppressing *BU1*. A, Gross morphology of WT and a plant suppressing *BU1* (*BU1*:RNAi-2, 7) at vegetative phase. Bar = 50 cm. B, Close-up view of the lamina joint region of WT and *BU1*:RNAi-2, 7. C, Relative expression level of *BU1* and its homologs (Os02g0747900, Os03g0171700, Os10g0403800) in *BU1*:RNAi-2, 7 to WT, analyzed by real-time PCR and normalized by *RUBQ2*. The error bars indicate SD. D, Gross morphology of WT and a plant suppressing *BU1* and its three homologues (*BU1F*:RNAi-3, 6) at vegetative phase. Bar = 50 cm. E, Close-up view of the lamina joint region of WT and *BU1F*:RNAi-3, 6. F, Relative expression level of *BU1* and its homologs in *BU1F*:RNAi-3, 6 to WT, analyzed by real-time PCR and normalized by *RUBQ2*. The error bars indicate SD. G, BL response at the lamina joint of WT, *BU1F*:RNAi-3, and -6. The error bars indicate SD (n>3).

**Figure 7.** BR signaling model in rice. Induction of *BU1* by BL is partially inhibited in *OsBRII1* mutant (*d61-2*) and *RGA1* mutant (*d1*). In addition, *BU1* is the primary response (upregulated) gene to BR signaling. Therefore, BU1 may be a novel mediator of a distinct pathway from OsBZR1, because OsBZR1 functions as the transcription repressor. Moreover, regulation of downstream genes by BU1 may be through a complex mechanism like the regulation of human Id protein.
Figure 1. Protein sequence analysis of BU1. A, Alignment (A) and phylogenetic tree (B) of Helix-Loop-Helix (HLH) domains for BU1 (Os06g0226500), its three homologous proteins, PRE1, and two bHLH proteins associated with BR signaling, BEE1 and BIM1. Upper five protein sequences are full-length. HLH domains are indicated below the sequences. Black and gray backgrounds indicate identical and similar amino acids, respectively. The bar corresponds to 0.1 amino acid substitutions per site.
Figure 2. Phenotypes of rice plants overexpressing BU1 (BU1:OX). A, Gross morphology of Wild-type (WT, left), BU1:OX-3 (center), -4 (right) at the heading stage. Bar = 50 cm. B, Relative expression level of BU1 in BU1:OX-3,4 to WT, analyzed by real-time PCR and normalized by RUBQ2. The error bars indicate SD. C, Lamina joint of second leaf from flag leaf in WT and BU1:OX-3,4. BU1:OX showed enhanced bending of the lamina joint. D and E, Grain morphology at the heading stage. WT and BU1:OX-3,4 (center, right) have larger grains than that of WT (left). The error bars indicate SD (n=10). Photograph (F) and length as a percentage (G) of internodes in WT and BU1:OX-3,4 (n=8). Uppermost internode of BU1:OX-3,4 was shorter, but two lower internodes was longer than WT. Bar = 50 cm.
**Figure 3.** Involvement of BU1 in BR signaling. A and B, Effect of brassinazole (BRZ). Etiolated seedlings of WT and BU1:OX-4 were grown in complete darkness with or without 10 μM of BRZ 2001 for 2 weeks, prior to measurement of the length of the 2nd lower internodes and mesocotyls. Arrows indicate nodes. Organs between arrowheads and seeds indicate mesocotyls. Bar = 5 cm. The error bars indicate SD (n = >9). C, Gross morphology of d61 (Osbr1) which has an active BR receptor OsBR11 (left), and d61 overexpressing BU1 (right). The erect leaf phenotype of d61 was suppressed by overexpression of BU1. Bar = 50 cm. D, Culm of d61 (left) and d61 overexpressing BU1 (right). Arrows indicate nodes. Bar = 10 cm.
Figure 4. Expression pattern of BU1 analyzed by real-time PCR. Total RNA used in these experiments were isolated from shoots of 2-week-old seedlings after a variety of chemical treatments for 24 h. Data is the average of three or four independent experiments and normalized by 18S rRNA. The error bars indicate SD. A, Response of BU1 to various plant hormones. IAA 10 μM, ABA 100 μM, GA 10 μM, BL 1 μM. B, Down-regulation of BU1 by CHX. WT (Nipponbare) plants were treated with mock, CHX, BL, and both with or without 100 μM CHX treatment for 3 h, before 1 μM BL or mock treatment for 24 h. C and D, Expression of BU1 in response to BL treatment in either d61-2 and d1 or WT (T65). Plants were treated with 1 μM BL for 24 h.
Figure 5. Expression pattern of BU1. A and C, Expression analysis of BU1 in various organs and seeds by real-time PCR. Data is the average of three or four independent experiments and normalized by RUBQ2. Total RNA was isolated from various organs (A) and embryo and endosperm of 10 dehusked seeds (C). LJ, lamina joint. YP, young panicle. DAH, day after heading. N.D., not detected. The error bars indicate SD.

B, Histochemical GUS staining of rice plants harboring BU1::GUS. (1, 2) Close-up view of second lamina joint region of 9-day-old seedling and 8th lamina joint region. (3) 9-day-old seedling. Leaf blade was stained. Leaf blade (4) and sheath (5) of 9-day-old seedling. (6) Transverse section of leaf blade. (7) Flower before anthesis. (8) Close-up view of stigma and ovule. (9) Transverse section of ovule. (10) Palea and lemma of young panicle. Stained blue line is a vascular bundle. (11) Embryo and (12) cross section of embryo 3 days after imbibition. Epithelial cells were stained.
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