Dynamics of Brassinosteroid Response Modulated by Negative Regulator LIC in Rice

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Abstract

Brassinosteroids (BRs) regulate rice plant architecture, including leaf bending, which affects grain yield. Although BR signaling has been investigated in Arabidopsis thaliana, the components negatively regulating this pathway are less well understood. Here, we demonstrate that Oryza sativa LEAF and TILLER ANGLE INCREASED CONTROLLER (LIC) acts as an antagonistic transcription factor of BRASSINAZOLE-RESISTANT 1 (BZR1) to attenuate the BR signaling pathway. The gain-of-function mutant lic-1 and LIC-overexpressing lines showed erect leaves, similar to BZR1–depleted lines, which indicates the opposite roles of LIC and BZR1 in regulating leaf bending. Quantitative PCR revealed LIC transcription rapidly induced by BR treatment. Image analysis and immunoblotting showed that upon BR treatment LIC proteins translocate from the cytoplasm to the nucleus in a phosphorylation-dependent fashion. Phosphorylation assay in vitro revealed LIC phosphorylated by GSK3–like kinases. For negative feedback, LIC bound to the core element CTCGC in the BZR1 promoter on gel-shift and chromatin immunoprecipitation assay and repressed its transcription on transient transformation assay. LIC directly regulated target genes such as INCREASED LEAF INCLINATION 1 (ILI1) to oppose the action of BZR1. Repression of LIC in ili1 transcription in protoplasts was partially rescued by BZR1. Phenotypic analysis of the crossed lines depleted in both LIC and BZR1 suggested that BZR1 functionally depends on LIC. Molecular and physiology assays revealed that LIC plays a dominant role at high BR levels, whereas BZR1 is dominant at low levels. Thus, LIC regulates rice leaf bending as an antagonistic transcription factor of BZR1. The phenotypes of lic-1 and LIC-overexpressing lines in erect leaves contribute to ideal plant architecture. Improving this phenotype may be a potential approach to molecular breeding for high yield in rice.

Introduction

Brassinosteroids (BRs) are plant steroid hormones that have been used to increase the yield of crops [1,2]. BRs function in multiple developmental and physiological processes, including vascular differentiation, reproductive development, photomorphogenesis, and stress responses [3–5]. BR-deficient and -insensitive mutants show dwarfism, dark-green leaves, reduced fertility, and altered photomorphogenesis in the dark [6–9]. In rice (Oryza sativa), leaf-angle response to BRs is a specific physiological process. For example, the erect leaves of BR-deficient rice allow for greater growth density and higher grain yield [10]. Thus, analysis of genes involved in rice BR signaling could shed light on the molecular mechanisms of BR-regulated growth in monocots and help identify feasible approaches to increase rice yield by genetic engineering.

The BR signaling pathway has been well studied in Arabidopsis. Most of the signaling components of this pathway, from the BR receptor BRI1 and co-receptor BAK1 to nuclear transcription factors BZR1 and BES1/BZR2, have been identified [11,12]. During the early events of BR signaling, BRI1 perceives BRs, thus inducing dissociation of the inhibitory protein BK11, which results in association with and transphosphorylation of the co-receptor BAK1 [13–17]. BR signal kinases (BSKs) mediate signal transduction from BRI1 to BSU1 phosphatase through association with and phosphorylation of BSU1 [18]. BSU1 positively regulates BR signaling by dephosphorylating the negative regulator BR-insensitive 2 (BIN2). This process facilitates accumulation of unphosphorylated BZR1 and BES1/BZR2 in the nucleus [19–23], which directly or indirectly activate the expression of BR-responsive genes and regulate plant growth [21,24,25]. BZR1 is also responsible for the negative feedback of BR biosynthetic genes such as CPD by directly repressing transcription [26]. BZR1 and BES1 are major transcription factors in the BR signaling pathway [27]. BZR1 binds to the BR-responsive element (BRRE, CGTTGT/CG) and mainly represses gene expression. BES1 binds to E-box by interacting with BIM1 or MYB30 to promote target gene expression [28–30]. BZR1 could also bind to E-box and BES1 to BRRE, so the functions of the family members may overlap [31,32]. These are key transcription factors activating the BR signaling pathway in plants. Phosphatase 2A (PP2A) dephosphorylates BZR1 and also BRI1 in mediating BR signaling. BRI1 degradation depends on PP2A–mediated dephosphorylation that is specified by methylation of the phosphatase, thus
Brassinosteroids (BRs) are phytohormones mediating multiple biological processes, such as development and stress response. They have been used in crops to produce high yield. In rice, the ideal plant architecture for high yield includes effective tillers, as well as height and leaf angle, which is modulated by BRs. Activation of BR1–mediated BR signaling is well understood, but much less is known about its inactivating mechanism. Here, we found a gain-of-function mutant lic-1 with the phenotype of the ideal plant architecture. The C3H-type transcription factor LIC antagonizes BZR1 to repress BR signaling in rice. We used BR to induce the negative regulator LIC and found that it functioned at high BR level, which may restrain plant development. LIC was phosphorylated by GSK3-like kinases. Phosphorylated LIC mainly localized in cytoplasm, whereas dephosphorylated LIC was in nucleus, which was regulated by BR treatment. LIC regulated transcription patterns of the downstream genes in an opposite direction to BZR1. BZR1 activated BR signaling, but the brake module of LIC repressed BR cascade amplification. LIC and BZR1 may balance BR signaling to control growth and development in rice.

leading to the termination of BR signaling [33–35]. However, how BR signals are repressed at the transcriptional level to elicit a “turn-off” pathway is less well known.

Rice, as a model monocot plant and one of the major crops, has been used to study the BR action mechanism. Both rice and Arabidopsis share primary BR biosynthesis and signal transduction pathways. A series of Arabidopsis orthologs of biosynthetic genes identified in rice include D2, D11, BRD1, BRD2, and CPD [36–38]. However, only a few members in the BR primary signaling pathway have been reported [39–41]. OsBR1 and OsBAK1 are cell-surface receptor kinases that perceive BR signals. Os GLYCOGEN SYNTHASE KINASE 1 (OsGSK1), an ortholog of AtBIN2, is a negative regulator of rice BR signaling. Although the direct targets of BZR1 and BES1 have been identified in Arabidopsis, only a few targets have been identified in rice [31,32]. The transcription factor OsBZR1, the closest ortholog of both BZR1 and BES1, has similar functions as its Arabidopsis orthologs [42]. OsBZR1 translocated from the cytoplasm to the nucleus in response to BR treatment in a process mediated by 14-3-3 proteins [42–44]. A pair of antagonizing HLH/bHLH factors, INCREASED LEAF INCLINATION (II1) and II1 BINDING bHLH (IB1), function downstream of OsBZR1 to regulate cell elongation and lamina joint bending [45]. These studies suggest a conserved BR signaling mechanism in rice and Arabidopsis. BZR1 is a key component of the transcription pathway that activates BR signaling in both species. However, how to halt BR signaling at the node of transcription factors including BZR1 remains unclear.

A CCCH-type zinc finger protein, LEAF AND TILLER ANGLE INCREASED CONTROLLER (LIC) is involved in sterol homeostasis in rice [10]. Here, we studied the phenotypes of a rice LIC gain-of-function mutant lic-1 and LIC-overexpressing rice lines to explore a novel mechanism of BR signaling. Both groups showed erect leaves and reduced BR sensitivity as compared with antisense lines. LIC was further characterized as an antagonistic transcription factor of BZR1 in regulating rice architecture. Furthermore, LIC is phosphorylated by GSK1/BIN2 (GSK3-like kinases), which affect translocation from the nucleus to cytoplasm. LIC may mediate a novel mechanism that represses the BR signaling pathway.

Author Summary

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Results

LIC Truncation Results in Erect Leaves in Rice

A T-DNA insertion line of lic-1 was obtained from the Rice Mutant Database (http://rmmd.ncgr.cn) [46]. Molecular analysis revealed that the T-DNA was inserted in the eighth exon near the 3’ terminus of LIC and was predicted to cause the deletion of 110 amino acids (Figure 1A and Figure S1A). The inserted gene encodes a truncated LIC protein containing the CCCH DNA binding domain, EELR activation domain and the putative phosphorylation sites (Figure S1B and S1C).

Segregation analysis of the heterozygous lic-1 with molecular evidence revealed an approximate 3:1 (76/24) ratio of lic-1 mutants to the wild-type, which indicates that lic-1 is a dominant mutant. The crossed progenies of lic-1 and the LIC antisense lines showed increased leaf angles that were similar to those of the antisense lines (Table S1). As compared with wild type, the lic-1 line showed reduced leaf angles from tillering stage (Figure S2A and S2B). All LIC-overexpressing lines also showed erect leaves at tillering stage (Figure S2A, Figure S3, and Table S2). During the seedling stage, the wild type and lic-1, as well as overexpression lines, did not differ in leaf angle. Therefore, the phenotype of the lic-1 mutant was consistent with the LIC-overexpression lines in terms of leaf angle.

BR biosynthetic genes D2 and D11 had repressed expression in antisense lines. In contrast, the expression of the biosynthetic gene BRD1, as well as the receptor gene BRRI1, was enhanced in lic-1 (Figure S4).

In rice, the physiologic processes of leaf bending and root growth are sensitive to BR [47,48]. In the wild type (WT), increased leaf angle depended on the concentration of BR (Figure 1B and 1C). The overexpressing lines and lic-1 showed reduced dependence on BR concentration in leaf bending. In contrast, the antisense line was more sensitive to BR dosage than the WT. In the antisense lines, the root growth patterns in response to 24-eBL (an active form of BR) were similar to leaf angle patterns (Figure S5A and S5B). Thus, LIC overexpression reduced the BR response, and LIC depletion caused hypersensitivity to BR in terms of leaf bending and root growth. Therefore, LIC may negatively regulate BR signaling in rice.

OsLIC is a Direct Target of OsBZR1

Bioinformatics analysis revealed the BZR1 binding site BRRE (CGGTGTCG) [26] present in the promoter of LIC (Figure 2A). EMSA was used to examine BZR1 binding to the cis-elements in the LIC promoter in vitro. When the purified BZR1 protein was incubated with the reaction mixture, a shifted band appeared in the upper part of the gel but not in the control MBP. The greater the amount of BZR1 in the incubation, the greater the amount of shifted band on the gel. When the competitive unlabeled probe (Co) was added to the system, the shifted band was suppressed. In contrast, neither mutated P1 (MP1, CGAAAA) nor P2 (CGTGTG) shifted under the same conditions (Figure 2B). We performed chromatin immunoprecipitation (ChIP) assay with WT rice (Figure S9). Real-time PCR revealed a fragment of the LIC promoter containing the P1 binding element significantly enriched as compared with the reference gene promoter (UBQ5) and control fragments (P2, P3 and P4; Figure 2C). In the RNAi lines of BZR1, LIC transcription was increased (Figure 2D). Thus, BZR1 binds to the cis-element in the LIC promoter, and knockdown of BZR1 leads to upregulation of LIC.

We crossed the BZR1 RNAi lines with erect leaves to the LIC antisense lines with increased leaf bending to explore the genetic relationship of the lines. By molecular identification (Figure S6A and S6B), phenotypic analysis revealed an increased leaf bending...
phenotype in the progenies, which was similar to that of the LIC antisense lines (Figure 2E and 2F). Therefore, BZR1 may functionally depend on LIC in terms of genetics.

LIC Is a Substrate of GSK3–Like Kinases, the Rice Orthologs of AtBIN2

Transformed LIC-GFP fusion protein was used to investigate subcellular localization. With BR treatment, GFP-tagged LIC was rapidly weakened in the cytoplasm within 30 min but was enhanced in the nucleus (Figure 3Aa and b). The ratio of GFP-tagged LIC in the nucleus to that in the cytoplasm (N/C ratio) was significantly increased with BR treatment. Although LICm, mimicking the C-terminus-truncated protein, was distributed in the nucleus and cytoplasm, the cytoplasmic signal of LICm was clearly weaker than that of intact LIC (Figure 3Ac and d; 3C). Digital signal assay demonstrated a lower ratio of LIC than truncated LICm in the nucleus. The LICm pattern showed a similar increased N/C ratio in response to BR treatment. In contrast, the truncated protein LICp, lacking the putative phosphorylation sites (designated P site in Figure 1A and Figure 3C) was localized only in the nucleus (Figure 3B). Western blot analysis revealed a greater LIC band in the nucleus of lic-1 as compared with the WT. Intensity of the nuclear band was enhanced by treatment with 24-eBL (1 μM) for both lic-1 and the WT. At the total protein level, the signal intensity of LIC in the WT and lic-1 was not significantly different after treatment (Figure 3D). Thus, the translocation of LIC from the cytoplasm to the nucleus may be regulated by BR treatment and depend on the phosphorylation status of LIC.

The GSK3-like kinase BIN2 phosphorylates BZR1 through the conserved GSK3 kinase phosphorylation sites (S/Txxxs/T) and promotes its cytoplasmic retention in Arabidopsis [49]. In rice, whole-genome screening analysis revealed two putative orthologs of BIN2, OsGSK1 and OsSKETHA [43]. Yeast two-hybrid assay revealed that LIC but not forms LICm and LICp interact with GSK1 and SKETHA, as well as AtBIN2.

The mutated form did not interact with them (Figure S7). Western blot analysis revealed that LIC was two bands and the larger one was increased by incubation with BIN2. Furthermore, the intensity of the larger band was reduced by the addition of λ-phosphatase 1 (Figure 4A), which agreed with the prediction that 5 typical phosphorylation sites of GSK3-like kinases (S/Txxxs/T) were deposited in the P site domain of LIC protein.

When plants were treated with 24-eBL (1 μM), the phosphorylated form of LIC was suppressed (Figure 4B), whereas the dephosphorylated form was increased. The P-LICm was weaker than that for LICm (Figure 4C). Western blot revealed dephosphorylated LIC accumulated in the nuclear fraction, with the phosphorylated form mainly in the cytoplasm (Figure 4D).

Transformed cells with GFP-tagged LIC showed the N/C ratio of LIC with digital fluorescence signals was 3.0 (Figure 5A and 5B). The nuclear distribution of LIC was enhanced with 24-eBL treatment. In contrast, in cells co-transformed with both GSK1 and LIC, less of LIC localized in the nucleus. Western blot analysis demonstrated that phosphorylated LIC was upregulated after incubation with GSK1 but downregulated with λ-phosphatase 1 (Figure 5C). This result suggested that GSK1 phosphorylated LIC, which might repress its localization in the nucleus.

LIC Regulates BZR1 in a Negative Feedback Loop

Expression pattern assay demonstrated BZR1 and LIC with overlapping and distinct expression patterns in different organs (Figure S10). The expression of LIC was distributed from the abaxial to adaxial sides in leaves. In contrast, the expression of BZR1 was dominant in the abaxial sides of leaves (Figure 6A).

The effect of BR on LIC transcription showed repression at low (1 nM) and activation at high (>100 nM; Figure 6B) 24-eBL concentrations. This matches the phenotype of root growth (Figure S5C). The peak of LIC transcription occurred with 1 μM 24-eBL. In contrast, the mRNA level of BZR1 was increased with low levels of 24-eBL (1 nM) and decreased with high levels (>100 nM;
Figure 6B). Western blot analysis demonstrated that the pattern of LIC protein level was similar to the mRNA pattern with low levels of 24-eBL. However, the reduced negative-peak occurred with a higher 24-eBL concentration (100 nM) than for the RNA (1 nM). Additionally, the increased protein expression was sustained with up to 10 μM 24-eBL (Figure 6C). Thus, LIC expression may be downregulated by a low level of BR but upregulated by higher concentrations.

In the mutant d2, BR deficiency caused LIC expression reduced to only 20% the WT level. BZR1 depletion resulted in increased LIC expression (Figure 6D). Time-course assay revealed LIC expression gradually increased from 15 min up to 3 h during BR treatment (1 μM 24-eBL) (Figure 6E). Thus, LIC expression may be downregulated by a low level of BR but upregulated by higher concentrations.

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Figure 2. LIC promoter is targeted to the BZR1 protein. (A) A diagram of the LIC promoter containing BZR1 binding site (black circles): CGTGCG. White rings represents the sequence CGTG. Black lines P1–4 indicate the sequences tested in ChIP assays. P1 contained CGTGCG and P2 contained CGTG. But both elements were absent in P3 and P4. (B) Gel shift assay with BZR1 protein and the fragment sequences of the LIC promoter. The arrow indicates shifted bands caused by BZR1 binding to the LIC promoter P1 (CGTGCG). The unlabeled P1 was a competitive probe (Co). BZR1 could not bind to P2 (CGTG) or mutated P1 (MP1, CGAAAA). MBP was a negative control. (C) ChIP assay revealed BZR1 enriched the LIC promoter fragment containing P1 in vivo. Data are mean ± SD (n = 3). (D) Phenotypes of the progeny of BZR1 R X AS2 and the parent lines BZR1R and AS2, as well as the wild-type in (E). Leaf angles were averaged in 15 plants. Data are mean ± SE. doi:10.1371/journal.pgen.1002686.g002
LIC may be a primary transcription factor targeting OsBZR1 to regulate the BR signaling pathway.

LIC and BZR1 Function Antagonistically in Regulating Downstream Genes

To determine the potential antagonistic functions of both genes, we analyzed the expression patterns of their potential downstream genes. BZR1 mainly binds IBH1 to affect the balance of a pair of antagonistic HLH/bHLH transcription factors ILI1 and IBH1 in rice [45]. In an LIC-depleted line (AS2), ILI1 expression was higher than in the WT, whereas IBH1 transcription was not significantly altered (Figure 8A). In an LIC-depleted line (AS2), ILI1 expression was higher than in the WT, whereas IBH1 transcription was not significantly altered (Figure 8A). The core motif sequence CTCGC of LIC target was present as a glomerate pattern in ILI1 but as a sparse pattern in IBH1. EMSA data indicated that the fragment containing the sequence B2 in ILI1 strongly bound to LIC. In contrast, the signal of C3 in IBH1 with a single core element was weaker (Figure 8B). ChIP analysis of the potential target ILI1 after BR treatment in the WT demonstrated significant changes (>2.5-fold) in binding in diverse regions such as a, d, e, f, and n, but not in regions such as b, c, h, i, and l (Figure 8C). IBH1 exhibited a similar pattern as ILI1 on ChIP analysis, but the copy number of the core element on the IBH1 fragments, such as c and k, was much lower than that for ILI1 (Figure 8D). Unexpectedly, the change appeared in the region without the core motif such as j, so other unknown motifs may be involved. To further explore the potential activity of the transcription factor with its targets ILI1 and IBH1, we used a protoplast transfection assay. LIC repressed the expression of ILI1pro:LUC but activated that of IBH1pro:LUC (Figure 8E). Competitive binding assay showed that the repression activity of LIC on ILI1 was weakened by co-expression of BZR1 (Figure 8F). Thus, LIC dominantly repressed ILI1 expression and weakly bound to IBH1 to enhance expression to balance the regulation activity of BZR1.
BZR1 mainly targets IBH1

Treatment with BR (1 spherated LIC relative to the control without BIN2 and PP1 (-P%) were anti-LIC antibody in the top panel. The amount of protein is shown with phosphorylation status of LIC is illustrated by autoradiography of an

Figure 4. LIC is phosphorylated by BIN2/GSK1. (A) Immunoblotting analysis to demonstrate that LIC was phosphorylated by BIN2. LIC phosphorylation was antagonized by λ-phosphatase 1 (PP1). The phosphorylation status of LIC is illustrated by autoradiography of an anti-LIC antibody in the top panel. The amount of protein is shown with Coomassie Blue staining in the bottom panel. The levels of unphosphorylated LIC relative to the control without BIN2 and PP1 (P%) were calculated after normalization against the intensity of Coomassie Blue staining, and these values are shown beneath the gel images. (B) Treatment with BR (1 μM) decreased the levels of phosphorylated LIC and increased that of unphosphorylated LIC. Rice plants were grown for 2 weeks and then soaked with 1 μM 24-eBL (+) or mock solution (−) for 3 h. LIC protein was analyzed by immunoblotting with an anti-LIC antibody (upper panel). The loading control with Coomassie Blue staining is shown in the bottom panel. (C) The mutated protein LICm caused decreased phosphorylation in the lic-1 mutant. The 24-eBL concentration was 1 μM. (D) Immunoblotting assay for LIC protein in the nuclear and cytoplasmic fractions. Dephosphorylated LIC was dominant in the nucleus (N), and phosphorylated forms were dominant in the cytoplasm. Nuclear and cytoplasmic protein fractions were extracted from 2-week-old rice seedlings. Histone 3 was a marker for the nuclear protein and β-actin for the cytoplasmic protein.

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Discussion

In this study, we identified LIC as a negative regulator of BZR1 to halt BR signaling to control leaf angle in rice. LIC antagonizes BZR1 by repressing its transcription in leaf bending (Figure 9). Like BZR1, the transcription factor LIC is phosphorylated by GSK1/ BIN2. LIC and BZR1 are a pair of antagonistic transcription factors that repress each other during transcription. However, their repression strength may depend on BR level. The gain-of-function mutant *lic-1* showed that LIC repressed *BZR1* transcription and leaf bending, which may mimic a BR signaling balance to switch on the “brake.” The transcriptional expression of LIC and its protein accumulation in the nucleus were induced by BR. LIC dominantly binds to *BZR1* and its target *ILI1* and weakly to *IBH1*. In contrast, *BZR1* mainly targets *IBH1* to affect the balance of a pair of antagonistic HLH/bHLH transcription factors, except to bind LIC. The “seesaw” mechanism of the antagonistic function may work at various BR levels during plant development. *BZR1* may function at a low level to promote signaling and LIC at higher levels for braking. Our data suggest that LIC is a component of the BR signaling pathway and mediates a novel braking module that represses BR signaling to control plant development.

LIC Is A Major Negative Regulator Mediating Signaling from GSK3 Kinases

Leaf bending is a specific phenotypic response to BR in rice [47]. LIC-depleted rice lines show increased leaf bending, which mimics the phenotypes of enhanced responses to BR, such as the OsBAK1-overexpressing lines [40]. Consistently, the gain-of-function mutant *lic-1* and overexpressing lines show erect leaves similar to OsBZR1- and OsBAK1-depleted lines. Reverse expression patterns of downstream genes such as *ILI1* and *IBH1* were found in the silenced lines of *BZR1* and LIC. Similarly like BZR1, LIC acts in an early BR response, because its expression was induced by BR treatment within 15 min. BR-induced LIC accumulation in the nucleus was a rapid response to BR and acts as an upstream component in BR signaling. Therefore, LIC functions negatively in the BR-mediated regulation of leaf bending.

LIC, with phosphorylation sites of GSK1/BIN2 kinases, interacts with BIN2 and its rice ortholog GSK1 and SKETHA and could be phosphorylated. The C-terminus-truncated LICm, as well as P-site-truncated LICp, show decreased interaction with GSK1/BIN2 and consequently display lower phosphorylation levels and greater accumulation in the nucleus to constitutively regulate downstream genes. The shuttle of LIC between the nucleus and cytoplasm was regulated by BR treatment and might depend on the phosphorylation status of LIC. This shuttle localization pattern depended on BIN2/GSK1, as seen with BZR1/BES1 [23,42,50]. Our results suggest that LIC directly mediates BR signaling from GSK3 kinases.

LIC Antagonizes BZR1 in Rice Leaf Bending

*BZR1* is a key component positively regulating BR signaling, whereas LIC plays a negative role in the signaling pathway. With BR treatment, LIC and BZR1 accumulate in the nucleus and regulate downstream genes. BZR1 represses BR-downregulated genes through the downregulation of BIN2 phosphorylation and decreases cytoplasmic retention mediated by 14-3-3 proteins during BR-mediated induction [42,50]. BZR1 and BES1 gain-of-function mutants in *Arabidopsis* are hypersensitive to BR. Depleted AtBES1 leads to reduced BR sensitivity [51]. The RNAi lines of *OsBZR1* are insensitive to BR and show erect leaves [42]. Our genetic analysis and molecular data suggested that LIC and BZR2 work on rice leaf bending in a genetic pathway, but their roles are opposite to each other. EMSA and ChIP data, as well as transcription assay data, indicated that the BZR1 protein directly represses LIC expression via the specific BRRE motif (CTGGCG). We found that LIC protein recognizes the BZR1 gene through the core element (CTCGG) to repress its transcription. The expression of both genes may be induced by BR treatment at various concentrations. BR treatment at low concentrations (10⁻⁷ M) induced the expression of BZR1 and promoted the dephosphorylation of BZR1 protein as an activation mechanism. However, BR treatment at high concentrations (up to 10⁻⁵ M) induced LIC expression. The repression of BZR1 transcriptional expression by LIC is enhanced by high BR levels. Therefore, LIC and BZR1 antagonize each other in controlling BR-mediated leaf bending.

LIC, with only one CCCH domain, binds DNA or RNA in vitro (Figure S1) [10]. It prefers to recognize the core sequence CTGGC, which is present in genes such as *BZR1* and *ILI1*, to regulate BR.
signaling in rice. Our finding of the motif binding to LIC with specificity will provide new insights into this family.

BZR1 is a transcription factor that represses the expression of downstream genes such as OsIBH1, which is responsible for leaf bending. ILI1/PRE1 and IBH1 promote or repress cell elongation downstream of BZR1 in rice and Arabidopsis [45,52]. Overexpression of ILI1 causes increased leaf bending, whereas overexpression of IBH1 results in erect leaves in rice. EMSA and ChIP results suggested that LIC greatly represses ILI1, the positive partner of OsIBH1. As well, LIC weakly binds to OsIBH1 promoter to enhance its transcriptional expression. This pattern is similar to BZR1 weakly binding to the promoter of ILI1, which is induced by BR [45]. In regulating downstream genes, LIC may play a major role in repressing positive regulators such as ILI1, and BZR1 may function to repress negative regulators such as IBH1. Therefore, a novel negative regulation module of BR signaling is parallel to and antagonizes the BZR1 signaling pathway to regulate leaf bending. In plant development, LIC and BZR1 show various spatial and temporal expression patterns. BZR1 acts in the presence of low levels of BR, whereas LIC is predominantly activated by high levels of BR and antagonizes BZR1 to prevent intense activation of the BR cascade. The novel negative regulation module of LIC and the positive one of BZR1 in mediating leaf bending may help in designing ideal plant architecture for improving photosynthesis efficiency during rice development. The approach may have potential in rice molecular breeding for high yield.

Materials and Methods

Plant Materials and Growth Conditions

Rice (Oryza sativa ssp. japonica var. Zhonghua 10) plants were grown in the field or in the greenhouse at 30°C/25°C (day/night) cycles. For the analysis of BR induction in leaf bending and root growth, rice seeds were sterilized with 1% NaClO and grown in half-strength Murashige and Skoog (MS) medium with the indicated concentrations of 24-eBL (Sigma-Aldrich, St. Louis, MO, USA) at 30°C under continuous light. Seedlings were examined 7 days after germination. For every transgenic rice plant, 3 lines were used.

Leaf-Bending Assay

Sterilized seeds were grown for 8 days in a dark chamber. Uniform seedlings were then sampled by excising segments of approximately 2 cm that contained the second-leaf lamina joint under dim light conditions. These were floated on distilled water containing various concentrations of 24-eBL. After incubation in a dark chamber at 30°C for 72 h, the angle between the lamina and the sheath was measured [47].

Total RNA Isolation and Quantitative RT–PCR Analysis

Total RNA was extracted from 2-week-old seedlings by using the Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). The first-strand cDNAs were synthesized by use of M_MLV reverse transcriptase (Promega) and used as RT-PCR templates. Quantitative RT–PCR was performed with the iCycler (Bio-Rad) and the SYBR Green PCR Master Mix (Invitrogen). Relative transcript levels were calculated by normalizing to the transcript level of the reference gene Actin (OsAct1).
tative real-time PCR analysis involved an Mx3000P (Stratagene) with a SYBR green detection protocol. RT-PCR was repeated at least 3 times for each harvested samples with gene-specific primers and \textit{ACTIN1} as the reference gene (see Table S1). The data were analyzed by the CT formula considering amplification efficiencies for every PCR [53].

Vector Construction and Plant Transformation

The cDNA of LIC from a rice cDNA library was amplified by PCR and ligated into pUN1301 binary vectors for overexpression. Full-length cDNAs of LIC, GSK1, \textit{SKETHA}, and \textit{AtBIN2} without the stop codon were amplified by PCR from rice or \textit{Arabidopsis} and cloned into pGADT7 or pGBDT7 vectors. All binary vector constructs were transformed into \textit{Agrobacterium tumefaciens} strain GV3101 or EHA105, then transformed into rice calli by \textit{A. tumefaciens}-mediated transfection [54,55]. Primers are in Table S4.

For tobacco transformation, full-length cDNAs of LIC and GSK1 were ligated into pBI121 and pRT105-3×FLAG vectors [56], respectively. The binary vector constructs were transformed into \textit{A. tumefaciens} strain GV3101 and then transformed into tobacco by \textit{A. tumefaciens}-mediated transfection.

Protoplast Transient Expression Assay

Full-length \textit{LIC} sequence was inserted into the pBI221 vector to generate pBI221-LIC. To generate the \textit{BZR1pro:LUC} reporter gene, the \textit{BZR1} promoter was amplified with the rice genomic DNA used as a template and then inserted into the pGEM-T Easy vector to produce pGEM-BZR1p. The \textit{BZR1} promoter was released from pGEM-BZR1p by digestion with \textit{HindIII} and \textit{BamHI} and inserted into the corresponding sites of the YY96 vector [57] to produce \textit{BZR1pro:LUC}. The \textit{ILI1pro:LUC} and \textit{IHB1pro:LUC} reporter genes were constructed as for \textit{BZR1pro:LUC}.

Figure 6. LIC and BZR1 expression patterns and their responses to BR. (A) RNA \textit{in situ} expression of LIC and BZR1 on the abaxial and adaxial sides of leaves (the bottom panel represents the negative control with sense probes). Bar = 10 μm. (B) LIC and BZR1 transcriptional expression response to various concentrations of BR. Data are mean ± SD (n = 5). *P < 0.05 and **P < 0.01 compared with no BR treatment as determined by Student’s t test. (C) Immunoblotting to show the response of LIC protein expression to BR. LIC was repressed by low concentrations of BR (<100 nM) and induced by high concentrations of BR (>200 nM). Coomassie Blue staining served as the loading control. The levels of LIC were calculated after normalization against the intensity of Coomassie Blue staining in 3 replicated experiments, and the quantified values are shown beneath the gel images. Data are mean ± SE. (D) LIC transcriptional expression with BR treatment in wild-type (WT) and BR-deficient mutant \textit{d2} and \textit{BZR1} RNAi transgenic lines (\textit{BZR1}R). LIC antisense line 2 (AS2) was a control. Data are mean ± SD (n = 3). (E) Time course response of transcription expression of LIC to BR (1 μM). LIC was rapidly induced by BR. Data are mean ± SD (n = 5). (F) BZR1 and CPD transcriptional response to BR treatment in the wild type and \textit{LIC} antisense lines. For BZR1, data are mean ± SD (n = 5). *P < 0.05 compared with no BR treatment. For CPD, data are mean ± SD (n = 3). doi:10.1371/journal.pgen.1002686.g006
Isolation of *Arabidopsis* protoplasts and PEG-mediated transfection were as described [58]. The reporter constructs BZR1pro:LUC, ILI1pro:LUC and IBH1pro:LUC; effectors; and 35S:GUS construct (internal control) were co-transformed into protoplasts. After transformation, the protoplasts were incubated at 23°C for 12–15 h, then pelleted and resuspended in 100 mM [Cl]-buffer (Promega). For the ß-glucuronidase enzymatic assay, 5 μL extract was incubated with 50 μL 4-methylumbelliferyl ß-D-glucuronide assay buffer (50 mM sodium phosphate, pH 7.0, 1 mM ß-D-glucuronide, 10 mM EDTA, 10 mM ß-mercaptoethanol, 10 μM ß-mercaptoethanol, 0.1% sarkosyl, 0.1% Triton X-100) at 37°C for 15 min, and the reaction was stopped by adding 945 μL of 0.2M Na2CO3. For luciferase activity assay, 5 μL extract was mixed with 50 μL luciferase assay substrate (Promega), and activity was detected with use of a Modulus Luminometer/Fluometer with a luminescence kit (Promega). The reporter gene expression was expressed as relative ratio of LUC to ß-glucuronidase.

**Yeast Two-Hybrid Screening**

The cDNA of LIC was cloned into the pGADT7 vector. The cDNAs of GSK1, SKETHA, and AtBIN2 were cloned into pGBKTD7 (Stratagene) and co-transformed with pGADT7 into yeast cells. Yeast that could grow on SD/-Leu/-Trp/-His medium with ß-galactosidase activity exhibited transactivation activity.

Western blot analysis involved extracts prepared from yeast cells as described [14]. The yeast cells were collected, ground to a fine powder in liquid nitrogen, and further ground in cold grinding buffer (50 mM HEPES [pH 7.4], 10 mM EDTA, 0.1% Triton X-100, 1 mM PMSF). After the addition of an equal volume of 2× sample buffer, the samples were boiled for 10 min, separated by 13% SDS–PAGE, and transferred to a polyvinylidene fluoride membrane. The blots were incubated with the antibodies mouse anti-Myc (Neo-Marker, UK) or mouse anti-HA (Santa Cruz, Germany), then goat anti-mouse IgG HRP-conjugated secondary antibody (Santa Cruz, Germany).

**ChIP and EMSA**

Chromatin immunoprecipitation (ChIP) was performed as described [26] with 3-week-old seedlings. The antibody polyclonal anti-BZR1 or anti-LIC was used for immunoprecipitation. Untagged purified LIC protein was used to inject rabbit, and polyclonal...
serum was affinity-purified with its target antigen. ChIP products were analyzed by quantitative real-time PCR, and enrichment was calculated as the ratio of transgenic to wild-type sample or BR-treated and control seedlings. Data are mean ± SD from 3 biological replicates. The primers for UBQ5 (LOC_Os04g57220) promoter were 5'-TATCCAACATGAATGCCACA-3' and 5'-CAGCACGAGATGAGTAAAACAA-3'. Sequences used in bioinformatics analysis are in Table S3.

EMSA was performed essentially as described [59]. Briefly, the OsBZR1 coding region was cloned into a maltose-binding protein (MBP) fusion vector [pETMALc-H vector, Pryor and Leiting, 1997] with the primers for OsBES1NAsp718, 5'-CTCGGTACCGGAGCTGGTGGGTATGACGTC-3', and OsBES1CHind3, 5'-CGCAAGCTTTCATTTCGCGCCGAGC-3'. The recombinant MBP–OsBZR1 was purified from Escherichia coli with amylose resin (NEB, http://www.neb.com) according to the manufacturer's instructions [60]. The coding sequence of LIC was cloned into the expression vector pGEX-4T-1 [10]. The construct was transformed into E. coli BL21 (DE3). Cells were grown at 30°C and induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After induction, cells were harvested by centrifugation and lysed by sonication. The recombinant LIC protein was purified by affinity chromatography using immobilized amylose resin (NEB). The purified LIC protein was used in EMSA experiments.}

Figure 8. Opposite regulation of downstream genes in BR signaling by LIC and BZR1. (A) Transcriptional expression patterns of ILI1 and IBH1 in the LIC antisense line (AS2). Data are mean ± SD (n = 3). (B) Gel shift assay to illustrate LIC binding to the different fragments of the ILI1 and IBH1 promoters. ILI1 B2 and IBH1 C3 contain the binding element S. ILI1 B1, B3, IBH1 C1 or C2 fragments contain no or less binding elements. (C) and (D) ChIP analysis of LIC binding to the ILI1 and IBH1 promoters by use of anti-LIC antibody. The binding was enhanced in the lic-1 mutant and in wild-type plants in the presence of BR. The black circle with white ring indicates the binding element S. B1–3 and C1–3 are the probes used in (B), and a–n (used in (C)) and a–k (used in (D)) indicate the sequences tested in ChIP assay. The UBQUITIN5 promoter was used as a control. (E) Transient transfection assay to illustrate that LIC repressed ILI1pro:LUC and activated IBH1pro:LUC reporter gene expression in Arabidopsis protoplasts (the 403-bp ILI1 promoter indicated as B2 in (C) and the 451-bp IBH1 promoter indicated as C3 in (D) were used). The inhibition of AtCPDpro:LUC reporter gene expression by BZR1 was the control. Data are mean ± SD. (F) Transient transfection assay indicated that LIC and BZR1 antagonistically regulate ILI1pro:LUC reporter gene expression. Data are mean ± SD. doi:10.1371/journal.pgen.1002686.g008
at a final concentration of 1 mM when the optical density (OD$_{600}$) of the cultured cells was 0.5–0.9. The fusion protein was purified with Glutathione Sepharose 4B (GE Healthcare). The nucleotide sequences of the double-stranded oligonucleotides for EMSA were for LIC P1 (‘5’-CGA CGT CGT GGC GGC GCG-5’ and ‘5’-GCC GGC CGC ACG ACG CGC-3’) and LIC P2 (‘5’-CCG CCC GGT GTG TGG GGC-3’ and ‘5’-CCC CCA CAC ACG CGC CCG-3’). The oligonucleotides were annealed and then labeled with the Biotin 3’ End DNA Labeling Kit (Pierce). Standard reaction mixtures (20 μL) for EMSA contained 2 μg purified proteins, 2 μL biotin-labeled annealed oligonucleotides, 2 μL 10× binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), 1 μL 50% glycerol, 1 μL 1% NP-40, 1 μL 1 M KCl, 1 μL 100 mM MgCl$_2$, 1 μL 200 mM EDTA, 1 μL 1 μg/μL poly (dId-cd) and 8 μL ultrapure water. The reactions were incubated at room temperature (25°C) for 20 min and loaded onto a 10% native polyacrylamide gel containing 45 mM Tris, 43 mM boric acid, 1 mM EDTA, pH 8.3. The gel was sandwiched and transferred to an N+ nylon membrane (Millipore) in 0.5× TBE buffer at 380 mA in a 4°C refrigerator for 60 min. The detection of biotin-labeled DNA by chemiluminescence followed the manual of the LightShift Chemiluminescent EMSA Kit (PIERCE).

Confocal Microscopy and Quantification of Protein Fluorescent Signal

GFP fluorescence was visualized under a confocal microscope (Zeiss LSM510 META, Germany) equipped with an argon laser (488 nm). GFP was excited by an Argon laser at 488 nm, and images were acquired using a 512b Roper Cascade EMCCD camera and MetaMorph software (Molecular Devices, Sunnyvale, CA). Images of LIC-, LICm-, and LICp-GFP were obtained with identical image acquisition settings. A series of images at different focal points along the z-axis were collected from the top to the bottom. Projection of the z-series of images results in a 3D view of the cell.

To quantify the effect of 24-eBL on OsLIC-GFP localization in the time-course experiment, images were obtained with a 500-ms exposure time. Quantification of the fluorescent protein signal involved use of ImageJ (http://rsb.info.nih.gov/ij). To measure the ratio of nuclear to cytoplasmic signals (N/C ratio) for LIC-GFP for each cell, small areas were drawn, and measurements of integrated densities were taken from representative areas within the nucleus, cytoplasm, and background (central vacuole) of each cell. Each sample of at least 20 cells was measured 3 times; the average N/C ratio were then calculated [42].

Western Blot Analysis and Kinase Assay In Vitro

Total protein samples were extracted from 2-week-old rice seedlings with 2× SDS loading buffer; cytoplasmic and nuclear fractions were extracted as described [20,61]. Tissues were lysed with use of a buffer (20 mM Tris-HCl, pH 7.0, 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl$_2$, 30 mM β-mercaptoethanol, 13-protease inhibitor cocktail, and 0.7% Triton X-100) and fractionated by centrifugation at 3000 x g. The supernatant was taken as the cytosolic fraction. The pellet was further washed with a resuspension buffer (20 mM Tris-HCl, pH 7.0, 25% glycerol, 2.5 mM MgCl$_2$, and 30 mM β-mercaptoethanol) and reconstituted as the nuclear fraction. All proteins were separated on SDS-PAGE gels, transferred to a nitrocellulose membrane, and probed with anti-LIC antibody. For the in vitro kinase assay, purified LIC-GST protein was incubated with BIN2/GSK1 protein or β-phosphorylase 1 at 30°C for 30 min and loaded onto SDS-PAGE gels. SDS-PAGE gels of 8% or 15% were used to analyze the total LIC level or its phosphorylation, respectively. The proteins were transferred to a nitrocellulose membrane until the 35-kDa protein marker ran out of the gel during electrophoresis.

RNA Hybridization In Situ

Tissues were fixed in 4% (w/v) paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium phosphate buffer; samples were vacuum-infiltrated for 30 min and then stored overnight at 4°C. The dehydrated samples after a graded ethanol series were embedded in Paraplast Plus (Oxford Labware, St. Louis, MO). A fragment of 232 bp was amplified from the second exon of LIC with the primers ‘5’-GGATCCGAAATCGGCGCATG-3’ and ‘5’-AAGCTTTTCG CAGGACCAGGAGCA-3’, subcloned into the pGEM-T-easy vector (Promega), and used as a template for RNA probe synthesis. A fragment of BZR1 was amplified with the primers ‘5’-ATCGAGGAGCGACTCTGGG-3’ and ‘5’-GTTGACGAGCTTGGAGGGGG-3’. Hybridization in situ with digoxigenin-labeled sense or antisense RNA of LIC and BZR1 was conducted as described [62].

Bioinformatics Analysis of the Putative Binding Motif

MEME software (http://meme.sbcsc.edu/meme/cgi-bin/meme. cgi) was used to find recurrent motifs among multiple sequences in A. thaliana microarray data for the LIC antisense lines that were up- or downregulated by at least 2-fold [10]. We extracted 1-kb genomic sequences upstream of 1,175 genes to screen the potential motifs. Differentially expressed genes were divided into those up- or downregulated. Randomly generated sequences of the same length were used as controls to remove false-positive results.

Accession Numbers

Sequence data from this article can be found in the GenBank or EMBL database under the following accession numbers: LIC, Os06g49080; GSK1, Os01g10840; SKETHA, Os06g35530; IL1, OsII1, Os04g54900; Os IBH1, Os04g0660100.
Supporting Information

Figure S1 Identification of lic-1 mutant and LIC-overexpressing lines. (A) A diagram of the T-DNA insertion site in the lic-1 mutant. LB represents the left border primer in T-DNA, LP and RP represent the left and right primers for LIC respectively. P1+P2 represent primers used to amplify the N-terminal fragment of LIC and P1+P3 represent primers used to amplify full-length LIC. (B) PCR of genomic DNA to amplify T-DNA with primers LB+RP and LIC with primers LP+RP. Italicized numbers 9, 17 and 22 indicate homozygous mutants. (C) PCR of cDNA to amplify full-length LIC and the N-terminal fragment of LIC in the lic-1 mutant. (D) Quantitative RT-PCR analysis of LIC RNA levels in antisense lines and overexpressing lines. Data are mean ± SD (n = 3).

Figure S2 Comparative morphology of the lic-1 mutant and the transgenic lines. (A) Gross morphologic features of LIC over-expressors and the lic-1 mutant (40 days old). LIC-overexpressing lines (OX1) and the lic-1 mutant showed dwarfism and erect leaves. The antisense line 2 (AS2) and BR-deficient mutant d2 are controls. Bar = 20 cm. (B) Quantification of leaf angles in the wild type, lic-1 mutant and OX1; AS2 and d2 are controls. Data are mean±SE of 50 measured plants.

Figure S3 Phenotypes of lic-1 mutant and LIC transgenic lines. lic-1 mutant and LIC-overexpressing line 2 (OX2) show erect leaves and antisense line 3 (AS3) an increased leaf angle.

Figure S4 BR marker genes expression in transgenic lines. Quantitative RT-PCR analysis of the mRNA level of BR synthetic genes D2, D11, BRD1 and the receptor gene BRI1 in the wild type, LIC antisense line 2 (AS2) and lic-1 mutant. Data are mean ± SD (n = 3). *P<0.05 and **P<0.01 compared with the wild type as determined by Student’s t test.

Figure S5 Rice root growth at different concentrations of BR. (A) BR sensitivity of the lic-1 mutant and the LIC-overexpressing lines in root growth. The upper panel represents treatment without BR, and the bottom panel represents 1 μM BR treatment; OX1, LIC-overexpressing line 1; AS2, LIC antisense line 2. Bar = 1 cm. (B) Quantification of primary root length under different concentrations of BR. Data are mean ± SD of root length in 30 plants. (C) BR promoted root growth at low levels (<1 nM) and restrained root elongation at high levels (>100 nM). Bar = 2 cm.

Figure S6 Identification of hybrid generations of a LIC antisense line and a B2R1 RNAi line. (A) Identification of the B2R1 RNAi vector and the LIC antisense vector in hybrid generations. H1, H2 and H3 represent hybrid generations and CK indicates the B2R1 RNAi line or the LIC antisense line as a positive control. (B) Quantitative RT-PCR analysis of LIC and B2R1 RNA levels in parent lines and hybrid generations. Data are mean ± SD (n = 3). (TIF)

Figure S7 Western blot analysis of protein expression in the yeast cells. (A) LIC interacted with AtBIN2 and rice orthologs in yeast cells. Left panel, LIC interacted with AtBIN2, OsGSK1 and OsSKETHA in a yeast two-hybrid assay; pGADT7-DWF1 and pGBDT7-GSR1 co-transformed yeast served as a positive control [63] and AD- and BD vector–co-transformed yeast as a negative control. Middle panel, mutated LIC failed to interact with BIN2/ GSK1/SKETHA, pGADT7-LICm- and pGBDT7-co-transformed yeast served as a negative control. Right panel, yeast cells transformed with a single protein served as a negative control. (B) Western blot analysis with an anti-HA tag antibody. Protein was extracted from yeast co-transformed with LIC/LICm/LICp and GSK1 or yeast co-transformed with the AD and BD vectors. (C) Immunoblotting analysis with an anti-Myc tag antibody. Protein was extracted from yeast co-transformed with BIN2/GSK1/SKETHA and LICm or yeast co-transformed the AD and BD vectors.

Figure S8 EMSA to test LIC binding to the predicted motifs. (A) Putative DNA motifs to which LIC binds (denoted as S1–14) as predicted by use of microarray chip gene promoters and MEME software (see Materials and Methods). (B) EMSA to illustrate LIC binding to S1–3. Lane 1 shows the band shifted by S1, lane 2 the band shift caused by S2, lane 3 the band shift caused by S3, and lanes 4–6 unlabeled S1–3 (denoted as Co1-3), which served as competitive probes that weakened the intensity of the shifted bands of S1–3. Lanes 7–9, mutated S1–3, denoted as M1 (GAAAAATG), M2 (TCGAAAAG) and M3 (CTAAAAAT) respectively, eliminated the shifted bands. (C) Putative DNA motifs to which LIC binds as predicted from ChIP sequences. Letter probability of every site is shown on the right. (D) LIC bound to the sequence CTCGC marked as S. M1 (ATCGCG), M2 (CTCAGC) and M3 (AAAGAAG) were the mutated probes. Co represented the competitive unlabeled S sequence.

Figure S9 Specificity of the anti-LIC antibody and the anti-BZR1 antibody used in the ChIP assay. Left, western blot analysis with the LIC antibody displayed one specific band for the total protein fraction; LIC protein was decreased in antisense lines and increased in overexpressing lines. Right, western blot with the BZR1 antibody displayed one specific band for wild-type proteins.

Figure S10 Expression patterns of LIC and B2R1. (A) Expression patterns of LIC and B2R1 in various organs in rice (S, shoot; R, root; ST, stem; P, panicle; L, leaf; LS, leaf sheath). Data are mean ± SD (n = 3). (B) LIC and B2R1 expression patterns during seed development and in leaves Data analyzed by use of electronic fluorescent pictographic software, http://www.bar.utoronto.ca/cfp/cgi-bin/cfpWeb.cgi). The color scale illustrates the microarray signal level. YL, young leaf; ML, mature leaf.

Figure S11 Phylogenetic tree of rice LIC (Os06g49080) and related proteins in other model species. The sequence of LIC was used in BLAST searches of NCBI databases [http://130.14.29.110/blast/, nr, est, httg, gss, and wgs databases, default values]. Midpoint-rooted neighbor-joining trees were constructed with full-length protein sequences by use of MEGA 3.1 [http://www.megasoftware.net/index.html] [64]. The variables were pseudo correction, pairwise deletion and bootstrap (1000 replications; random seed). Blue box: genes of dicots; red box: genes of monocots.

Table S1 Number of seeds per panicle and leaf angle for the progenies of antisense line 2 and lic-1 hybrid lines.

Table S2 Phenotypes of LIC transgenic rice lines and gain-of-function mutants.

Table S3 Sequences from the ChIP assay for motif searches.
Table S4  Primers used in this study.

(DOC)

Acknowledgments

We thank Dr. Jianming Li (University of Michigan) for the gift of the AtBIN2-GST expression vector, Drs. Yanhai Yin (Iowa State University) and Chengai Chu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for the gift of the OsBZR1-MBP expression vector, and Rongxi Jiang and Wei Luo for assistance in gene transformation in rice and field management. The authors are grateful to Dr. Zhiyong Wang (Stanford University) for commenting on the manuscript.

Author Contributions

Conceived and designed the experiments: KC CZ YX. Performed the experiments: CZ. Analyzed the data: CZ. Wrote the paper: CZ KC.

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