Plant Physiology®

Research Article | Articles

Rice Leaf Angle and Grain Size Are Affected by the OsBUL1 Transcriptional Activator Complex

Seonghoe Jang, Gynheung An, Hsing-Yi Li

Published January 2017. DOI: https://doi.org/10.1104/pp.16.01653

Abstract

Rice atypical HLH protein *Oryza sativa BRASSINOSTEROID UPREGULATED 1-LIKE1* (*OsBUL1*) is preferentially expressed in the lamina joint where it controls cell elongation and positively affects leaf angles. *OsBUL1* knockout mutant (*osbul1*) and transgenic rice for double-stranded RNA interference (**dsRNAi**) of *OsBUL1* produced erect leaves with smaller grains, whereas *OsBUL1* overexpressors and an activation tagging line of *OsBUL1* exhibited increased lamina inclination and grain size. Moreover, *OsBUL1* expression was induced by brassinolide (**B** *osbul1* did not respond to **BL** treatment. To understand the molecular network of OsBUL1 function in rice, v isolated a novel OsBUL1-interacting protein, LO9-177, an uncharacterized protein containing a KxDL motif, and functionally studied it with respect to the lamina inclination and grain size of rice. OsBUL1 COMPLEX1 (OsBC1) is basic helix-loop-helix (**bHLH**) transcriptional activator that interacts with OsBUL1 only in the presence of LO9-177 forming a possible trimeric complex for cell elongation in the lamina joint of rice. Expression of *OsBC1* is also upregulated by **BL** and has a similar pattern to that of *OsBUL1*. Transgenic rice plants expressing *OsBC1* under th control of *OsBUL1* promoter showed increased grain size as well as leaf bending, while transgenic lines for **dsRNA** and/or expressing a dominant repressor form of *OsBC1* displayed reduced plant height and grain size. Together, these results demonstrated that a novel protein complex consisting of OsBUL1, LO9-177, and OsBC1 is associate with the **HLH-bHLH** system, providing new insight into the molecular functional network based on **HLH-bHLH** proteins for cell elongation.

Plant architecture is an important factor for efficient photosynthesis and high yield (Sakamoto et al., 2006). In ric (*Oryza sativa*), the degree of lamina inclination is an important trait that determines architecture. The degree of lamina angles depends on cell division, expansion, and cell wall composition in the lamina joint (Nakamura et al. 2009; Zhang et al., 2009; Zhao et al., 2010; Ning et al., 2011). Erect leaf phenotypes are desired to avoid shade when plants are grown at high planting density (Van Camp, 2005).

Mutations in brassinosteroid (BR) biosynthesis genes (Hong et al., 2002, 2003; Sakamoto et al., 2006; Wu et al., 2008) and BR signaling genes (Bai et al., 2007) change lamina angle. Other phytohormones are also involved in controlling the lamina joint inclination. Ethylene participates in BR-induced lamina inclination. Indole-3-acetic acid influences lamina joint inclination at high concentrations and has a synergistic interaction with BR (Wada et al., 1981; Cao and Chen, 1995). A gain-of-function mutant for *OsGH3-1* encoding an indole-3-acetic acid amido synthetase showed increased leaf angles due to stimulated cell elongation at the lamina joint (Zhao et al., 2013). Reduced expression of *SPINDLY*, a negative regulator of gibberellin signaling, also causes increased lamina inclination (Shimada et al., 2006).

Transcription factors that determine the angle have been identified. Mutations in *OsLIGULELESS1*, encoding a *SQUAMOSA* promoter binding domain protein, exhibit an erect leaf phenotype (Lee et al., 2007). Transgenic plant overexpressing rice *ILI1-BINDING HLH1* (*OsIBH1*) also show erect leaves (Zhang et al., 2009). Inducing expression of genes encoding atypical helix-loop-helix (HLH) proteins such as *BRASSINISTEROID UPREGULATED1* (*BU1*), *INCREASED LAMININAR INCLINATION1* (*ILI1*), and *POSITIVE REGULATOR OF GRAI LENGTH1* (*PGL1*) conferred a higher lamina angle degree (Tanaka et al., 2009; Zhang et al., 2009; Heang and Sassa, 2012a).

Increased lamina angles are observed in transgenic rice plants overexpressing *LAX PANICLE* (Komatsu et al., 2003), a T-DNA insertion mutant of *OsWRKY11* (Wang et al., 2005), and RNA interference transgenic lines for *SHORT VEGETATIVE PHASE* group MADS-box genes such as *OsMADS22*, *OsMADS55*, and *OsMADS47* (Lee e al., 2008). Decreased expression of rice *LEAF AND TILLER ANGLE INCREASED*, encoding a CCCH-type zinc-finger protein, also results in increased lamina inclination through regulating BR signaling (Wang et al., 20 *INCLINATION2*, encoding a VERNALIZATION INSENSITIVE3-like protein, acts as a repressor of cell divisi regulation of collar development (Zhao et al., 2010). Enhancing expression of a gene encoding BAHD acyltransferase-like protein produces slender grains with enlarged leaf angles (Feng et al., 2016). Additionally, a gain-of-function epiallele of rice *RELATED TO ABSCISIC ACID INSENSITIVE3/VIVIPAROUS1* (*VP1*) *6*, encoding a B3 DNA-binding domain-containing protein, caused larger lamina inclination but smaller grain size by modulating BR homeostasis (Zhang et al., 2015).

Basic helix-loop-helix (bHLH) proteins are a group of important transcription factors that play diverse roles in both animals and plants. They occupy key positions in phytochrome signal transduction cascades, contributing to stomata differentiation, cell fate determination, and BR-response gene expression (Bernhardt et al., 2005; Duek and Fankhauser, 2005; Serna, 2007; Bhattacharya and Baker, 2011). In particular, several bHLH proteins

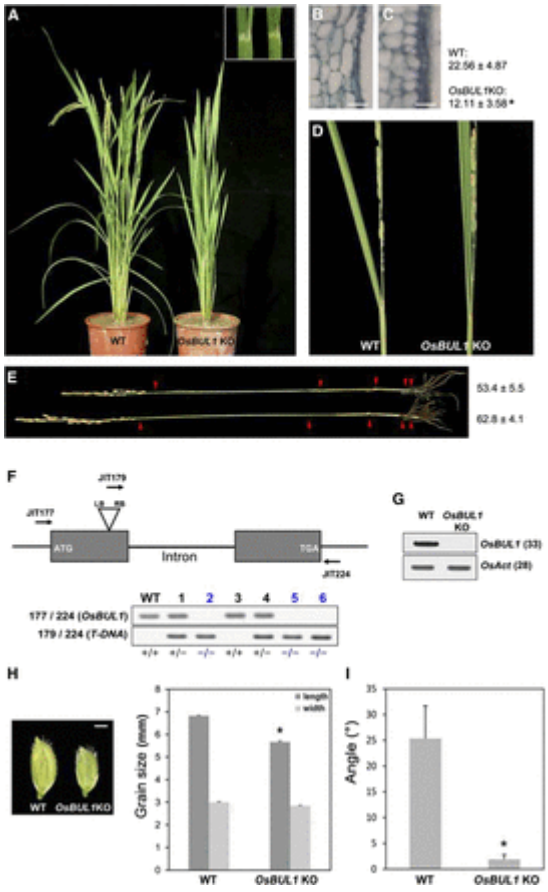
including BRI1 ENHANCED EXPRESSION1 (BEE1) to BEE3 (**Friedrichsen et al., 2002**) and BES1-INTERACTI MYC-LIKE1 (BIM1) to BIM3 (**Yin et al., 2005**) are implicated in BR signaling in Arabidopsis (*Arabidopsis thaliana*). Recently, a group of typical **bHLH** proteins, ACTIVATORS OF CELL ELONGATIONs (ACEs), were also reported act as positive regulators (**Ikeda et al., 2012**), whereas another **bHLH** protein, ANTAGONIST OF PGL1 (APG), w found to function as a negative regulator of cell elongation (**Heang and Sassa, 2012a**, **2012b**, **2012c**). Interestingly some atypical **HLH** proteins, such as PACLOBUTRAZOL RESISTANCEs (PREs), BU1, ILI1, PGL1, and PGL2 (**Hyun and Lee, 2006**; **Lee et al., 2006**; **Tanaka et al., 2009**; **Wang et al., 2009**; **Zhang et al., 2009**; **Heang and Sassa, 2012a**, **2012b**), also contribute to cell elongation, while others, including ATBS1-INTERACTING FACTORs (AIFs), IBH1, and OsIBH1, suppress cell elongation (**Wang et al., 2009**; **Zhang et al., 2009**; **Ikeda et al., 2013**). Recently, a triantagonistic **bHLH** system was reported to explain the relationship between **bHLH** and atypical **HLH** proteins in controlling cell elongation in Arabidopsis (**Ikeda et al., 2012**), although AIFs were missing in the model.

In this work, we functionally characterized *OsBUL1* that encodes an atypical **HLH** protein belonging to the PRE group using gain-of-function and loss-of-function approaches in rice. To better understand the network of OsBUL1 function and to identify components of the network, we searched for OsBUL1 interacting proteins, and LO9-177, a KxDL motif-containing protein, was isolated. *LO9-177* also showed cell elongation activity under the control of *OsBUL1* promoter, and the protein was found to be a molecular mediator between OsBUL1 and OsBC1, a typical **bHLH** transcription factor that promotes increased lamina angles. Our findings reveal one more layer in the cell elongation machinery based on the atypical **HLH**-**bHLH** network by providing a novel complex positively acting on lamina inclination and/or grain size through cell elongation of rice.

RESULTS

OsBUL1 Knockout Rice Produces Erect Leaves with Small Grains

We identified an erect leaf mutant from a T-DNA insertion mutant population in rice (**Fig. 1, A–E**; **Jeon et al., 2000**; **Jeong et al., 2002**, **2006**). Genotyping revealed that T-DNA was inserted in the first exon of Os02g51320 (The gene encodes an atypical **HLH** protein and is highly homologous to BU1 (**Tanaka et al., 2009**). We na gene *O. sativa BU1-like 1* (*OsBUL1*). Intact *OsBUL1* mRNA was not detected in the mutant line, demonstrating tha *osbul1* is a null allele (**Fig. 1G**). The mutant exhibited reduced lamina angles (**Fig. 1, A and D**). Average length of cells in the lamina joint was reduced by 46.3% compared to wild-type control (**Fig. 1, B and C**). Plant height was also reduced due to reduced internode length (**Fig. 1E**). The mutant showed small spikelets and consequently produced small grains (**Fig. 1H**). Leaf angle was also reduced in the mutant (**Fig. 1I**). Transgenic rice plants generated by double-stranded RNA interference (**dsRNAi**) using *OsBUL1* coding sequence showed similar phenotypes (Supplemental Fig. S1, A–C), indicating that the mutant phenotypes were due to mutations in *OsBUL1* In addition to the *OsBUL1* transcript, transcript levels of *OsBUL1* homologous genes such as *OsBU*, *OsBUL2*, *OsBUL3*, and *OsILI1* were also less abundant in the **dsRNAi** plants, indicating a multigene knockdown effect (Supplemental Fig. S1, D and E).



Download figure

Open in new tab

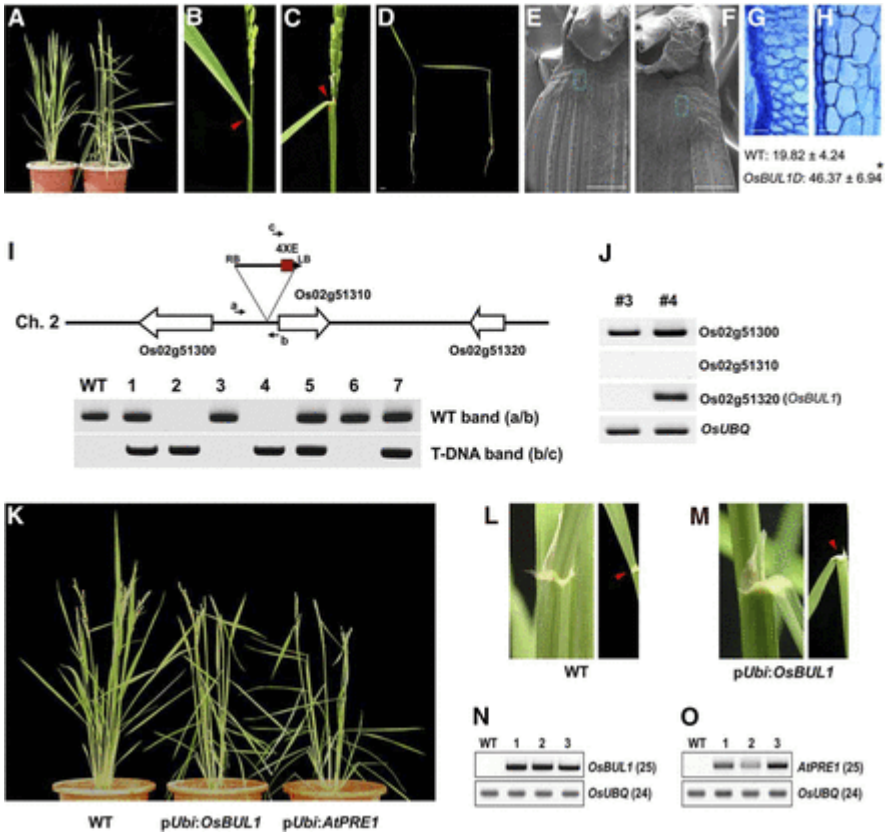
Download powerpoint

Figure 1.

OsBUL1 null mutants show erect leaves. A and D, *OsBUL1* knockout mutant plants exhibit erect leaves. The lamina joint a second leaf from the wild type (left) and *OsBUL1*KO is shown in the box (A). B and C, Comparison of cells in the lamina joi by longitudinal sections in wild-type (B) and *OsBUL1*KO (C) plants. Length of cells in the lamina joint of wild-type control ar *OsBUL1*KO plants is presented. Values are given as means ± SD (μm; $n > 12$; $^*P < 0.01$, Student's t test). Bar = 20 μm in B and C. E, Mild reduction of plant height by retarded elongation of lower internodes is observed in *OsBUL1*KO plants (top). Arrowheads indicate each node of plants, and plant height of each genotype is presented as means ± SD (cm, $n > 12$). F, *OsBUL1*KO plant is a T-DNA insertion mutant, and #2, #5, and #6 plants are *OsBUL1* KO homozygous plants. Genotyping primers were as follows: (JIT177) 5′-GGAATTCATGTCGAGCAGAAGGTCGTCGCGTG-3′, (JIT179) 5′-CCACAGTTTTCGCGATCCAGACTG-3′, and (JIT224) 5′-CGCTTGCTGCTGCTGCTTGCCGATC-3′. +/+ Means wild type/wild type segregant, and +/− and −/− indicate heterozygote and homozygote for T-DNA insertion, respectively. G, *OsBUL1* transcripts are not detected in *OsBUL1*KO plants. The numbers in the parentheses indicate PCR cycles. H, The mutant plants produce smaller grains. Bar = 2 mm. Error bars indicate SD ($n = 30$; $^*P <$ 0.0001, Student's t test). I, Leaf angles between wild-type and *OsBUL1*KO plants. The third leaf from the top of the main stem was used for measurement and values are given as means ± SD in the graph ($n = 18$; $^*P < 0.001$, Student's t test).

Isolation of *OsBUL1D*, a Gain-of-Function Mutant of *OsBUL1*

We isolated an activation tagging line in which *OsBUL1* expression was significantly increased (Fig. 2). The mutant plants displayed increased lamina inclination. The leaf-bending phenotype was observed even at the seedling stage (Fig. 2D). Scanning electron microscopy and sectional anatomy revealed that the lamina joint area of the mutant was expanded and the cells in the region were enlarged (Fig. 2, F and H). Indeed, average length of cells in the lamina joint of *OsBUL1D* was more than 2-fold longer than that of wild-type control (Fig. 2, G and H). The heterozygous plants were selfed, and the progeny showed a 3:1 ratio of the mutant to the wild type, indicating that the mutant is dominant. T-DNA was inserted 8.1 kb downstream of *OsBUL1* near Os02g51310 on chromosome 2 (Fig. 2I). While Os02g51320 transcript level was dramatically increased, expression of nearby genes was not significantly affected (Fig. 2J).



Download figure

Open in new tab

Download powerpoint

Figure 2.

Larger lamina angles caused by an *OsBUL1* activation tagging line and recapitulation of the increased lamina inclination phenotype by *OsBUL1* or its Arabidopsis homolog, *PRE1* overexpression. A, A gain-of-function mutant of *OsBUL1* (*OsBUL1D*; right) and the wild type. B to D, Compared to the wild type (B), *OsBUL1D* showed increased leaf angles (C) in a flag leaf and young leaves (right in D). Arrowheads indicate the lamina joint of flag leaf (B and C). Bar = 1 cm in D. E to H, Magnified images show that the lamina joint area is extended in *OsBUL1D* (F and H) compared to the wild type (E and G) due to enlarged cells. G and H, Longitudinal

sections of the lamina joint area in the wild type (G) and *OsBUL1D* (H). Length of cells in the lamina joint of wild-type control and *OsBUL1D* plants is presented. Values are given as means ± SD (μm; $n > 15$; $^*P < 0.001$, Student's t test). Bars = 1 mm (E and F) and 25 μm (G and H). I, Genotyping of the *OsBUL1D* line. 4XE means four tandem copies of CaMV *35S* enhancer. Primer sequences are (a) 5′-TGCCACCTCAGTAAAAACCGGACAC-3′, (b) 5′-CGATGACAAGTTGAGGGAGCTTTGG-3′, and (c) 5′-CGTCCGCAATGTGTTATTAAG-3′. J, Higher expression level of *OsBUL1* (Os02g51320) in the *OsBUL1D*. K, Overexpression of *OsBUL1* or its Arabidopsis homolog, *PRE1* in wild-type rice phenocopies the gain-of-function mutant of *OsBUL1*. L and M, Compared with the wild type (L), *OsBUL1* overexpressors show increased lamina inclination (M). Arrowheads indicate the lamina joint of flag leaf (L and M). N and O, Expression of *OsBUL1* and *AtPRE1* in transgenic rice plants shown in K. The numbers in the parentheses indicate PCR cycles.

The *OsBUL1D* mutant plants produced grains with increased size (Supplemental Fig. S2). Recapitulated phenotyp of *OsBUL1D* was observed in transgenic rice overexpressing *OsBUL1* by maize (*Zea mays*) *Ubi1* promoter (**Fig. 2, K to N**; Supplemental Fig. S2). These results are consistent with a previous report stating that grain size was increased by cell elongation in transgenic rice plants expressing *PGL2* (the same gene as *OsBUL1*) under the control of *chitinase* promoter (**Heang and Sassa, 2012b**).

To investigate whether *OsBUL1* is functionally conserved in dicot plants, we produced rice plants overexpressing Arabidopsis *PRE1* that are homologous to rice *OsBUL1.* These transgenic plants showed similar phenotypes to *OsBUL1*-overexpressing plants (**Fig. 2, K and O**; Supplemental Fig. S2). We expressed the *OsBUL1* gene in Arabidopsis and tobacco (*Nicotiana benthamiana*) using CaMV *35S* promoter. The transgenic plants showed phenotypes of elongated petioles due to cell elongation (Supplemental Fig. S3). Transgenic Arabidopsis with reduced expression of *PRE1* showed short petioles with tiny cells (Supplemental Fig. S3, A and B).

OsBUL1 Is Induced by Exogenous Brassinolide and Is Involved in BR Signaling

Transgenic rice plants carrying GUS driven by the 2.2-kb *OsBUL1* promoter were generated and their GUS activity was examined. GUS expression was detected in seedlings, lamina joints, nodes, panicles, and floral organs including palea, lemma, and anthers (**Fig. 3, A–H**). Expression was not found in leaf sheath, leaf blade, and internode. The results coincided with the expression pattern of *OsBUL1* transcripts analyzed by qRT-PCR (**Fig. 3I**).

Download figure

Open in new tab

Download powerpoint

Figure 3.

Spatiotemporal expression of *OsBUL1*. A to H, GUS staining of various tissues from p*OsBUL1*:*GUS* transgenic rice plants: seedlings (A), spikelets with different developmental stages (B), a spikelet after removing palea and lemma (C), young panicle (D), lamina joint (E and F), anthers (G), and node (H). Bar = 1 mm in A to C. I, Relative expression level of *OsBUL1* in various organs at different developmental stages. J, Induction of *OsBUL1* expression by GA_3 and **BL** at the 24-h time point after treatment. E indicate SD of three biological replicates.

We examined whether *OsBUL1* expression is regulated by phytohormones that play roles in cell elongation. The level of *OsBUL1* transcripts was increased 19.5-fold by 24-h brassinolide (**BL**) treatment. However, gibberellin (GA did not influence the gene expression (**Fig. 3J**). Whereas dark-grown wild-type seedlings had longer shoots, roots, and coleoptiles compared to light-grown seedlings, *osbul1* seedlings had shorter shoots when they were grown under dark conditions compared to light conditions (Supplemental Fig. S4). *OsBUL1* transcripts are more abundant in plants grown in the dark (Supplemental Fig. S4C). Lamina inclination assay revealed that *osbul1* did not respond to various concentrations of **BL** (Supplemental Fig. S5, A and B). These observations indicate that *OsBUL1* is linke to **BR** signaling (Supplemental Fig. S4A)

OsBUL1 Interacts with LO9-177

To understand functional roles of OsBUL1 at the molecular level, we performed yeast two-hybrid screening of a ri cDNA library prepared from aboveground parts of rice plants. The screening resulted in identification of LO9-177 (**Fig. 4A**), which is an uncharacterized conserved protein with a predicted monomeric mass of 13.7 kD, containing characteristic KxDL motif toward its C terminus (**Hayes et al., 2011**). The spatiotemporal expression pattern of *LO 177* overlapped with that of *OsBUL1* (**Fig. 3I**; Supplemental Figs. S11B and S12, A–D). They were colocalized in th nucleus as well as the cytoplasm (Supplemental Figs. S9A).

Download figure

Open in new tab

Download powerpoint

Figure 4.

OsBUL1 interacts with LO9-177. A, Yeast two-hybrid systems demonstrated that OsBUL1 interacts with LO9-177, a small protein containing the KxDL motif. Also, OsBUL1 forms a homodimer and interacts with OsBUL3 and OsIBH1 but not with OsBU1, OsBUL2, and ILI1 (OsILI1). B, His pull-down assays with bacterial recombinant proteins.OsBUL1-GST fusion proteins were pulled down only with LO-177:His (6x His residues) fusion proteins. C, **BiFC** assays showed yellow florescent signals from reconstructed YFP in both the cytoplasm and nucleus of rice protoplasts containing YFPn:OsBUL1 and YFPc:LO9-177. The blue color is autofluorescence from chloroplasts, and nuclear localization signal (NLS)-RFP marker was used for nuclear labeling. Merged fluorescence signals include reconstructed YFP by **BiFC**, RFP by NLS-RFP, and autofluorescence in chloroplasts. The negative controls that were conducted by cotransfecting unfused YFP fragments together with a single complementary **BiFC** interactor-protein resulted in no detectable signals (Supplemental Fig. S9C). Bar = 5 μm.

OsBUL1 formed homodimers and selectively interacted with OsBUL3 among OsBUL1 homologs including OsBU OsBUL2, OsBUL3, and OsILI1 (the same as ILI1). OsBUL1 also interacted with OsIBH1, an OsILI1-interacting protein (**Zhang et al., 2009**; **Figure 4A**; Supplemental Fig. S7A). Of note, OsIBH1 interacted with OsBUL1 and its homologs but could not form a homodimer (Supplemental Fig. S7A).

In vitro pull-down assays using *Escherichia coli*-expressed recombinant proteins, GST-tagged OsBUL1 (GST:OsBUL1) interacted with His-tagged LO9-177 (TRX:LO9-177:His), but not with TRX:His (**Fig. 4B**). Interaction between OsBUL1 and LO9-177 was also verified in rice cells by bimolecular fluorescence complementation (**BiFC**) assays using nYFP:LO9-177 and cYFP:OsBUL1 (**Fig. 4C**).

To study the functional roles of *LO9-177*, a plasmid for overexpression of *LO9-177* by *OsBUL1* promoter was constructed and introduced into rice plants. They showed phenotypes of increased lamina angles with elongated cells in the lamina joint and grain size (**Fig. 5, A and D**; Supplemental Fig. S6). Transgenic plants overexpressing *LO9-177* by the maize *ubi1* promoter displayed similar phenotypes (**Fig. 5B**). On the contrary, transgenic lines expressing *LO9-177* **dsRNAi** showed reduced leaf angles with mildly reduced length of cells in the lamina joint (**Fig. 5, C and D**). These indicate that *LO9-177* functions similar to *OsBUL1*.

Download figure

Open in new tab

Download powerpoint

Figure 5.

Transgenic rice plants for functional characterization of *LO9-177*. A to C, Transgenic rice plants expressing *LO9-177* driven by *OsBUL1* promoter (A) and constitutive ubiquitin promoter (B) caused increased leaf angles while **dsRNAi** lines displayed erect leaves (C). The full-length *LO9-177* coding region (372 bp) was used for **dsRNAi** construction, and endogenous expression level of *LO9-177* was examined by RT-PCR using primers, 5′-AGACTCGATAGGGGAGAGGA-3′ and 5′-TCGGCAAAAGTGCAGACAAAAC-3′. The third leaf from the top of the main stem was used for lamina angle measurement and values for leaf angles are given as means ± SD (n = 10 to 14; *P < 0.001, Student's t test). RNA was extracted from collars for cDNA synthesis. The numbers in parentheses indicate PCR cycles. D, Longitudinal sections of the lamina joint area in the wild type, p*OsBUL1*:*LO9-177*, and **dsRNAi** of *LO9-177* plants. Length of cells in the lamina joint of wild-type control, p*OsBUL1*:*LO9-177*, and **dsRNAi** of *LO9-177* plants is also presented. Values are given as means ± SD (n > 15; *P < 0.01; **P < 0.05, Student's t test). Bars = 20 μm.

LO9-177 Interacts with OsBC1, a bHLH Protein

We screened the rice library using the LO9-177 protein as a bait and isolated a novel **bHLH** protein (Os09g33580) that we named OsBUL1 COMPLEX1 (OsBC1; **Fig. 6A**). Expression patterns of *OsBC1* and *LO9-177* were similar t each other in several organs including collars and panicles (Supplemental Figs. S11B and S12). LO9-177 was able to form a homodimer and interact with OsBUL1 homologs such as OsBU1, OsBUL2, and OsBUL3, except OsILI1. Moreover, LO9-177 did not interact with OsIBH1 and APG, which was reported to interact with PGL2 as a **bHLH** protein (**Fig. 6A**; **Heang and Sassa, 2012b**). GST pull-down assays with GST:OsBC1 and TRX:LO9-177:His confirmed the interaction between them (**Fig. 6B**). OsBC1 was localized in the nucleus whereas LO9-177 was four in both the nucleus and cytoplasm (Supplemental Fig. S9, A and B). **BiFC** assays using rice protoplasts displayed florescent signals in the nucleus with speckles from reconstructed YFP by nYFP:OsBC1 and cYFP:LO9-177 (**Fig. 6C**), suggesting the interaction may occur near active transcription sites (**Reddy et al., 2012**). Interestingly, OsBC1 was able to interact with OsIBH1 although it could not interact with OsBUL1 or OsBUL1 homologs (Supplemental Fig. S7B).

Download figure

Open in new tab

Download powerpoint

Figure 6.

LO9-177 interacts with OsBC1. A, LO9-177 is able to make a homodimer. It interacts with OsBUL1 and its homologs, such as OsBU1, OsBUL2, and OsBUL3, except ILI1 (OsILI1), with different affinities and with a typical **bHLH** protein, OsBC1, but does not interact with OsIBH1 and APG. B, GST pull-down assays showed LO9-177:His fusion proteins were pulled down only together with GST:OsBC1 fusion proteins. C, Reconstructed yellow fluorescence from YFPn:OsBC1 and YFPc:LO9-177 was detected or nucleus with speckles of transfected rice protoplasts. The negative controls that were conducted by cotransfecting unfused fragments together with a single complementary **BiFC** interactor protein resulted in no detectable signals (Supplemental Fi Bar = 5 μm.

PDF

Help

OsBUL1, LO9-177, and OsBC1 Form a Complex

LO9-177 interacted with both OsBUL1 and OsBC1 while OsBUL1 did not directly interact with OsBC1. A yeast three-hybrid system was used to evaluate whether LO9-177 is a molecular mediator between OsBUL1 and OsBC1 The experiment revealed that OsBUL1 interacts with OsBC1 in the presence of LO9-177, indicating formation of a trimeric complex consisting of OsBUL1, LO9-177, and OsBC1 (**Fig. 7, A–C**). Interaction between OsBUL1 and AP(was not enhanced by LO9-177 (**Heang and Sassa, 2012b**). We confirmed the formation of a complex by in vitro pull-down assays using recombinant proteins obtained from *E. coli* culture. GST:OsBC1 fusion proteins were pullec down together with TRX:OsBUL1:His fusion proteins only in the presence of LO9-177 (**Fig. 7C**). Notably, OsBC1

has transcriptional activation activity and forms a homodimer (Supplemental Fig. S8). GUS staining of rice plants harboring p*OsBC1*:*GUS* construct indicated that the gene is preferentially expressed in anthers and lamina joint. This expression pattern is similar to that of *OsBUL1* (**Fig. 8**). In addition, we examined the expression of *OsBUL1* and *OsBC1* in *oslg1-1* mutant that is defective in collar formation (**Lee et al., 2007**). In the mutant, both *OsBUL1* and *OsBC1* transcripts were rarely detectable in the lamina joint area, suggesting that both genes are preferentially expressed in the lamina joint rather than in the sheath or blade of mature leaves (Supplemental Fig. S2, G and H).

Download figure

Open in new tab

Download powerpoint

Figure 7.

OsBUL1 interacts with OsBC1 through LO9-177. A, Yeast three-hybrid systems demonstrated that OsBUL1 interacts with OsBC1 only in the presence of LO9-177. However, coexpression of LO9-177 did not affect the interaction between OsBUL1 and APG. B, Schema showing the positive interaction between OsBUL1 and OsBC1 mediated by LO9-177 in yeast cells. C, His pull-down assays demonstrated that OsBC1 can be pulled down with OsBUL1 only in the presence of LO9-177.

Download figure

Open in new tab

Download powerpoint

Figure 8.

Histochemical GUS staining of rice plants harboring p*OsBC1*:*GUS*. A to C, Closed and open spikelets. D to F, Lamina joint parts of 10-d-old rice seedlings (D) and mature plants at heading stage (E and F).

Expression of *OsBC1* Driven by *OsBUL1* Promoter Increased Lamina Inclination and Grain Size

We examined the functional activity of *OsBC1* by expressing the gene under the control of *OsBUL1* promoter in ric
Transgenic plants containing p*OsBUL1*:*OsBC1* showed phenotypes of increased lamina angles and grain size (**Fig 9, A–C**). Epidermal cells of mature grains from the transgenic plants were enlarged, and the expression level of genes involved in cell elongation such as expansins was higher compared to the wild type (Supplemental Fig. S14)
Conversely, *OsBC1*-**dsRNAi** transgenic rice lines exhibited semidwarf phenotype with small grains (**Fig. 9, A–C**) bu did not show any obvious reduction in lamina inclination. Panicle branches were widely spread in the p*OsBUL1*:*OsBC1* plants, whereas they were short and compact in the *OsBC1*-**dsRNAi** lines (**Fig. 9C**). Interestingly transgenic Arabidopsis overexpressing *OsBC1* under the control of the *35S* promoter produced narrow and elongated leaves that were composed of elongated cells (Supplemental Fig. S10).

Download figure

Open in new tab

Download powerpoint

Figure 9.

Observation of functional activity of *OsBC1* at locations where *OsBUL1* is expressed. A, Transgenic rice plants containing p*OsBUL1*:*OsBC1* had dramatically increased angles of leaves and panicle branches, whereas **dsRNAi** of *OsBC1* lines were semidwarf without obvious changes of leaf angles. RNAs were extracted from young panicles for cDNA synthesis for RT-PCR. The 273-bp fragment of *OsBC1* coding region amplified by primers, 5′-GACCACTCTCAGAAGATGGAAG-3′ and 5′-CTACTGGAAAGAGCACATG-3′, was used for **dsRNAi** construction. B, The grain size of transgenic plants with p*OsBUL1*: also increased, while **dsRNAi** lines of *OsBC1* produced small grains (*$P < 0.001$, Student's *t* test). Bar = 5 mm. C, Angles f panicle branches are also increased in transgenic plants with p*OsBUL1*:*OsBC1*, while **dsRNAi** lines of *OsBC1* produced c panicle architecture. Red arrowheads indicate the lamina joint of flag leaf in each genotype. Bar = 5 cm. The third leaf from the top of the main stem was used for lamina angle measurement, and values for leaf angles are given as means ± SD ($n = 8$ to 12; *$P <$ 0.001, Student's *t* test). D, Expression of *OsBC1*-SRDX, a repressor form of *OsBC1* under the control of *OsBUL1* promoter, rendered rice plants to exhibit similar phenotypes to *OsBC1* **dsRNAi** lines. RNAs were isolated from collars for cDNA synthesis and RT-PCR using primers, 5′-CATCCCTGAAGATGCCTCAATG-3′ and 5′-TATGCGAATCCTAGTTCCAGTTCGAGATC-3′.

We also generated a construct for chimeric OsBC1 repressor by inserting oligomers coding for the SRDX domain (LDLDLELRLGFA; **Hiratsu et al., 2003**) in front of the stop codon of *OsBC1*. Fusion of the SRDX domain to a transcriptional activator can convert it into a repressor that overrides activation of endogenous transcription factors, resulting in a dominant-negative phenotype. On the contrary, the SRDX fusion to a native repressor enhances transcriptional repression, causing overexpression phenotypes (**Ikeda and Ohme-Takagi, 2009**). Transgenic rice plants expressing the transgene under the control of the *OsBUL1* promoter showed phenotypes similar to those of

OsBUL1 dsRNAi lines (Fig. 9D), indicating that OsBC1 may act as a transcriptional activator in lamina joints and panicles of rice.

DISCUSSION

In this study, we screened rice T-DNA mutant pools to identify plants showing abnormal lamina angles, and a T-DN tagging line for *OsBUL1* KO was isolated and characterized. Previously, atypical HLH proteins similar but different from OsBUL1 have been reported to play roles in cell elongation in an antagonistic manner with other atypical HLH and bHLH proteins in Arabidopsis and rice (Wang et al., 2009; Zhang et al., 2009; Ikeda et al., 2012). Arabidopsis AIFs, as atypical HLH proteins, act as negative regulators of BR signaling and interacting antagonists of PREs (Wang et al., 2009), another group of atypical HLH proteins including OsBUL1. Moreover, Arabidopsis PREs are able to interact with IBH1 as rice ILI1 does with OsIBH1 to have antagonistic effects on cell elongation (Zhang et al., 2009). Recently, a triantagonistic model has been suggested for PRE1, IBH1, and ACEs/CRYPTOCHROME INTERACTING BASIC-HELIX-LOOP-HELIX5 (CIB5) in Arabidopsis without AIFs (Ikeda et al., 2012). Based on thi model, PRE1 positively regulates cell elongation by forming heterodimers with IBH1 that suppresses IBH1 inhibitio of ACEs. AIFs, like IBH1, are also known to interact not only with PREs but also with ACE1, implying a complicated network of HLH-bHLH proteins in cell elongation (Ikeda et al., 2013; Supplemental Fig. S13).

OsBUL1 is upregulated by exogenous BL similar to *OsBU1* (Tanaka et al., 2009), and the *OsBUL1D* and *OsBUL1* overexpressing rice exhibited increased lamina inclination and grain size resulting from cell elongation. On the contrary, *osbul1* did not respond to BL treatment in lamina bending assays and produced erect leaves with small spikelets. This phenotypic alteration is well matched with the spatial expression pattern of *OsBUL1*.

To understand the molecular functional networks of OsBUL1 in rice, the OsBUL1 bait protein was screened against the rice cDNA library using yeast two-hybrid methods, and a small uncharacterized protein, LO9-177, was isolated as an OsBUL1-interacting protein. Although a bHLH protein, APG was reported to interact with OsBUL1 as a negative regulator of cell elongation (Heang and Sassa, 2012a, 2012b, 2012c), we could not detect a pos interaction between APG and OsBUL1 or its homologs including BU1 (OsBU1), OsBUL2, OsBUL3, and ILI yeast systems (Supplemental Fig. S7B). Phenotypic analyses of transgenic rice using gain-of-function and loss-of-function approaches demonstrated that *LO9-177* is a genetic factor affecting lamina inclination as well as encoding an interacting protein of OsBUL1. Mild lamina inclination phenotype of *LO9-177* overexpressing plants might be du to the amount of interacting proteins for functional complex formation in the lamina joint. Actually, the KxDL domain in LO9-177 is regarded as being critical in selective interaction with a protein or protein complex (Hayes et al., 2011). In mammals, the KXD protein containing a KxDL motif interacts with biogenesis of lysosome-related organelles complex-1 subunit 1 (BLOS1) whose mutation is responsible for Hermansky-Pudlak syndrome and transgenic *KXD* knockout mouse showed similar symptoms of Hermansky-Pudlak syndrome (Yang et al., 2012). Thus, LO9-177 was used as a bait for screening of interacting proteins and interestingly a bHLH protein, OsBC1 was isolated as a LO9-177 interactor. LO9-177 was able to interact with atypical HLH proteins such as OsBU1,

OsBUL1, OsBUL2, and OsBUL3 (but not OsIBH1), but did not interact with APG showing interaction specificity a complexity among HLH/bHLH and LO9-177 proteins. Despite LO9-177 being localized both in the nucleus and the cytoplasm, the interaction with OsBC1 occurs only in the nucleus, which is likely due to subcellular localization of OsBC1, a nuclear protein. Next, we tested the possibility of formation of a complex consisting of OsBUL1, LO9-1 and OsBC1. Yeast two- and three-hybrid systems together with pull-down assays suggested that the three proteins form a complex.

In order to examine the functional activities of *OsBC1*, we generated transgenic rice containing p*OsBUL1*:*OsBC1* and the transgenic lines exhibited a significant increase in lamina angles and grain size. In contrast, **dsRNAi** and p*OsBUL1*:*OsBC1*-SRDX lines showed reduced height and produced smaller grains, implying that OsBC1 is a transcriptional activator that plays a positive role in cell elongation and the transcriptional activity of OsBC1 was shown in yeast cells (Supplemental Fig. S8). However, the lamina angle of *OsBC1* knockdown plants was not affected significantly, suggesting that the loss-of-function effect of *OsBC1* may be compensated for by the function of other homologous genes. For example, Arabidopsis ACEs/CIBs, which are **bHLH** transcription factors, play a similar role in cell elongation (**Ikeda et al., 2012**). Of note, *OsBC1* as well as *OsBUL1* are upregulated by **BL** (Supplemental Fig. S11). Although it is not obvious yet whether OsBC1 is a homolog of Arabidopsis ACEs, it seem that the triantagonistic model suggested in Arabidopsis research (**Ikeda et al., 2012**) can be applied to rice in cell elongation (**Fig. 10**): Atypical **HLH** proteins including OsBUL1 interact with OsIBH1, *OsIBH1*-overexpressing transgenic rice produced small cells (**Zhang et al., 2009**), and we identified OsBC1, a transcriptional activator containing a **bHLH** domain for cell elongation that interacts with OsIBH1. In Arabidopsis, there is another group of atypical **HLH** proteins that includes AIF1, AIF2, AIF3, and AIF4 whose mRNAs are expressed ubiquitously. They a as negative regulators in cell elongation by interacting with both PREs and ACE1 (**Ikeda et al., 2013**). Identificatio and functional characterization of rice AIF homologs will provide more information about plant cell elongation through the HLH-bHLH regulation systems in plants.

Download figure

Open in new tab

Download powerpoint

Figure 10.

Simplified working model for cell elongation through a network of **HLH-bHLH** proteins. Previous studies (**Tanaka et al., 2009**; **Wang et al., 2009**; **Zhang et al., 2009**; **Ikeda et al., 2012**) and data provided by this study were used together for the construction of the model. The formation of a hexameric complex was drawn based on yeast two- and three-hybrid results with pull-down assays in this study. The arrows and T-shaped lines signify positive and negative actions, respectively. The dashed arrows were drawn b the up-regulation of *OsBUL1* and *OsBC1* by **BL**.

In addition, we provide evidence of a novel complex consisting of OsBUL1, LO9-177, and OsBC1 that has a positiv effect on cell elongation. Moreover, each component in the complex is capable of making a homodimer implying tha assembly of a hexameric complex might be possible (**Fig. 10**).

Regulation of plant cell elongation is governed by multiple signals and is also important for normal development an adaptation to various environmental conditions. The balance among atypical HLH and bHLH proteins seems to be critical for cell elongation, and the fine-tuning of the balance or stabilization of molecular activity required for cell elongation by OsBC1 is likely achieved by the formation of a complex with LO9-177 and atypical HLHs including OsBUL1 (**Fig. 10**). By better understanding the machineries controlling the balance of HLH-bHLH proteins, we may manipulate the lamina inclination of crop plants, which is an important agronomic trait for improved productivity.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

OsBUL1 activation tagging line, PFG_3A_01926, and *OsBUL1*null mutant line, PFG_2D_01383, are japonica rice cultivars Dongjin and Hwayoung, respectively. Transgenic rice plants were produced with Dongjin or Tainung67 (TNG67) japonica rice cultivars. Rice plants (*Oryza sativa*) were grown in the field under natural long days or in the greenhouse with 28°C day/25°C night cycles. Lamina angles were measured between a stem and a leaf blade at the second or the third leaf from 8 to 12 individual plants per line. To assess the leaf angles of mature rice plants in the paddy field, leaves were numbered from top to bottom. Thus, each flag leaf of the main tiller was regarded as the first leaf. Arabidopsis (*Arabidopsis thaliana*) strain, Columbia (Col), and tobacco (*Nicotiana benthamiana*) plants were used for transformation and grown in a growth chamber under long days with 23°C day/20°C night cycles.

Lamina Joint Inclination Bioassay

Sterilized seeds were germinated and grown for 8 d in a growth chamber. The lamina joint inclination assays were performed as previously described (**Jeong et al., 2007**). Seedlings were sampled by excising approximately 2-cm segments that contained lamina joints at the same position from each plant under dim light conditions. They were floated on distilled water containing various concentrations of **BL**. After incubation at 28°C, the angle induced between the lamina joint and the sheath was measured.

Vector Construction and Transformation

The whole/parts of open reading frames (ORFs) of *OsBUL1*, *PRE1*, *LO9-177*, *OsBC1*, and *OsBC1*-SRDX were cloned into pGA3426 or its derivatives for overexpression and/or **dsRNAi** purposes in rice. For expression by *OsBUL1* promoter, the ubiquitin promoter of pGA3426 was replaced with the 2.2-kb *OsBUL1* promoter. Vector pGA3383 was used for analyzing *OsBUL1* promoter activity using the GUS reporter in rice (**Kim et al., 2009**). Constructed plasmids were individually transformed into embryonic calli of Dongjin or TNG67 rice cultivars by *Agrobacterium tumefaciens*-LBA4404 mediation as described previously (**Jeon et al., 2000**). pGA643 vect pJawohl8-RNAi silencing vector (kindly provided by I.E. Somssich, Max Planck Institute for Plant Breeding Research) were used for Arabidopsis and tobacco transformation by floral dipping and tissue culture via *A. tumefaciens*-mediated DNA delivery (**Clough and Bent, 1998**; **Jang et al., 2002**), respectively.

Hormone Treatment

Eight-day-old rice (*O. sativa* cv TNG67) seedlings grown in the growth chamber were treated with brassinolide (1 μM, **BL** from Sigma-Aldrich) or gibberellin (100 μM GA_3 from Sigma-Aldrich). Whole parts above roots were harvested for RNA extraction at the 24 h time point after treatment.

Total RNA Isolation and Quantitative RT-PCR Analysis

Total RNAs of all the materials harvested were isolated using RNeasy plant mini kit (Qiagen) or Trizol solution (Invitrogen) according to the manufacturer's instructions. DNase-treated RNA was subjected to reverse transcriptase reactions using oligo(dT) primer and Superscript III reverse transcriptase (Invitrogen) according to t manufacturer's protocol. Subsequent PCR was performed with the first-strand cDNA mixture and EX-Taq polymerase (Takara Bio). qPCR was performed on a CFX96TM real-time system (Bio-Rad) using Maxima SYBR Green qPCR Master Mix (Thermo). For PCR, each sample was analyzed in triplicate. The run protocol was as follows: denaturation at 95°C for 10 min and annealing/extension repeated 45 times (95°C for 15 s and 60°C for 30 s, data acquisition was performed). Housekeeping genes such as *OsUBQ* (**Komiya et al., 2008**), *OsAct* (**Caldana et al., 2007**), *AtPEX4* (**Gregis et al., 2013**), and *AtACT* (**Jang et al., 2009**) were included in the reactions as intern controls for normalizing the variations in the amount of cDNA used (**Guénin et al., 2009**). The threshold cycle (C_T) was automatically determined for each reaction by the system set with default parameters. The specificity of the qRT-PCR was determined by curve analysis of the amplified products using the standard method installed in the system. Information on primers used is presented in Supplemental Table S1.

GUS Staining

For promoter analysis, about 2.2 kb of *OsBUL1* 5′ region was amplified with primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGCGCGCGATGATTTCGTGACATG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGTCAACAGCTAGCCTCTTCTACCAAACAC-3′ and cloned into pDONR207 by BP reaction (Invitrogen). For *OsBC1* promoter, an ~3-kb fragment of *OsBC1* 5′ region was amplifie with primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTACTTAATTTAGTGTCATGTAAG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCAATGCCCTTGGTGTCCTAGATG-3′ and cloned into pDONR207. The entry clones for *OsBUL1* promoter and *OsBC1* promoter were used for LR reaction with pGA338 Gateway vector for GUS fusion, respectively. The resulting plasmids were transformed into rice, and GUS staining was performed according to the method described previously (**Jefferson, 1989**).

Histological Analyses

Lamina joint samples were fixed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, dehydrated th graded ethanol series, replaced with xylene, and embedded in Paraplast plus (Sigma-Aldrich). Paraffin sec μm) were cut and stained with filtered 1% toluidine blue. Anthers stained with GUS were embedded using Epon81 resin (Fluka) and polymerized at 60°C. Cross sections (3 μm) were cut with a rotary microtome (Leica). The sectior were photographed under a light microscope (Olympus BX51).

Yeast Assays and BiFC Assays

Rice (*O. sativa* cv TNG67) cDNA library was constructed using poly(A)$^+$ mRNAs extracted from whole aboveground parts including leaves, culms, and panicles at different developmental stages. The HybriZAP-2.1 XR cDNA library construction kit was used, and the initial plaque-forming units (pfu) of the constructed library was 1.8×10^6. Screening of the library with baits such as pBD:OsBUL1 and pBD:LO9-177 was performed as described previously

(Jang et al., 2002). For yeast three-hybrid assays, we used an adaptor vector, pBridge (Clontech), together with prey vectors. *Eco*RI/*Xho*I fragment of amplified *OsBUL1* full ORF (using 5′-GGAATTCATGTCGAGCAGAAGGTCGTCGCGTG-3′ and 5′-GCCTCGAGTCAGGAGCGGAGGATGCTGCGGAT 3′; restriction enzyme sites are underlined) was cloned into *Eco*RI/*Sal*I sites fused to the binding domain (BD) and PspOMI/*Bgl*II fragment of amplified *LO9-177* ORF (using 5′-GAGGGGCCCTCATGGAGAAGTCGCCGCCGGAG-3 and 5′-GCCAGATCTTTAATCAAGCGGACTTTCAAG-3′; restriction enzyme sites are underlined) was inserted into *Not*I/*Bgl*II sites of the pBridge vector for independent expression in yeast cells. Verification of interactions with X-Ga filter assays were also conducted as reported by Jang et al. (2015). For BiFC assays in rice, each cDNA of *OsBUL1*, *LO9-177*, and *OsBC1* was cloned into pVYCE vector or pVYNE vectors (Citovsky et al., 2006; Tzfira et al., 2005) for addition of half YFP to the each cDNA. YFP and CFP fusion for cellular localization of each protein was also conducted as previously described (Jang et al., 2008). Isolation and transfection of Arabidopsis and rice protoplasts were as described by Wu et al. (2009) and Zhang et al. (2011). Images of cells with fluorescence were taken by confocal microscopy (LSM 510 META NLO DuoScan; Carl Zeiss).

Protein Pull-Down Assays

The cDNAs encoding OsBUL1, LO9-177, and OsBC1 were cloned into the *Eco*RI/*Xho*I sites of the pGEX6P-1 for GST fusion and amplified *OsBUL1* ORF with *Eag*I/*Xho*I ends and *LO9-177* ORF with *Bam*HI/*Xho*I ends were also inserted into *Not*I/*Xho*I and *Bam*HI/*Xho*I sites of pET201 vector (Bhalerao et al., 1999), respectively, for His fusion. The nucleotide sequences for the fusion proteins were confirmed by sequencing (Sequencing Core Facility, Scientific Instrument Center, Academia Sinica, Taiwan). Constructed plasmids were introduced into *Escherichia co* BL21 (DE3), and cells transformed by construct of GST fusion were cultured to an OD_{600} followed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside. After overnight induction at 16°C, cells were collected and homogenized with lysis buffer (10 mM Tris, pH 8.0, 5 mM DTT, 1% Triton X-100, and 150 mM NaCl). The soluble GST fusion proteins were extracted and immobilized on glutathione-MagBeads (GenScript) for subsequent pull-down assays. For His pull-down assays, cells transformed by His-fusion construct were cultured until the OD_{600} reached 0.6, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to start induction. After ove induction at 16°C, cells were harvested and homogenized with lysis buffer (10 mM Na_2HPO_4, 10 mM NaH_2 mM NaCl, and 20 mM imidazole). The soluble thioredoxin (TRX)-tagged His fusion proteins were extracted and immobilized on Ni-charged MagBeads (GenScript), and the recombinant proteins were purified according to the manufacturer's instructions. In vitro pull-down assays were performed by incubation with a combined mixture of proteins in a binding buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 0.25% Triton X-100, and 35 mM β-mercaptoethanol) for 4 to 6 h at 4°C with rotation. Collection of the beads was achieved using a MagRack 6 (GE Healthcare) followed by washing six times with binding buffer. The pulled-down proteins were separated on a 12% SDS-polyacrylamide gel and detected by western blotting using anti-GST or anti-His antibody.

In Situ Hybridization

Young spikelets of rice (cv TNG67) were collected and fixed, dehydrated, embedded, and sliced (10 μm thickness and hybridization was performed as previously described (Ko et al., 2014). For preparation of digoxigenin-labeled RNA probes, DNA fragments containing the end part of the coding region to 3′ untranslated region or only 3′ untranslated region from each gene, *LO9-177* (352 bp) and *OsBC1* (326 bp), were amplified using following primers: *LO9-177*-For, 5′-GAACAATGCTTTGCGGAGGTGTC-3′ and *LO9-177*-Rev, 5′-CATAGGACTACAAGGTTACACAAC-3′; *OsBC1*-For, 5′-CAATCTCATGCCATCATGGAC-3′; and *OsBC1*-Rev, 5′-CAGACAAGGGGATGGACTCG-3′. Each amplified DNA fragment was cloned into the pGEM-T vector (Promega), and each sense and antisense probe was synthesized by T7 and SP6 RNA polymerases, respectively. Hybridization was performed at 62 or 63°C with 20 ng of digoxigenin-labeled RNA probes.

Accession Numbers

Genes in this article can be found in the GenBank/EMBL or RiceGE databases under the following accession numbers: ACE1 (At1g68920), ACE2 (At1g10120), ACE3 (At3g23690), AIF1 (At3g05800), AIF2 (At3g06590), AIF3 (At3g17100), AIF4 (At1g09250), APG (Os05g04740.1), CIB1 (At4g34530), CIB5 (At1g26260), PRE1 (At5g39860), PRE2 (At5g15160), PRE3 (At1g74500), PRE4 (At3g47710), PRE5 (At3g28857), PRE6 (At1g26945), IBH1 (At2g43060), LO9-177 (Os03g43910), OsBC1 (Os09g33580), OsBU1 (BU1, Os06g12210), OsBUL1 (Os02g51320), OsBUL2 (Os03g07540), OsBUL3 (Os10g26410), OsBUL4 (Os10g26460), OsBUL5 (Os11g39000.1), OsBUL6 (Os03g07510.1), OsEXPA1 (Os04g15840), OsEXPA2 (Os01g60770), OsEXPA3 (Os05g19570), OsEXPA4 (Os05g39990), OsILI1 (ILI1, Os04g54900), OsIBH1 (Os04g56500), OsXTH1 (Os04g51460), and OsXTR1 (Os11g33270).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *OsBUL1* **dsRNAi** lines phenocopy *OsBUL1* null mutation.

Supplemental Figure S2. Increased *OsBUL1* expression confers larger grains and leaf angles in rice.

Supplemental Figure S3. Introduction of rice *OsBUL1* into dicot plants Arabidopsis and tobacco.

Supplemental Figure S4. Morphological changes of *OsBUL1*KO seedlings under light or dark conditions.

Supplemental Figure S5. Brassinolide response on wild-type and *OsBUL1*KO rice.

Supplemental Figure S6. Expression level of *LO9-177* is positively linked to grain size.

Supplemental Figure S7. Protein interactions using yeast two-hybrid systems.

Supplemental Figure S8. OsBC1 has autotranscriptional activation activity.

Supplemental Figure S9. Subcellular localization of proteins.

Supplemental Figure S10. Transgenic Arabidopsis overexpressing *OsBC1*.

Supplemental Figure S11. Expression analyses of *LO9-177* and *OsBC1*.

Supplemental Figure S12. In situ hybridization of genes in spikelets.

Supplemental Figure S13. A phylogenetic tree showing the relationships among atypical **HLH** and typical **bHLH** proteins.

Supplemental Figure S14. *OsBC1* affects cell size in plants.

Supplemental Table S1. A list of primers used in this study.

Acknowledgments

We thank Pei-Chun Liao and Wei-Chih Lin for rice transformation, and Hsing-Hui Lee and Ya-Chen Liu for assistance with pull-down assays. We also thank members of core facility laboratories of Academia Sinica for microscopy, DNA sequencing, and in situ hybridization and Miranda Loney for help with English editing.

Footnotes

- www.plantphysiol.org/cgi/doi/10.1104/pp.16.01653

- The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Seonghoe Jang (florigen@gate.sinica.edu.tw).

- S.J. designed the research; S.J. and H.-Y.L. performed research and analyzed data; G.A. and S.J. generated rice T-DNA tagging mutants; S.J. wrote the article.

- ↵1 This work was supported in part by a core grant from BCST/ABRC of Academia Sinica.

Glossary

BR
brassinosteroid

HLH
helix-loop-helix

bHLH
basic helix-loop-helix

dsRNAi
double-stranded RNA interference

BL
brassinolide

BiFC

bimolecular florescence complementation

ORF

open reading frame

Received October 25, 2016.

Accepted November 18, 2016.

Published November 22, 2016.

REFERENCES

Bai M-Y, Zhang L-Y, Gampala SS, Zhu S-W, Song W-Y, Chong K, Wang Z-Y (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. *Proc Natl Acad Sci USA* **104**: 13839–13844 Abstract/FREE Full Text Google Scholar

Bernhardt C, Zhao M, Gonzalez A, Lloyd A, Schiefelbein J (2005) The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the Arabidopsis root epidermis. *Development* **132**: 291–298 Abstract/FREE Full Text Google Scholar

Bhalerao RP, Salchert K, Bakó L, Okrész L, Szabados L, Muranaka T, Machida Y, Schell J, Koncz C (1999) Regulatory interaction of PRL1 WD protein with Arabidopsis SNF1-like protein kinases. *Proc Natl Acad Sci USA* **96**: 5322–5327 Abstract/FREE Full Text Google Scholar

Bhattacharya A, Baker NE (2011) A network of broadly expressed HLH genes regulates tissue-specific cell fates. *Cell* **147**: 881–892 CrossRef PubMed Google Scholar

Caldana C, Scheible W-R, Mueller-Roeber B, Ruzicic S (2007) A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. *Plant Methods* **3**: 7 Google Scholar

Cao H, Chen S (1995) Brassinosteroid-induced rice lamina joint inclination and its relation to indole-3-acetic acid and ethylene. *Plant Growth Regul* **16**: 189–196 CrossRef Google Scholar

Citovsky V, Lee L-Y, Vyas S, Glick E, Chen M-H, Vainstein A, Gafni Y, Gelvin SB, Tzfira T (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *J Mol Biol* **362**: 1120–1131 CrossRef Pu... Google Scholar

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735–743 CrossRef PubMed Google Scholar

Duek PD, Fankhauser C (2005) bHLH class transcription factors take centre stage in phytochrome signalling. *Trends Plant Sci* **10**: 51–54 CrossRef PubMed Google Scholar

Feng Z, Wu C, Wang C, Roh J, Zhang L, Chen J, Zhang S, Zhang H, Yang C, Hu J, et al. (2016) SLG controls grain size and leaf angle by modulating brassinosteroid homeostasis in rice. *J Exp Bot* **67**: 4241–4253 Abstract/FREE Full Text Google Scholar

↲Friedrichsen DM, Nemhauser J, Muramitsu T, Maloof JN, Alonso J, Ecker JR, Furuya M, Chory J (2002) Three redundant brassinosteroid early response genes encode putative bHLH transcription factors required for normal growth. *Genetics* **162** 1445–1456 **Abstract/FREE Full Text** **Google Scholar**

↲Gregis V, Andrés F, Sessa A, Guerra RF, Simonini S, Mateos JL, Torti S, Zambelli F, Prazzoli GM, Bjerkan KN, et al. (2013) Identification of pathways directly regulated by SHORT VEGETATIVE PHASE during vegetative and reproductive developm in Arabidopsis. *Genome Biol* **14**: R56 **CrossRef** **PubMed** **Google Scholar**

↲Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessi of adopting a systematic, experimental conditions-specific, validation of references. *J Exp Bot* **60**: 487–493 **Abstract/FREE Full Text** **Google Scholar**

↲Hayes MJ, Bryon K, Satkurunathan J, Levine TP (2011) Yeast homologues of three BLOC-1 subunits highlight KxDL proteins a conserved interactors of BLOC-1. *Traffic* **12**: 260–268 **CrossRef** **PubMed** **Google Scholar**

↲Heang D, Sassa H (2012a) Antagonistic actions of HLH/bHLH proteins are involved in grain length and weight in rice. *PLoS One* **7**: e31325 **CrossRef** **PubMed** **Google Scholar**

↲Heang D, Sassa H (2012b) An atypical bHLH protein encoded by POSITIVE REGULATOR OF GRAIN LENGTH 2 is involved i controlling grain length and weight of rice through interaction with a typical bHLH protein APG. *Breed Sci* **62**: 133–141 **CrossRef** **PubMed** **Google Scholar**

↲Heang D, Sassa H (2012c) Overexpression of a basic helix-loop-helix gene Antagonist of PGL1 (APG) decreases grain length of rice. *Plant Biotechnol* **29**: 65–69 **Google Scholar**

↲Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M (2003) Dominant repression of target genes by chimeric repressors that incluc the EAR motif, a repression domain, in Arabidopsis. *Plant J* **34**: 733–739 **CrossRef** **PubMed** **Google Scholar**

↲Hong Z, Ueguchi-Tanaka M, Shimizu-Sato S, Inukai Y, Fujioka S, Shimada Y, Takatsuto S, Agetsuma M, Yoshida S, Watanabe Y, et al. (2002) Loss-of-function of a rice brassinosteroid biosynthetic enzyme, C-6 oxidase, prevents the organized arrangement and polar elongation of cells in the leaves and stem. *Plant J* **32**: 495–508 **CrossRef** **PubMed** **Google Scholar**

↲Hong Z, Ueguchi-Tanaka M, Umemura K, Uozu S, Fujioka S, Takatsuto S, Yoshida S, Ashikari M, Kitano H, Matsuoka M (2003 A rice brassinosteroid-deficient mutant, ebisu dwarf (d2), is caused by a loss of function of a new member of cytochrome P45 *Plant Cell* **15**: 2900–2910 **Abstract/FREE Full Text** **Google Scholar**

↲Hyun Y, Lee I (2006) KIDARI, encoding a non-DNA Binding bHLH protein, represses light signal transduction in *Arabi thaliana*. *Plant Mol Biol* **61**: 283–296 **CrossRef** **PubMed** **Google Scholar**

↲Ikeda M, Fujiwara S, Mitsuda N, Ohme-Takagi M (2012) A triantagonistic basic helix-loop-helix system regulates cell elongatior in Arabidopsis. *Plant Cell* **24**: 4483–4497 **Abstract/FREE Full Text** **Google Scholar**

↲Ikeda M, Mitsuda N, Ohme-Takagi M (2013) ATBS1 INTERACTING FACTORs negatively regulate Arabidopsis cell elongation the triantagonistic bHLH system. *Plant Signal Behav* **8**: e23448 **CrossRef** **PubMed** **Google Scholar**

↲Ikeda M, Ohme-Takagi M (2009) A novel group of transcriptional repressors in Arabidopsis. *Plant Cell Physiol* **50**: 970–975 **Abstract/FREE Full Text** **Google Scholar**

↲Jang S, An K, Lee S, An G (2002) Characterization of tobacco MADS-box genes involved in floral initiation. *Plant Cell Physiol* **43**: 230–238 **Abstract/FREE Full Text** **Google Scholar**

↲Jang S, Marchal V, Panigrahi KCS, Wenkel S, Soppe W, Deng X-W, Valverde F, Coupland G (2008) Arabidopsis COP1 shap the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *EMBO J* **27**: 1277–1288 **Abstract/FREE Full Text** **Google Scholar**

↲Jang S, Torti S, Coupland G (2009) Genetic and spatial interactions between FT, TSF and SVP during the early stages of flor induction in Arabidopsis. *Plant J* **60**: 614–625 **CrossRef** **PubMed** **Google Scholar**

↲Jang S, Choi S-C, Li H-Y, An G, Schmelzer E (2015) Functional characterization of *Phalaenopsis aphrodite* flowering genes PaFT1 and PaFD. *PLoS One* **10**: e0134987 **Google Scholar**

↲Jefferson RA (1989) The GUS reporter gene system. *Nature* **342**: 837–838 **CrossRef** **PubMed** **Google Scholar**

↲Jeon J-S, Lee S, Jung K-H, Jun S-H, Jeong D-H, Lee J, Kim C, Jang S, Yang K, Nam J, et al. (2000) T-DNA insertional mutagenesis for functional genomics in rice. *Plant J* **22**: 561–570 **CrossRef** **PubMed** **Google Scholar**

↲Jeong D-H, An S, Kang H-G, Moon S, Han J-J, Park S, Lee HS, An K, An G (2002) T-DNA insertional mutagenesis for activati tagging in rice. *Plant Physiol* **130**: 1636–1644 **Abstract/FREE Full Text** **Google Scholar**

↲Jeong D-H, An S, Park S, Kang H-G, Park G-G, Kim S-R, Sim J, Kim Y-O, Kim M-K, Kim S-R, et al. (2006) Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. *Plant J* **45**: 123–132 **CrossRef** **PubMed** **Google Scholar**

↲Jeong D-H, Lee S, Kim SL, Hwang I, An G (2007) Regulation of brassinosteroid responses by phytochrome B in rice. *Plant Ce Environ* **30**: 590–599 **PubMed** **Google Scholar**

↲Kim S-R, Lee D-Y, Yang J-I, Moon S, An G (2009) Cloning vectors for rice. *J Plant Biol* **52**: 73–78 **CrossRef** **Google Scholar**

↲Ko S-S, Li M-J, Sun-Ben Ku M, Ho Y-C, Lin Y-J, Chuang M-H, Hsing H-X, Lien Y-C, Yang H-T, Chang H-C, Chan M-T (2014) The bHLH142 transcription factor coordinates with TDR1 to modulate the expression of EAT1 and regulate pollen developme in rice. *Plant Cell* **26**: 2486–2504 **Abstract/FREE Full Text** **Google Scholar**

↲Komatsu K, Maekawa M, Ujiie S, Satake Y, Furutani I, Okamoto H, Shimamoto K, Kyozuka J (2003) LAX and SPA: major regulators of shoot branching in rice. *Proc Natl Acad Sci USA* **100**: 11765–11770 **Abstract/FREE Full Text** **Google Scholar**

↲Komiya R, Ikegami A, Tamaki S, Yokoi S, Shimamoto K (2008) Hd3a and RFT1 are essential for flowering in rice. *Dev* **135**: 767–774 **Abstract/FREE Full Text** **Google Scholar**

↲Lee J, Park J-J, Kim SL, Yim J, An G (2007) Mutations in the rice liguleless gene result in a complete loss of the auricle, ligule, and laminar joint. *Plant Mol Biol* **65**: 487–499 **CrossRef** **PubMed** **Google Scholar**

↲Lee S, Choi SC, An G (2008) Rice SVP-group MADS-box proteins, OsMADS22 and OsMADS55, are negative regulators of brassinosteroid responses. *Plant J* **54**: 93–105 **CrossRef** **PubMed** **Google Scholar**

↲Lee S, Lee S, Yang K-Y, Kim Y-M, Park S-Y, Kim SY, Soh M-S (2006) Overexpression of PRE1 and its homologous genes activates gibberellin-dependent responses in *Arabidopsis thaliana*. *Plant Cell Physiol* **47**: 591–600 **Abstract/FREE Full Text** **Google Scholar**

↲Nakamura A, Fujioka S, Takatsuto S, Tsujimoto M, Kitano H, Yoshida S, Asami T, Nakano T (2009) Involvement of C-22-hydroxylated brassinosteroids in auxin-induced lamina joint bending in rice. *Plant Cell Physiol* **50**: 1627–1635 **Abstract/FREE Full Text** **Google Scholar**

Ning J, Zhang B, Wang N, Zhou Y, Xiong L (2011) Increased leaf angle1, a Raf-like MAPKKK that interacts with a nuclear protein family, regulates mechanical tissue formation in the lamina joint of rice. *Plant Cell* **23**: 4334–4347 Abstract/FREE Full Text Google Scholar

Reddy AS, Day IS, Göhring J, Barta A (2012) Localization and dynamics of nuclear speckles in plants. *Plant Physiol* **158**: 67-77 FREE Full Text Google Scholar

Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S et al. (2006) Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. *Nat Biotechnol* **24**: 105–109 CrossRef PubMed Google Scholar

Serna L (2007) bHLH proteins know when to make a stoma. *Trends Plant Sci* **12**: 483–485 CrossRef PubMed Google Scholar

Shimada A, Ueguchi-Tanaka M, Sakamoto T, Fujioka S, Takatsuto S, Yoshida S, Sazuka T, Ashikari M, Matsuoka M (2006) Th rice SPINDLY gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. *Plant J* **48**: 390–402 CrossRef PubMed Google Scholar

Tanaka A, Nakagawa H, Tomita C, Shimatani Z, Ohtake M, Nomura T, Jiang C-J, Dubouzet JG, Kikuchi S, Sekimoto H, et al. (2009) BRASSINOSTEROID UPREGULATED1, encoding a helix-loop-helix protein, is a novel gene involved in brassinosterc signaling and controls bending of the lamina joint in rice. *Plant Physiol* **151**: 669–680 Abstract/FREE Full Text Google Scholar

Tzfira T, Tian G-W, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. *Plant Mc Biol* **57**: 503–516 CrossRef PubMed Google Scholar

Van Camp W (2005) Yield enhancement genes: seeds for growth. *Curr Opin Biotechnol* **16**: 147–153 CrossRef PubMed Google Scholar

Wada K, Marumo S, Ikekawa N, Morisaki M, Mori K (1981) Brassinolide and homobrassinolide promotion of lamina inclination rice seedlings. *Plant Cell Physiol* **22**: 323–325 Abstract/FREE Full Text Google Scholar

Wang D, Zhang H, Hu G, Fu Y, Si H, Sun Z (2005) Genetic analysis and identification of alarge leaf angles (lla) mutant in rice. *Chin Sci Bull* **50**: 492–494 Google Scholar

Wang H, Zhu Y, Fujioka S, Asami T, Li J, Li J (2009) Regulation of Arabidopsis brassinosteroid signaling by atypical b loop-helix proteins. *Plant Cell* **21**: 3781–3791 Abstract/FREE Full Text Google Scholar

Wang L, Xu Y, Zhang C, Ma Q, Joo S-H, Kim S-K, Xu Z, Chong K (2008) OsLIC, a novel CCCH-type zinc finger protein with transcription activation, mediates rice architecture via brassinosteroid signaling. *PLoS One* **3**: e3521 CrossRef PubMed Google Scholar

Wu CY, Trieu A, Radhakrishnan P, Kwok SF, Harris S, Zhang K, Wang J, Wan J, Zhai H, Takatsuto S, et al. (2008) Brassinosteroids regulate grain filling in rice. *Plant Cell* **20**: 2130–2145 Abstract/FREE Full Text Google Scholar

Wu F-H, Shen S-C, Lee L-Y, Lee S-H, Chan M-T, Lin C-S (2009) Tape-Arabidopsis Sandwich - a simpler Arabidopsis protopla isolation method. *Plant Methods* **5**: 16 CrossRef PubMed Google Scholar

Yang Q, He X, Yang L, Zhou Z, Cullinane AR, Wei A, Zhang Z, Hao Z, Zhang A, He M, et al. (2012) The BLOS1-interacting protein KXD1 is involved in the biogenesis of lysosome-related organelles. *Traffic* **13**: 1160–1169 CrossRef PubMed Google Scholar

Yin Y, Vafeados D, Tao Y, Yoshida S, Asami T, Chory J (2005) A new class of transcription factors mediates brassinosteroid-regulated gene expression in Arabidopsis. *Cell* **120**: 249–259 CrossRef PubMed Google Scholar

Zhang L-Y, Bai M-Y, Wu J, Zhu J-Y, Wang H, Zhang Z, Wang W, Sun Y, Zhao J, Sun X, et al. (2009) Antagonistic HLH/bHLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and Arabidopsis. *Pl Cell* **21**: 3767–3780 Abstract/FREE Full Text Google Scholar

Zhang X, Sun J, Cao X, Song X (2015) Epigenetic mutation of RAV6 affects leaf angle and seed size in rice. *Plant Physiol* **169** 2118–2128 Abstract/FREE Full Text Google Scholar

Zhang Y, Su J, Duan S, Ao Y, Dai J, Liu J, Wang P, Li Y, Liu B, Feng D, Wang J, Wang H (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods* **7**: 30 Google Scholar

Zhao S-Q, Hu J, Guo L-B, Qian Q, Xue H-W (2010) Rice leaf inclination2, a VIN3-like protein, regulates leaf angle through modulating cell division of the collar. *Cell Res* **20**: 935–947 CrossRef PubMed Google Scholar

Zhao S-Q, Xiang J-J, Xue H-W (2013) Studies on the rice LEAF INCLINATION1 (LC1), an IAA-amido synthetase, reveal the effects of auxin in leaf inclination control. *Mol Plant* **6**: 174–187 CrossRef PubMed Google Scholar

View Abstract

Previous Next

Back to top

Table of Contents

Print
Download PDF
Article Alerts
Email Article
Citation Tools
Request Permissions

Share
Tweet
Like 0

PDF
Help

We recommend

Antagonistic HLH/bHLH Transcription Factors Mediate Brassinosteroid Regulation of Cell Elongation and Plant Developme in Rice and Arabidopsis
Xuehui Sun et al., Plant Cell

Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint.
C Yamamuro et al., Plant Cell

So Inclined: Phosphate Status and Leaf Angle in Rice
Jennifer Mach, Plantae

A tripartite growth regulatory cascade of basic helix-loop-helix transcription factors.
Nancy R Hofmann, Plant Cell

Helix–loop–helix/basic helix–loop–helix transcription factor network represses cell elongation in Arabidopsis through an apparent incoherent feed-forward loop
Miroslava K. Zhiponova et al., Proc Natl Acad Sci U S A

The pleiotropic ABNORMAL FLOWER AND DWARF1 affects plant height, floral development and grain yield in rice
Deyong Ren et al., Journal of Integrative Plant Biology

Shade-induced stem elongation in rice seedlings: Implication of tissue-specific phytohormone regulation
Huihui Liu et al., Journal of Integrative Plant Biology

Tumor clonality and resistance mechanisms in EGFR mutation-positive non-small-cell lung cancer
Future Oncology

In this issue

Plant Physiology
Vol. 173, Issue 1
Jan 2017
Table of Contents
Table of Contents (PDF)
Cover (PDF)
About the Cover
Index by author

More in this TOC Section

Similar Articles

Our Content

Home
Current Issue
Plant Physiology Preview

Archive
Focus Collections
Classic Collections
The Plant Cell
Plant Direct
Plantae
ASPB

For Authors

Instructions
Submit a Manuscript
Editorial Board and Staff
Policies
Recognizing our Authors

For Reviewers

Instructions
Journal Miles
Policies

Other Services

Permissions
Librarian resources
Advertise in our journals
Alerts
RSS Feeds