The auxin response factor, OsARF19, controls rice leaf angles through positively regulating OsGH3-5 and OsBRI1

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ABSTRACT

Auxin and brassinosteroid (BR) are important phytohormones for controlling lamina inclination implicated in plant architecture and grain yield. But the molecular mechanism of auxin and BR crosstalk for regulating lamina inclination remains unknown. Auxin response factors (ARFs) control various aspects of plant growth and development. We here report that OsARF19-overexpression rice lines show an enlarged lamina inclination due to increase of its adaxial cell division. OsARF19 is expressed in various organs including lamina joint and strongly induced by auxin and BR. Chromatin immunoprecipitation (ChIP) and yeast one-hybrid assays demonstrate that OsARF19 binds to the promoter of OsGH3-5 and brassinosteroid insensitive 1 (OsBRI1) directing their expression. OsGH3-5-overexpression lines show a similar phenotype as OsARF19-O1. Free auxin contents in the lamina joint of OsGH3-5-O1 or OsARF19-O1 are reduced. OsGH3-5 is localized at the endoplasmic reticulum (ER) matching reduction of the free auxin contents in OsGH3-5-O1. osarf19-TDNA and osgh3-5-Tos17 mutants without erected leaves show a function redundancy with other members of their gene family. OsARF19-overexpression lines are sensitive to exogenous BR treatment and alter the expressions of genes related to BR signalling. These findings provide novel insights into auxin and BR signalling, and might have significant implications for improving plant architecture of monocot crops.

Key-words: OsARF19; OsBRI1; rice (Oryza sativa L.).

INTRODUCTION

Leaf angle (lamina inclination or lamina joint bending) is the degree of bending between the leaf blade and leaf sheath or the inclination between leaf blade and culm. Leaf angle is an important agronomic trait for determining plant architecture and grain yield. The regulatory mechanism of rice leaf angle depends on the coordination of several phytohormone signals including brassinosteroids (BRs) and auxin, which may be related to differential cell elongation by adaxial and abaxial cells in the lamina joint (Yokota & Mor2007; Sakamoto et al. 2013). A conserved mechanism of BR regulation of plant development in Arabidopsis and rice acts through a pair of antagonizing HLH/bHLH (basic helix-loop-helix factors) transcription factors that act downstream of Brassinazole-resistant 1 (BZR1, Zhang et al. 2009a,b). OsGRAS19, a new member of the GRAS family [GA INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR)], is involved in the BR signalling pathway. OsGRAS19-overexpressing plants displayed larger leaf angles (Chen et al. 2013). However, LC2 (Leaf Inclination2) controls leaf angles by inhibiting the adaxial cell division (Zhao et al. 2010); ILA1 (Increased Leaf Angle1) affects leaf angles by regulating vascular bundle formation and cell wall composition in the leaf lamina joint (Ning et al. 2011), which is distinct from BR-dependent pathways. In a recent report, Loose Plant Architecture 1 (LPA1), the functional rice ortholog of AtIDD15/SHOOT GRAVITROPISM 5 (SGR5), was shown to regulate the leaf angle by controlling the adaxial growth of lamina joint, demonstrating the complicated nature of the rice leaf angle regulatory mechanism (Wu et al. 2013).

Besides the above recent reports, auxin was also shown to control normal leaf development in various aspects (Davies et al. 1995). Wada et al. (1981) discovered that auxin has an impact on lamina inclination. Auxin early response genes, such as AUX/IAA and GH3, the auxin receptor TIR1, and auxin response factor (ARF), were reported in succession related to regulating leaf angle. Overexpression of OsIAA1 increased lamina joint angle and dwarfism (Song et al. 2009). In overexpressing lines of MiR393a/b and related OsAFB2- or OsTIR1-suppressed lines, flag leaf inclination was enlarged (Bian et al. 2012). Interestingly, OsTIR1 and OsAFB2 were shown to interact with OsIAA1 by using yeast two-hybrid and bimolecular fluorescence complementation assays (Bian et al. 2012). The auxin early response gene GH3 encodes an indole-3-acetic acid-amido synthetase and functions in maintaining the auxin homeostasis through conjugating excess IAA to
various amino acids (Hagen & Guilfoyle 1985; Staswick et al. 2002). A gain-of-function rice mutant, _l1D_ (OsGH3-13), displays an increased number of tillers, enlarged leaf angles, and dwarfism (Zhang et al. 2009a,b). The rice gain-of-function mutant, _lcl-D_ (Leaf inclination1, alias OsGH3-1), and overexpressing lines of OsGH3-1 all show enlarged leaf angles due to stimulated cell elongation at the lamina joints (Zhao et al. 2013). The other OsGH3 gene family member, OsGH3-2, also acts in leaf inclination (Du et al. 2012). Increased leaf angle was observed in the OsGH3-2 overexpressing rice lines. These reports showed that auxin homeostasis also has a crucial role in leaf inclination control. In a recent report, a loss-of-function mutant of _auxin response factor 11_ (OsARF11) in rice, _osarf11-1_ , showed a reduction in plant height and the leaf angle of flag leaves compared with wild type (WT) (Sakamoto et al. 2013). Furthermore, in the _osarf11-1_ mutant, expression of _OsBRI1_ , _OsIAA1_ , _OsSAUR13_ and _OsGH3-1_ was decreased about 70% of WT rice, suggesting that OsARF11, even multiple OsARF proteins, may function in the transcription regulation of these genes.

ARFs are important for normal growth and development in plants. In _Arabidopsis_ , they control tissue and organ development in four aspects. (1) Lateral root (LR) initiation (Okushima et al. 2005, 2007; Wilmoth et al. 2005; Fan et al. 2012): _ARF7_ and _ARF19_ regulate LR formation via direct activation of lateral organ boundary domains (LBD)16 and LBD29. (2) Leaf development: _ARF5/MONOPTEROS_ (MP) is critical to leaf initiation and vein pattern formation (Garrett et al. 2012). (3) Floral organ formation: _Arabidopsis_ _ARF6_ and _ARF8_ regulate floral organ development via repression of class 1 knox genes (Tabata et al. 2010). (4) _ARF10_ affects shoot regeneration (Qiao et al. 2012). While a lot of ARFs have been investigated in the dicot, _Arabidopsis_ , comparatively less is known in monocots such as rice. Besides the above-mentioned OsARF11 implicated in the regulation of leaf angle, functions of OsARF1, OsARF12 and OsARF16 have been investigated. OsARF1 functions in regulating the crown root (adventitious root, AR) formation via binding to the promoter of the _LBD_ gene, _OsCRL1_ (Waller et al. 2002; Inukai et al. 2005; Attia et al. 2009). OsARF12 acts in regulating root elongation, affecting iron accumulation and phosphate homeostasis (Qi et al. 2012; Wang et al. 2014). OsARF16 participates in phosphate starvation response (Shen et al. 2013). Here, we explore the biological functions of OsARF19, which works in controlling leaf angle trough _OsGH3-5_ and _OsBRI1_.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Rice plants (_Oryza sativa_ L.) were grown in standard culture solution (Wang et al. 2009) in a greenhouse with a light/dark cycle of 12/12 h at 30/24 °C. _Nicotiana benthamiana_ plants were grown on vermiculite containing Murashige and Skoog salt (MS) nutritional liquid in a growth chamber (light/dark cycle of 12/12 h at 25/18 °C). Six-week-old _N. benthamiana_ plants were used for transient _Agrobacterium tumefaciens_ -mediated expression.

**Overexpression of OsARF19 and OsGH3-5 in *Nipponbare* (NIP)**

The open reading frame (ORF) of _OsARF19_ and _OsGH3-5_ was amplified using the primers _OVARF19U/L_ and _OVGH3-5U/L_ shown in Supporting Information Tables S2 and S3, respectively. _OsARF19_ PCR products were cloned into _pcAMBIA1300_ containing a CaMV35S promoter to create an _OsARF19-overexpression construct_. _3OS-OsARF19_ , while _OsGH3-5_ PCR products were recombinated in the pHTFWG2 vector to create _3OS-OsGH3-5-GFP_ vector. The two vectors above were introduced into _A. tumefaciens_ strain EHA105 using electroporation and transformed into WT rice NIP (Hei et al. 1994). Overexpression analysis of _OsARF19_ and _OsGH3-5_ genes was monitored by RT-PCR using the primers _RTARF19U/L_ and _OsGH3-5-qRT-U/L_ as listed in Supporting Information Tables S2 and S3, respectively.

**Identification of osarf 19 and osgh3-5 mutants**

The identification of T-DNA insertion sites in the mutant _osarf19_ (PGF-1B-11635.R) was carried out according to http://signal.salk.edu/cgi-bin/RiceGE. Right border primer Ngus-RB was used to confirm integration of T-DNA in _osarf19_ and the gene-specific primers arf19U/L were used to identify WT band of _OsARF19_. The homozygous lines of the _TOS17_ insertion line for _OsGH3-5_ , _osgh3-5_ (NC0973), were identified by PCR using primer _TOS17-tail16_ to confirm the integration of _TOS17_ in mutant lines and gene-specific primers _GH3-5-LP/RP_ to identify the corresponding WT band. The PCR insertion products were ligated with pUCm-T vector (Sangon, Shanghai, China) and transformed into _Escherichia coli_ DH5α, and the flanking sequences of the T-DNA insertion site were sequenced (Sangon). To confirm the transcription level of the _OsARF19_ and _OsGH3-5_ genes in _NIP_ , _osarf19_ and _osgh3-5_ mutant, RT-PCR was performed using the primers _RTARF19U/L_ and _OsGH3-5-qRT-U/L_ as listed in Supporting Information Tables S2 and S3, respectively. Primer sequences for the PCR and RT-PCR are listed in Supporting Information Tables S2 and S3.

**β-glucuronidase (GUS) staining**

Construction of _OsARF19_ and _OsGH3-5_ promoter-β-glucuronidase (_OsARF19pro:GUS_ and _OsGH3-5pro:GUS_) transgenic rice and GUS staining of seedlings were performed as described by Qi et al. (2012). The construction of _OsARF19_ and _OsGH3-5pro:GUS_ was performed according to a published method (Cheng et al. 2007). Primers _ProARF19U/L_ in Supporting Information Table S2 and _ProGH3-5-GUSU/L_ in Supporting Information Table S4 were used for amplification of the promoter region. _OsARF19pro:GUS_ and _OsGH3-5pro:GUS_ were introduced into the _A. tumefaciens_ strain EHA105 and transformed into
NIP. The DR5:GUS auxin reporter described by Ulmasov et al. (1997a,b) was transformed into NIP. OsARF19-O1, OsARF19-O2, Dongjin (DJ) and osarf19 for detecting auxin distribution.

Quantitative RT-PCR (qRT-PCR) analysis
Total RNA was isolated from leaves or roots of 14-day-old seedlings with various treatments. RNA extraction, reverse transcription and qRT-PCR were according to Wang et al. (2010). The sequence of the related primers for qRT-PCR is listed in Supporting Information Tables S2 and S7.

Measurement of IAA contents
The IAA concentrations of lamina joints in NIP, OsARF19-overexpression lines, OsGH3-5-overexpression lines, DJ and osarf19 plants were measured by gas chromatography-selected reaction monitoring mass spectrometry (GC-MS), as described by Ljung et al. (2005). Germinated seeds were grown in standard culture solution for 7 d. About 0.5-cm-long lamina joint was excised. Furthermore, five independent biological replicates of each 20 mg sample were purified after addition of 250 pg of 13C6-IAA internal standard using ProElu C18 (www.dikma.com.cn), and auxin contents were measured with FOCUS GC-DSQII (Thermo Fisher Scientific Inc, Waltham, MA, USA). For auxin influx measurement, the germinated seeds of NIP, OsARF19-O1 and OsARF19-O2 were grown in normal culture solution for 7 d.

Scanning electron microscopy
Scanning electron microscopy was conducted as described previously (Zhao et al. 2010). About 1-cm-long lamina joints of the third leaf from 2-month-old plant were excised from NIP and OsARF19-O1 plants. Samples were observed with an S-3000N scanning electron microscope (Hitachi, Tokyo, Japan).

Microscopy of cross and longitudinal sections
Lamina joints (from flag leaf of 3-month-old NIP and OsARF19-O1 plants) were collected for cross and longitudinal sections. Microscopic analysis was performed as described elsewhere (Qi et al. 2012). Ten independent sections were microscopically examined and photographed to measure the cell layers and cell lengths (Zhao et al. 2010).

ChIP-PCR analysis
Rice (NIP) protoplasts with transiently expressed 35S:OsARF19-GFP were performed for ChIP-PCR assay as described in Du et al. (2009) and Zhang et al. (2011). The ChIP-IT® Express kit was used for target DNA enrichment according to the manufacturer’s description (Active Motif, Carlsbad, CA, USA). The related primers in PCR of targets DNA are listed in Supporting Information Table S4.

Yeast one-hybrid assay
Yeast one-hybrid assays were carried out using the MATCH-MAKER One-Hybrid Library Construction and Screening Kit (Takara, Dalian, China) according to the manufacturer’s manual. Full-length cDNA sequences of OsARF19 were PCR amplified using the primers OsARF19-orf-ADUL shown in Supporting Information Table S5. OsGH3-5 and OsBRI1 promoter fragments containing WT AuxRE or mutated AuxRE were obtained by DNA renaturation (primers shown in Supporting Information Table S5).

Cell wall isolation and cellulose measurement
Shoots from 1-month-old NIP, OsARF19-overexpression lines and OsGH3-5-overexpression lines were collected for cell wall isolation and cellulose measurement. The assays were conducted as described previously (Zhang et al. 2012).

Agrobacterium-mediated transfection of N. benthamiana leaves and polyethylene glycol-mediated transformation of rice protoplast
Full-length cDNA of OsARF19 was ligated into the binary vector pCAMBIA1300 under the control CaMV35S promoter (35S) resulting in 35S:OsARF19 vector. The promoter region of OsGH3-5 was ligated into the gFP vector resulting in OsGH3-5pro:GFP. The primer sequences of OVARF19U/L and GH3-5-sGFPU/L are listed in Supporting Information Table S6. The above vectors were introduced into the A. tumefaciens strain EHA105 using electroporation respectively. Agrobacterium-mediated infection of N. benthamiana was performed as described previously (Chen et al. 2008; Qi et al. 2012). After 2 d, the fluorescence was visualized under a Nikon microscope equipped with NIS-Elements Basic Research 3.0 software (http://www.nis-elements.com). For the subcellular localization of OsGH3-5, 35S:OsGH3-5:GFP vector was infected to N. benthamiana through Agrobacterium-mediated transfection, and was introduced into rice protoplast by polyethylene glycol-mediated transformation, by Qi and Chen, respectively (Chen et al. 2011; Qi et al. 2012).

BR sensitivity tests
The coleoptile elongation test was performed as described previously (Duan et al. 2006). Germinated seeds of NIP, OsARF19-O1 and OsARF19-O1-O2 were grown in standard culture solution with 0, 0.001, 0.01, 0.1 and 1 μM 24-eBL for 7 d under dark condition. Then coleoptile lengths were photographed and measured. Primary root (PR) inhibition analysis was carried out as described previously with slight modification (Zhang et al. 2012). Germinated seeds were grown in normal culture solution supplemented with various concentrations of 24-eBL for 1 week, and then the root length was measured. Leaf angles were measured after 8 d in a dark growth and 3 d incubation with various concentrations of 24-eBL as described in Zhang et al. (2012).
Accession numbers

Sequence data from this article can be found in the Rice Genome Annotation Project or GenBank under the following accession numbers: OsARF19, LOC_Os06g48950, AK103312; OsGH3-5, LOC_Os05g50890, AK071721; OsGH3-1, LOC_Os01g57610, OsGH3-2, LOC_Os01g55940, OsGH3-13, LOC_Os11g32520, OsIAA1, LOC_Os01g08320; OsARF23, LOC_Os11g32110; OsTIR1, LOC_Os05g05800; OsBRI1, LOC_Os05g52050; OsBZR1, LOC_Os07g39220; OsD2, LOC_Os01g03040; OsDWARF, LOC_Os03g40540; OsACTIN, LOC_Os03g50885.

RESULTS

Phenotypes of rice OsARF19 gain and loss-of-function lines

From our previous work, it was known that OsARF19 is an active transcription factor interacting with many OsIAAs in the nucleus (Shen et al. 2010). To understand the biological function of OsARF19, we constructed 11 OsARF19-overexpression rice lines (Fig. 1a,b). Compared with WT/NIP, 9 of 11 OsARF19-overexpression lines revealed dwarfism, narrow leafs, thin seeds and enlarged leaf angles (Supporting Information Table S1a,b). T5 generations of OsARF19-O1 and OsARF19-O2 in the OsARF19-overexpression rice lines were further analysed in detail. The plant height of OsARF19-O1 or -O2 was lower than 30% of WT/NIP (Fig. 1c); the flag leaf width of OsARF19-O1 or -O2 was narrower than 20% of WT/NIP (Fig. 1d) and the grain breadth of OsARF19-O1 or OsARF19-O2 was thinner than 18% of WT/NIP (Fig. 1e). Further quantitative measurement showed that the flag leaf angle at the heading stage and the second/third leaf at the vegetative period were about 38–20° compared with the WT/NIP, which typically reveals almost 120° (Fig. 1f–h). These altered morphologies of OsARF19-O1 and -O2 suggested that OsARF19 might act in rice growth and development. Especially, the enlarged leaf angle, which is an important agronomic trait, in respect to the rice yield, motivated us to study the OsARF19 in depth.

To understand how OsARF19 controls rice growth and development, a T-DNA inserted mutant of OsARF19 (osarf19) found to own an insertion in the eighth exon of OsARF19 gene in WT/DJ was identified and analysed (Supporting Information Fig. S1a-c and Tables S1 & S2). Our analysis showed that osarf19 did not simply show opposite phenotypes of OsARF19-O1 mentioned. This is probably because there are 25 members in ARF family of rice, and OsARF19 belongs to one of class IIa subfamilies including nine highly homologous OsARFs (Wang et al. 2007). Therefore, the OsARF19 gene seems to share with other OsARFs a high degree of redundancy. Nonetheless, in osarf19 mutant, the LR number was clearly decreased to 30% of the WT/DJ (Fig. 1f). The results are consistent with that of the arf7/arf19 double mutant revealing fewer LRs in Arabidopsis (Okushima et al. 2005).

Osarf19-O1 shows increases of adaxial cell division at the lamina joint and reduction of cellulose contents

The lamina joint plays a significant role in leaf inclination formation and enlarged leaf inclination (Cao & Chen 1995; Yamamuro et al. 2000). To understand the involvement of OsARF19 in controlling rice leaf angle in the alteration of lamina joint, the collar length of lamina joint was measured. The collar lengths of adaxial surfaces in the lamina joint of OsARF19-O1 and OsARF19-O2 were increased by 60 and 55%, respectively, compared with the WT/NIP, while the
OsARF19 binds to the OsGH3-5 promoter and up-regulates OsGH3-5 expression

In rice, the function of the three early auxin response genes, OsGH3-1, OsGH3-2 and OsGH3-13, was shown to result in increasing leaf angles (Zhang et al. 2009a,b; Du et al. 2012; Zhao et al. 2013). ARFs are transcription factors controlling OsGH3 gene expression such as the MicroRNA160-ARF17-GH3.2 (or GH3.3, GH3.5 and GH3.6) pathway in Arabidopsis (Mallory et al. 2005) and the MicroRNA167-ARF8-GH3.2 pathway in rice (Yang et al. 2006). To investigate whether these OsGH3 genes are direct targets of OsARF19, sequence analysis of auxin response elements (AuxRE) of OsGH3 promoters was performed, indicating that there were several AuxRE elements in the OsGH3-2, -5 and -13 promoters besides OsGH3-1 (Supporting Information Fig. S2). Further, ChIP assays were carried out by transiently transforming 35S:OsARF19-sGFP into rice protoplast. The DNA elements pulled down by 35S:OsARF19-sGFP were enriched and analysed by PCR using three primers included in OsGH3-1, -2, -5 and -13 promoter elements, respectively (Fig. 4a and Supporting Information Table S4). Our experiments showed that the promoter regions of OsGH3-2, -5 and -13 were enriched in the ChIP assays with OsGH3-5 being the most prominent. Furthermore, OsGH3-1 was not enriched there, indicating OsGH3-1 was not a direct target of OsARF19 but was indirectly induced by OsARF19 due to unknown mechanism.

To further confirm the binding of OsARF19 to the OsGH3-5 promoter, we performed yeast one hybrid using in vitro-expressed OsARF19. As shown in Fig. 4b (Supporting Information Figs S2 & S5), OsARF19 bound to the AuxRE-containing DNA fragments (tggctc) of OsGH3-5 promoter but failed to bind AuxRE-mutated motifs (aaaaaa). The results further demonstrated OsARF19 as an upstream regulating factor of OsGH3-5, directly binding to the OsGH3-5 promoter.

To demonstrate that OsARF19 controls rice leaf angles through OsGH3 genes, the transcriptional level of several early auxin response OsGH3 genes was quantified by qRT-PCR analysis (Fig. 4c). The transcripts of OsGH3-1, -2, -5 and -13 were all up-regulated in OsARF19-O1 or OsARF19-O2 compared with WT/NIP, while these OsGH3 genes were down-regulated in osarf19 mutant compared with WT/DJ.

To test whether the binding of OsARF19 to the OsGH3-5 promoter affects expression of the OsGH3-5 protein, the vectors of 35S:OsARF19-over and OsGH3-5pro:GFP (green fluorescent protein) were co-transformed into leaves of N. benthamiana (Fig. 4d and Supporting Information Table S6). Fluorometric analysis of GFP showed that OsARF19 indeed significantly enhanced the fluorescence intensity of OsGH3-5pro:GFP. The result indicated that OsARF19 also promoted the expression of OsGH3-5 on the protein level.

OsGH3-5-overexpression lines show similar phenotypes as OsARF19-O1 or OsARF19-O2

In order to gain genetic information on the relationship between OsARF19 and OsGH3-5, we constructed 12 OsGH3-5-overexpression rice lines (Supporting Information Fig. S3a,b). Ten of the OsGH3-5-overexpression lines in T3 generation revealed similar phenotypes with OsARF19-O1, characterized by enlarged leaf angles, dwarfism, narrow leaves collar lengths in abaxial side were not significantly different from WT/NIP (Fig. 2a,b).

To clarify the cellular mechanism of OsARF19 controlling rice leaf angles, we further observed the microstructure of leaf lamina joint of rice WT/NIP and OsARF19-O1. Like in the lzc mutant, there is a bulge or cell protuberances in the adaxial surface of lamina joint, while OsARF19-O1 has a smooth adaxial surface as in WT/NIP (Fig. 2c). The altered cell elongation and cell division in the lamina joint were therefore the two main reasons for the enlarged leaf angles (Duan et al. 2006; Zhao et al. 2010, 2013; Sakamoto et al. 2013). Observation of the cross and longitudinal sections revealed that the increased cell layers at the adaxial surface of the OsARF19-OI lamina joint were increased by about 45 and 60%, WT/NIP, respectively (Fig. 2e,h), illustrating that the enlarged leaf angle resulted from enhanced cell division but not cell elongation, that is, no alteration of cell size. In addition, the enlargement of cross sections showed that the vascular bundles in OsARF19-O1 plant were smaller than those in the WT/NIP (under panel of Fig. 2d).

According to the microstructure characteristic of OsARF19-O1 with smaller vascular bundles, we measured the cellulosic content of the leaf in NIP, OsARF19-O1 and OsARF19-O2. The cellulosic contents in both OsARF19-overexpression lines were decreased about 30% compared with the WT/NIP (Fig. 2h). The result was similar with that of ILAI, which regulates leaf angle through altering vascular bundle formation and cell wall composition in the lamina joint (Ning et al. 2011).

OsARF19 is constitutively expressed in various organs including the lamina joint and is induced by auxin

To understand whether the OsARF19 functions in rice growth and development, we evaluated the expression pattern of OsARF19 in various organs using the GUS reporter gene fusion. Ten positive transgenic lines were obtained and three lines were used for further investigation. GUS staining was found to be prominent in root including PR (or AR) and LR (Fig. 3a–c). OsARF19 was expressed at a lower level in the leaf relative to shoot (Fig. 3d,e,i,l). OsARF19 expression was also observed in the anther, stigma of the flower, glume, seed and lamina joint (Fig. 3f–h,j,k). qRT-PCR further confirmed that OsARF19 was expressed at different levels in various tissues (Fig. 3l), consistent with the GUS staining results. Further, we found that OsARF19 expression was induced by auxin treatments (Fig. 3m), suggesting that its function might be regulated by auxin. The expression pattern of OsARF19 matches well with the phenotypes of OsARF19-O1 or OsARF19-O2 mentioned above, further implying that OsARF19 may function in the various aspects of growth and development of rice organs and tissues.
Figure 2. Microstructure analysis of lamina joints in WT/NIP, OsARF19-O1 and OsARF19-O2. (a) Comparison of the lamina joint of flag leaves from 3-month-old seedlings of NIP, OsARF19-O1 and OsARF19-O2. (b) Collar lengths of adaxial and abaxial surfaces of the flag leaves. (c) Scanning electron microscopy images of the adaxial surfaces in the lamina joint of the flag leaves. (d) Cross section of lamina joints of flag leaves in NIP and OsARF19-O1. Upper panel, 4 x amplification; lower panel, 10 x amplification. (e) Adaxial cell layers of cross sections. (f) Cellulose contents in WT/NIP, OsARF19-O1 and OsARF19-O2. (g) Longitudinal section of lamina joint of the flag leaves in NIP and OsARF19-O1. (h) Cell layers of the longitudinal sections of lamina joints. Ten biological repeats were used for each test. Error bars indicate SD (n = 10). ** indicate significant difference at P < 0.01. Bars = 200 μm.
Figure 3. Expression patterns of the OsARF19 gene. (a, b) Primary roots (expression pattern of OsARF19 in adventitious roots was the same as in primary root). (a) Elongation region. (b) Meristem region to root cap. (c) Lateral root and root hair. (d) Shoot. (e) Cross section of leaf sheath. (f–h) Glume and flower. (f) Glume. (g) Anther. (h) Ovary and stigma. (i) Leaf. (j) Seed germinated for 3 d. (k) Lamina joint of top leaf of 1-month-old seedling. OsARF19pro:GUS was transformed into rice WT/NIP. Ten positive transgenic rice lines were used for analyses of GUS expression. A total of 7-day-old roots, shoots, leaves and other tissues were photographed. All bars = 400 μm. (l) qRT-PCR analysis of OsARF19 expression in WT/NIP rice. L: leaf, F: flower, R: root, LR: lateral root, Sh: shoot, S: seed, LJ: lamina joint. OsACTIN gene was used as an internal reference. Three independent biological replicates were used in the experiments. Error bars indicate SD (n = 3). * indicates significant difference at P < 0.05 and ** indicates P < 0.01. (m) OsARF19 expression under auxin treatments. One-week-old WT/NIP seedlings were grown in solution containing 10 μM IAA and 1 μM 2,4-D for 3 h, respectively. qRT-PCR analysis was performed as described in Fig. 3i.
Figure 4. OsARF19 directly activates expression of OsGH3-5. (a) ChiP-PCR analysis. The ChiP of OsARF19 assays was performed using rice Nipponbare protoplasts expressing the 35S:OsARF19-GFP fusion. Products of ChiP assays were amplified using three specific primers (listed in Supporting Information Table S4) with the AuxRE elements in OsGH3-2, -5 and -13 promoters or without the AuxRE in OsGH3-1 promoter, respectively. (b) Yeast one-hybrid (YOH) analysis of OsARF19 and OsGH3-5 promoter. The bait vector containing the OsGH3-5 promoter fragment P3 or the mutated P3 (P3M)-fused HIS2 reporter gene, and the prey vector containing OsARF19 fused GAL4 activation domain were co-transformed into yeast cells (Y187). Yeast cells were grown on (SD–Trp–Leu–His) media supplemented with 25 mM 3-amino-1, 2, 4-triazole (3AT) or without 3AT (-3AT) to suppress background growth or not. AD + GH3-5-P3/P3M and OsARF19 + pHis2 were used as negative controls. The core region of P3 and P3M was indicated on the right. (c) Relative mRNA levels of OsGH3 genes in the leaf lamina joints of WT/NIP, OsARF19-O1, OsARF19-O2, WT/DJ and osarf19. qRT-PCR analysis using three independent biological replicates was performed according to Wang et al. (2010). Error bars indicate SD (n = 3). ** indicates significant difference at P < 0.01.

In order to investigate further OsGH3-5 function, the expression pattern of OsGH3-5 was also analysed. Both the experiments OsGH3-5promoter:β-glucuronidase (GUS) staining and qRT-PCR were examined (Supporting Information Fig. S5). As shown in Supporting Information Fig. S5, OsGH3-5 is expressed in lamina joints and other tissues matching the expression pattern of OsARF19 in Fig. 3. These tissue-specific expression patterns were consistent with the phenotypes of OsGH3-5-overexpression lines and osgh3-5. OsGH3-5 expression in lamina joints was significantly induced by auxin treatments (Fig. 6d,e), indicating that OsGH3-5 was auxin responsive and thus regulated by auxin.

Furthermore, the auxin reporter DR5:GUS was expressed in WT/NIP and OsGH3-5-O1, respectively (Fig. 6f). DR5:GUS in OsGH3-5-O1 was markedly shallower than in WT/NIP. Moreover, free auxin contents in lamina joints in WT/NIP, OsGH3-5-O1 and OsGH3-5-O2 were measured using GC-MS (Fig. 6g). The results also showed that the auxin contents in OsGH3-5-O1 or -O2 were lower than those in WT/NIP. These results imply that the OsGH3-5 might function in auxin conjugation.

OsARF19-O1 and OsARF19-O2 show decreased free IAA contents at lamina joints

In order to explore if the transcription factor OsARF19 upstream of OsGH3-5 affects the leaf angle through regulating free IAA contents, we analysed IAA contents and IAA distribution in the lamina joints of rice NIP, OsARF19-O1, OsARF19-O2, DJ and osarf19 using GC-MS and DR5:GUS analyses, respectively (Fig. 7a,b). The results show that IAA contents in the lamina joint of OsARF19-O1 or -O2 were reduced to ~20% of WT/NIP while in the osarf19 mutant they were enhanced by ~20% compared with the WT/DJ. DR5:GUS staining in the lamina joint of OsARF19-O1 or -O2 was significantly weaker than that in WT/NIP while in the osarf19 mutant signals were stronger than in WT/DJ. Decreased IAA contents in OsARF19-O1 or -O2 were consistent with those in OsGH3-5-overexpression lines.

To further reveal the molecular mechanism of OsARF19 in controlling leaf angles, the expression of genes involved in leaf inclination and in auxin signalling was analysed in the lamina joints of rice NIP, OsARF19-O1, OsARF19-O2, DJ and osarf19 (Fig. 7c). The transcript abundances of the genes auxin/indole-3-acetic acid 1 (OsIAA1) were induced in
OsARF19-O1 or -O2 compared with WT/NIP, while they were reduced in osarf19. Importantly, both genes, OsARF23 (1) and transport inhibitor response 1 (OsTIR1), interacting with OsIAA1 showed opposite trends compared with

Figure 5. Characterization of OsGH3-5-overexpression lines. (a) Phenotypes for 3-month-old seedlings of NIP, OsGH3-5-O1 and OsGH3-5-O2. (b) Leaf angles of NIP, OsGH3-5-O1 and OsGH3-5-O2. Bar = 10 cm. (c) Statistical analyses of flag leaf angle. (d) Cellulose contents in WT/NIP, OsGH3-5-O1 and OsGH3-5-O2. Ten biological replicates were used in each test. Error bars indicate SD (n = 10). ** indicates significant difference at P < 0.01.
OsARF19 expression in OsTIR1-suppressed lines, osafb2 or its upstream OsMiRNA393-0Va and -OVb was also up-regulated (Supporting Information Fig. S6), suggesting that both OsARF19 and OsTIR1 are negatively regulating each other. These results agree with those OsTIR1-suppressed transgenic rice lines and OsIAA1-overexpression transgenic rice lines, which exhibited increase of leaf inclination (Song et al. 2009; Bian et al. 2012).

**OsARF19-overexpression lines are sensitive to exogenous BR and altered expression of BR response and biosynthesis genes**

BR signalling plays an important role in controlling the leaf angle size (Yokota & Mori 1992; Sakamoto et al. 2013). To understand whether OsARF19 is involved in BR signalling, we first performed three classical BR sensitivity experiments including coleoptile elongation, root growth and degree of leaf inclination using various concentrations of 24-eBL treatments (Fig. 8). Comparison of coleoptile elongation between WT/NIP, OsARF19-O1 and OsARF19-O2 revealed that the elongated coleoptile length became more pronounced with the increased BR concentration (Fig. 8a,d). In contrast to coleoptile elongation, PR growth was inhibited by BR treatments (Fig. 8b,d). The PR length of OsARF19-O1 or -O2 showed also a significant increase by 8–30% compared with that of WT/NIP under various concentration of 24-eBL treatments (Fig. 8c,d). Taken together, the above data indicate that OsARF19-O1 and -O2 were more sensitive to BR than WT/NIP, suggesting that OsARF19 could be related to BR signalling.

Furthermore, ChIP-PCR assays and yeast one hybrid confirmed that OsARF19 binds to OsBRI1 promoter using three different primers (Supporting Information Fig. S7 and Tables S4 & S5). Furthermore, the expressions of genes controlling leaf angle on BR response and biosynthesis in WT/NIP, OsARF19-O1, were analysed by qRT-PCR under control and BR treatment condition (Fig. 8e). The expression of the BR response genes OsBRI1 and Brassinazole-resistant 1 (OsBZR1) in WT/NIP was lower than that in OsARF19-O1 under control condition (Yamamuro et al. 2000; Bai et al. 2007). Furthermore, under BR treatment, their expression in WT/NIP and OsARF19-O1 was all decreased. However, the decreased ranges in OsARF19-O1 were higher than those in WT/NIP, further demonstrating that OsARF19-O1 was sensitive to exogenous BR. Further, BR biosynthetic genes OsD2 (CYP90D2), OsDWARF (CYP85A4) and OsD11 (CYP724B1) were all significantly up-regulated in OsARF19-O1 than in WT/NIP under normal growth (control) condition (Hong et al. 2002, 2003; Tanabe et al. 2005). Furthermore, they were dramatically down-regulated in WT/NIP and OsARF19-O1 under BR treatment compared with control condition, implying that these genes were modulated by OsARF19 under control condition but feedback-regulated by BR treatment. These results further confirmed that OsARF19 is involved in regulating BR signalling.

**DISCUSSION**

**OsARF19 functions in regulating leaf angles through directly altering OsGH3-5 expression**

ARFs in Arabidopsis are involved in transcriptional control of several GH3 genes. Such ARF7 positively regulates the expression of the GH3-2/YDK1 gene, which functions in hypocotyl and root elongation (Stowe-Evans et al. 1998; Takase et al. 2004); ARF8 binds to AtGH3 genes involved in the adenylation of IAA, which results in the formation of IAA amino acid conjugates (Tian et al. 2004); ARF17 increases GH3-2/YDK1, GH3-3, GH3-5 and DFL1/GH3-6 mRNA levels and leads to dramatic developmental tissue and organ defects (Mallory et al. 2005). In rice, the knowledge of the transcriptional control of genes involved in auxin homeostasis is still scarce. In this study, OsARF19-overexpression rice lines showed multiple phenotypes, including increased leaf angles, decreased height and reduced leaf width, suggesting that OsARF19 is critical in the regulation of rice architecture (Fig. 1). Our experimental data demonstrated the important role of OsARF19 in leaf inclination regulation through modulating adaxial cell division of the collar (Figs 2 & 3). We further uncovered that OsARF19 directly binds to the promoter of OsGH3-5 using ChIP assay, and positively regulated OsGH3-5 expression (Fig. 4). Genetic phenotypes of OsGH3-5-overexpression lines and expression pattern of OsGH3-5 further confirmed these results (Fig 5 and Supporting Information Fig. S5). Previous reports and our results together indicate that ARF regulation of GH3 genes is conserved between dicot and monocot. Our findings strongly support that OsARF19 functions in regulating leaf angles through directly altering OsGH3-5 expression.
Alteration of auxin level in the lamina joint might result in enlarged leaf angles

In rice, OsGH3 genes were classed into two groups, group I and group II. OsGH3-5 belongs to group I including OsGH3-3, -5, -6 and -12, which show high homology to AtGH3-10 and -11 in Arabidopsis. AtGH3-11 (JAR1/FIN219) has been reported to adenylate jasmonic acid (JA) (Staswick et al. 2002), while AtGH3-10 function is unknown. This study showed that OsGH3-5 is involved in auxin response (Fig. 6d,e). Auxin responses play an important role in plant growth and development by forming local

![Graph showing auxin distribution and expression of genes implicated in the increase of leaf angles in auxin signalling.](http://doc.rero.ch)
concentration gradients. As shown in Figs 6a-g and 7a,b, we found that free auxin levels at lamina joint of OsGH3-5 and OsARF19-overexpression lines were significantly reduced, suggesting that lowering auxin contents might alter lamina inclination. Particularly, free auxin contents in OsARF19-O1 were significantly decreased (Fig. 7a,b), implying that OsARF19 directly acts with OsARF19 (Shen et al. 2010), which binds to the OsGH3-5 promoter, positively regulating OsGH3-5. In the OsARF19-OsGH3-5 pathway, miR393 negatively regulates expression of OsTIR1, and OsTIR1 interacts with the OsIAA1 proteins (Bian et al. 2012); OsIAA1 also interacts with OsARF19 (Shen et al. 2010), which binds to the OsGH3-5 promoter, positively regulating OsGH3-5. Finally, OsGH3-5 overexpression impairs auxin response and reduces free auxin contents in the lamina joint (Fig. 6). In the OsARF19-OsBRI1 pathway, OsARF19 binds to the OsBRI1 promoter, positively regulating OsBRI1 to activate a BR signal transduction pathway, which regulates the expression of the related gene OsBZ1 and its downstream genes (Zhu et al. 2013) to affect lamina inclination. Therefore, apparently both described above co-modulate plant architecture, suggesting that the regulatory mechanism of the rice leaf angle might be more complicated than thought. Noteworthy, OsARF23(1) also interacts with OsIAA1 and antisense-OsARF23(1) line showed similar phenotype with OsARF19-O1 (Attia et al. 2009; Song et al. 2009). Whether OsARF23(1) also binds to OsGH3-5 as well as to OsARF19, and negatively regulates their expression during control of leaf angle, needs to be further investigated in the future.

Taken together, our physiological experiments, genetic analysis, cellular biological observation and molecular biological evidence support the finding that OsARF19 functions in bridging the molecular network of auxin and BR signalling. Moreover, underlying mechanisms might turn out to be useful for engineering rice architecture to culture high rice yield. In high-density plantings, characteristic semi-dwarf phenotype with erect leaves and shorter panicles was an ideal phenotype to improve grain yield (Morinaka et al. 2006; Sakamoto et al. 2006; Wu et al. 2008). osarf19/osgh3-5 double mutant and osgh3-5/osgh3-1 or -2 or -13 double or multiple

**OsARF19 regulates BR signalling through OsBRI1**

In previous reports, the AuxRE, TGTCTC, was shown to act as a crosstalk point for BR and auxin signalling: ARFs target the AuxRE to regulate the expression of auxin response genes and BR response genes for controlling plant growth and development (Ulmasov et al. 1997a,b; Goda et al. 2002; Nakamura et al. 2003; Nemhauser et al. 2004; Walcher & Nemhauser 2012). Auxin and BR signalling share several genes, which are involved in plant growth and development-related processes (Vert et al. 2008). In Arabidopsis, both ARF2 and ARF7 were demonstrated to be involved in auxin–BR interaction through diverse ways (Vert et al. 2008; Zhou et al. 2013). In rice, OsARF11 targets the AuxRE of the BR receptor kinase gene, OsBRI1, suggesting that ARF11 can control the degree of BR perception (Sakamoto et al. 2013). OsARF19 and OsARF11 have a close relationship in an evolutionary tree, belonging both to the class II subfamily of OsARF gene family in rice (Wang et al. 2007; Shen et al. 2010). Furthermore, OsARF19-O1 is sensitive to BR treatments, and has a phenotype revealing increased leaf angles (Figs 1 & 8), which is opposite to the osarf11-Tos17 insertion mutant (Sakamoto et al. 2013). We show that OsARF19 directly binds to the OsBRI1 promoter and controls the expression of OsBRI1 (Supporting Information Fig. S7). These data suggest that OsARF19 functions as a bridge linking auxin and BR signalling during regulation of lamina inclination.

**OsARF19 links to both auxin and BR signalling**

Although several mutants revealing increased leaf angles have been reported, the molecular mechanism remained to be revealed, especially in respect to the molecular crosstalk between auxin and BR signalling. Here, we summarize that both pathways – OsARF19-OsGH3-5 and OsARF19-OsBRI1 – are involved in regulating leaf inclination (Fig. 9).

In the OsARF19-OsGH3-5 pathway, miR393 negatively regulates expression of OsTIR1, and OsTIR1 interacts with the OsIAA1 proteins (Bian et al. 2012); OsIAA1 also interacts with OsARF19 (Shen et al. 2010), which binds to the OsGH3-5 promoter, positively regulating OsGH3-5. Finally, OsGH3-5 overexpression impairs auxin response and reduces free auxin contents in the lamina joint (Fig. 6). In the OsARF19-OsBRI1 pathway, OsARF19 binds to the OsBRI1 promoter, positively regulating OsBRI1 to activate a BR signal transduction pathway, which regulates the expression of the related gene OsBZ1 and its downstream genes (Zhu et al. 2013) to affect lamina inclination. Therefore, apparently both described above co-modulate plant architecture, suggesting that the regulatory mechanism of the rice leaf angle might be more complicated than thought. Noteworthy, OsARF23(1) also interacts with OsIAA1 and antisense-OsARF23(1) line showed similar phenotype with OsARF19-O1 (Attia et al. 2009; Song et al. 2009). Whether OsARF23(1) also binds to OsGH3-5 as well as to OsARF19, and negatively regulates their expression during control of leaf angle, needs to be further investigated in the future.

Taken together, our physiological experiments, genetic analysis, cellular biological observation and molecular biological evidence support the finding that OsARF19 functions in bridging the molecular network of auxin and BR signalling. Moreover, underlying mechanisms might turn out to be useful for engineering rice architecture to culture high rice yield. In high-density plantings, characteristic semi-dwarf phenotype with erect leaves and shorter panicles was an ideal phenotype to improve grain yield (Morinaka et al. 2006; Sakamoto et al. 2006; Wu et al. 2008). osarf19/osgh3-5 double mutant and osgh3-5/osgh3-1 or -2 or -13 double or multiple

**Figure 8.** Morphological response of OsARF19-O1 and -O2 to 24-epibrassinolide (24-eBL). (a) Coleoptile elongation response to 24-eBL treatments. Panels from left to right show coleoptiles of 7 d seedlings of WT/NIP, OsARF19-O1 and OsARF19-O2 grown in the presence of 0, 0.001, 0.01, 0.1 and 1 µm 24-eBL under dark condition, respectively. Bar = 2 cm. (b) Primary root (PR) response to 24-eBL treatments. Panels from left to right show each 2 cm lamina joints from top to bottom of 8 d seedlings of WT/NIP, OsARF19-O1 and OsARF19-O2 grown in the presence of 0, 0.001, 0.01, 0.1 and 1 µm 24-eBL for 3 d under dark condition, respectively. Bar = 2 cm. (c) Leaf angle of response to 24-eBL treatments. Ten rice lines were measured for each treatment. Bars indicate standard deviation (n = 10). * indicates significant difference at P < 0.05 and ** indicates P < 0.01. (e) Comparison of the expression of genes related to leaf angle in BR signalling among WT/NIP, OsARF19-O1 and OsARF19-O2.

**Figure 9.** A proposed model for the role of OsARF19 in the regulation of rice leaf angle. ↓ indicates positive and negative modes of regulation; [IAA] indicates auxin concentration.
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Figure S1. Identification of osraf9 mutant.
Figure S2. Analysis of AuxRE elements in promoters of OsGH3 genes and OsBRI1.
Figure S3. Identification of OsGH3-5-overexpression lines.
Figure S4. Identification of the ochg3-3 mutant.
Figure S5. Expression pattern of OsGH3-5 in rice NIP.
Figure S6. OsARF19 expression in OsMiRNA393-overexpression lines and osafb2 (OsTIR1-RNAi) mutant.
Figure S7. ChIP assay and yeast one hybrid of OsARF19 and OsBRI1.
Table S1. Statistical data of phenotypical characterization in WT/NIP, OsARF19-overexpression lines, WT/DJ and osarf19.

Table S2. Primer sequences for OsARF19 gene.
Table S3. Primer sequences for OsGH3 genes.
Table S4. Primer sequences for ChIP-PCR analysis.
Table S5. Primer sequences for yeast one-hybrid assay.
Table S6. Primer sequences for co-expression analysis.
Table S7. Primer sequences for qRT-PCR analysis.