Down-regulation of a LBD-like gene, OsIG1, leads to occurrence of unusual double ovules and developmental abnormalities of various floral organs and megagametophyte in rice

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Abstract

The indeterminate gametophyte1 (ig1) mutation was first characterized to modulate female gametophyte development in maize (Zea mays). However, the function of its rice orthologue, OsIG1, remains unknown. For this, we first analysed OsIG1 localization from differential tissues in rice. Real-time quantitative PCR (qRT-PCR) and histochemical staining results demonstrated that the expression signal of OsIG1 was strongly detected in young inflorescence, moderately in mature flower and weakly in leaf. Furthermore, RNA in situ hybridization analyses exhibited that OsIG1 was strongly expressed in inflorescence meristems, floral meristems, empty-glume- and floret- primordia, especially in the primordia of stamens and immature ovules, and the micropylar side of the mature ovary. In OsIG1-RNAi lines, wrinkled blade formation was accompanied by increased leaf inclination angle. Cross-section further showed that the number of bulliform cells located between the vasculatures was significantly increased, indicating that OsIG1 is involved in division and differentiation of bulliform cell and lateral growth during leaf development. OsIG1-RNAi suppression lines showed pleiotropic phenotypes, including degenerated palea, glume-like features and open hull. In addition, a single OsIG1-RNAi floret is characterized by frequently developing double ovules with abnormal embryo sac development. Additionally, down-regulation of OsIG1 differentially affected the expression of genes associated with the floral organ development including EG1, OsMADS6 and OsMADS1. Taken together, these results demonstrate that OsIG1 plays an essential role in the regulation of empty-glume identity, floral organ number control and female gametophyte development in rice.

Key words: Embryo sac, empty glume, female gametophyte, OsIG1, ovule.

Introduction

The LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE (LBD/ASL) proteins (hereafter referred to as LBD) were identified as plant-specific transcription factors harbouring a conserved LATERAL ORGAN BOUNDARIES (LOB) domain (Ori et al., 2000; Semiarti et al., 2001; Chalfun-Junior et al., 2005; Borghi et al., 2007). The LOB domain includes a zinc-finger motif responsible for DNA binding and a leucine-zipper-like motif mediating
protein–protein interactions (Husbands et al., 2007). The LBD family comprises 43 members in Arabidopsis (Iwakawa et al., 2002; Shuai et al., 2002), 35 members in rice (Oryza sativa; Yang et al., 2006), and 57 in poplar (Zhu et al., 2007), respectively. Previous studies indicated that LBD proteins play a vital role in determining of leaf adaxial–abaxial polarity in Arabidopsis and maize (Lin et al., 2003; Xu et al., 2003; Evans, 2007) through repressing the expression of KNOTTED-LIKE HOMEBOX (KNOX) genes in lateral organs (Ort et al., 2000; Semiarti et al., 2001; Chalfun-Junior et al., 2005; Borghi et al., 2007; Evans, 2007). Additionally, several LBD genes, such as RTCS in maize and Crown rootless1 in rice, were implicated in the modulation of crown and lateral root development in an auxin-dependent pathway (Inukai et al., 2005; Liu et al., 2005; Okushima et al., 2007; Lee et al., 2009).

It has also been demonstrated that LBD proteins are involved in reproductive organogenesis as well as vegetative growth (Xu et al., 2003; Evans, 2007). For instance, in maize, the ramosa2 gene containing an LOB domain was revealed to determine florescence branch formation (Bortiri et al., 2006). The maize indeterminate gametophyte1 (ig1) gene was found to regulate female gametophyte development (Evans, 2007). The ig1 mutant fails to restrict the proliferative phase in the embryo sac, resulting in an indeterminate number of eggs, polar nuclei and synergids (Evans, 2007). The rice LBD gene degenerated hull1 (DHI) is required for glume formation, as demonstrated by the phenotype of its T-DNA insert mutant with degenerated hull and naked pistils and stamens (Li et al., 2008). Despite substantial investigations, little is known about the links between LBD proteins and floral organ development.

In angiosperms, the ovule is located within the pistil, which consists of one or more carpels. As the female reproductive structures, ovules are eventually developed into seeds to generate new population of offspring. By far, only a few genes which regulate ovule and carpel development have been characterized in plants (Lopez-Dec et al., 1999; Pagnussat et al., 2004; Skinner et al., 2004). Most of the isolated ovule identity genes belong to MADS-box genes. In Arabidopsis thaliana, cell fate in proliferating ovule primordia is specified by the MADS box gene STK (SEEDSTICK; Pinyopich et al., 2003). In rice, a loss-of-function mutant of D-lineage MADS-box gene OsMADS13 had the ovule replaced with carpelloid structures (Dreni et al., 2007; Yamaki et al., 2011). Both the YABBY gene DROOPING LEAF (DL) and MADS-box gene OsMADS3 were shown to genetically interact with OsMADS13 in the regulation of ovule identity and floral meristem determinacy (Li et al., 2011a). Furthermore, OsMADS6 and OsMADS58 are genetically associated with rice AGAMOUS (AG) subfamily genes to regulate the identity of stamens, carpels and ovules (Dreni et al., 2011; Li et al., 2011b). However, LBD family proteins potentially involved in the regulation of ovule development have not been identified.

Although rice displays a high degree of conserved synteny with maize, it remains unclear whether LBD genes are also involved in mediating female gametophyte development and other unknown functions in rice. In this study, we characterized a LBD gene OsIG1 from rice (O. sativa), an orthologue of maize IG1, and uncovered its novel roles in regulating floral organ development, in addition to its conserved function in female gametophyte development, using genetic and molecular approaches. We further revealed that OsIG1 might participate in mediating floral development through regulating the expression of some developmentally associated genes including jasmonic acid-synthesizing gene EGI (EXTRA GLUMEL), AGAMOUS-LIKE6-like gene OsMADS6 and E-class gene OsMADS1. Hence, our results allowed us to infer previously undescribed regulatory roles for OsIG1 in rice.

### Materials and methods

#### Plant materials and growth conditions

Rice transgenic lines in a Nipponbare (O. sativa ssp. japonica) background and wild-type (WT) Nipponbare were used throughout this study. The plants were grown in farm fields at Sichuan University, Chengdu, China. Primary transformants (T0) were first grown in the artificial climate incubators (Binder, Tuttlingen, Germany) under standard conditions (28 °C day/20 °C night, 12 h light/12 h dark) and transplanted into the field 5 weeks later.

#### Plasmid construction and rice transformation

To generate an RNAi construct, a 485 base pair (bp) OsIG1 fragment (Os01g0889400; positions 461–810 from the ATG of the coding region plus 135 bp of the 3’ untranslated region) was amplified by RT-PCR. An inverted-repeat fragment was constructed into vector pSK int and transferred into the pHb plasmid. To produce the OsIG1 promoter::GUS fusion construct, the open-reading-frame sequence of β-glucuronidase (GUS) gene was obtained from pBII121 by digesting with BamHI and SacI. Then the obtained fragment was inserted into pHb and the resulting construct was denoted GUS-Phb. About 2.1 kb of the OsIG1 gene fragment upstream of the translational start codon was isolated by PCR from Nipponbare genomic DNA (primers are listed in Supplementary Table 1). Products were digested with EcoRI and BamHI and ligated into EcoRI/BamHI-digested GUS-Phb to generate plasmid pOsIG1-GUS. The constructs were used to transform Nipponbare callus using engineered Agrobacterium tumefaciens (strain EHA101) as described previously (Hiei et al., 1994).

#### Tissue preparation and microscopic analyses

Fresh tissues from WT and RNAi plants were analysed with a stereoscopic Leica M420 microscope (Leica Microsystems, Bannockburn, IL, USA) equipped with Leica DFC280 digital camera system. For histological analysis, samples were fixed in FAA solution (3.7% formaldehyde/50% ethanol/5% glacial acetic acid), dehydrated with a series of ethanol solutions, embedded in paraffin and cut into sections 8 μm thick. The sections were stained with ferravanadium hematoxylin. To evaluate the viability of OsIG1-RNAi pollen grains, anthers from transgenic mature flowers were dissected in a drop of 1% I2/KI solution. The sections and pollen grains stained were photographed using a Leica DFC280 camera. For scanning electron microscopy, samples were fixed in 2.5% glutaraldehyde solution. Fixed samples were dehydrated with graded ethanol series, dried by critical-point drying, sputter-coated with platinum and observed using a scanning electron microscope [JSM-5900LV; SEMTech (STS), North Billerica, MA, USA].

#### RT-PCR and real-time quantitative PCR analysis

Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by treatment with DNase I to digest
genomic DNA (TaKaRa, Dalian, China). About 1 μg of total RNA from each sample was used for first-strand cDNA synthesis (Toyobo, Tokyo, Japan). Triplicate quantitative assays were performed using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) with an ABI PRISM Step One Plus real-time PCR system (Applied Biosystems) according to the manufacturer’s instructions. The relative expression level was determined based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by using rice actin and ubiquitin as internal reference genes. Primers used for real-time quantitative PCR (qRT-PCR) analysis are listed in Supplementary Table 1.

In situ hybridization and histochemical staining

The hybridization and immunological detection were performed as described by Dai et al. (2007). The gene-specific regions of OsIG1 were amplified and cloned into pGEM-T Easy (Promega, Madison, WI, USA) for synthesis of an RNA probe; the primers used are shown in Supplementary Table 1. Digoxigenin-labelled sense and antisense probes were synthesized with T7 or SP6 RNA polymerase (Roche, Indianapolis, IN, USA), respectively. GUS staining was performed according to the method described previously (Sieburth and Meyerowitz, 1997).

Subcellular localization analysis

The coding sequence of the OsIG1 gene was directionally cloned into the expression vector pART27-mcs-GFP (kindly provided by Professor Shuqing Cao, Hefei University of Technology, Hefei, China), containing the green fluorescent protein (GFP) coding sequence under the control of a 35 S promoter. This generated the pART27-OsIG1-GFP construct. Plasmid vectors pART27-OsIG1-GFP and pART27-GFP were introduced into protoplasts of Arabidopsis according to the methods described previously (Yoo et al., 2007). After incubation for 16–20 h at 23 °C, protoplasts were observed under a Leica TCS SPII confocal microscope using 488 and 633 nm excitation wavelengths and three-channel measurement of emission: 435 nm (blue/DAPI), 522 nm (green/GFP) and 680 nm (red/chlorophyll).

Results

The expression pattern of OsIG1

According to the previous information (Evans, 2007) and database (http://rapdb.dna.afrc.go.jp/), the genomic sequence of OsIG1 (Os01g0889400) spans 3399 bp, and contains three introns and four exons (Supplementary Fig. 1A). It has a deduced coding protein of 270 amino acids with a conserved LOB domain containing C block, GAS block and the predicted coiled-coil structure (Supplementary Fig. 1B; Evans, 2007). Phylogenetic analysis showed that OsIG1 is closely related to maize IAL1, rice IAL1, and Arabidopsis AS2 (Supplementary Fig. 1C; Evans, 2007).

To investigate the function of OsIG1, we analysed the OsIG1 expression pattern using qRT-PCR, promoter-GUS fusions and in situ hybridization. First, qRT-PCR analysis demonstrated that OsIG1 was strongly expressed in the young inflorescence, moderately in mature flower and weakly in leaf (Fig. 1A). OsIG1 expression was lowest in the root and stem relative to other tissues examined (Fig. 1A). To further confirm this result, a 2.1 kbp promoter region of OsIG1 was used to direct GUS expression in transgenic rice. As a result, GUS activity was mainly detected in young inflorescence (Fig. 1B), empty glumes and floret organs (Fig. 1C–E), such as in palea, lemma (Fig. 1C) and pistil (Fig. 1D) during early spikelet development stages. Additionally, GUS activity in the lodicules (Fig. 1E), filaments (Fig. 1E) and leaf (Fig. 1G) was observed as well. Furthermore, the elongating internode also showed a strong GUS signal in the transgenic plant driven by the OsIG1 promoter (Fig. 1F), whereas very weak signal was detected in root (Fig. 1G). Overall, the expression patterns of OsIG1 detected by qRT-PCR were consistent with the GUS signal shown in the transgenic lines.

To more precisely determine the spatial and temporal patterns of OsIG1 expression in floral organs, we performed RNA in situ hybridization analysis. Strong signals of OsIG1 were detected in the inflorescence meristems (Fig. 1H, I), floret primordia (Fig. 1J–L) and the empty glume (Fig. 1J–N). During organogenesis of florets, OsIG1 transcripts were also detected in the primordia of stamens and ovules (Fig. 1L, M). Notably, OsIG1 showed constitutive expression in nucellus (Fig. 1P) and later the detected signal was restricted to the micropylar end of the mature ovaries (Fig. 1Q). During embryogenesis, the OsIG1 transcripts were detected throughout various stages of embryo development (Fig. 1R–T). As controls, no signals were detected in floral meristems and floral organ primordia with the sense probe (Fig. 1U, V).

To determine experimentally subcellular localization, OsIG1 was fused in frame with GFP and transiently expressed in Arabidopsis mesophyll protoplasts. As expected, we found that the OsIG1-GFP fusion protein was unambiguously localized in the nucleus (Fig. 2), confirming that OsIG1 is a nuclear protein.

Alteration of floral development in OsIG1-RNAi repression plants

Because of the obvious expression of OsIG1 in the floral tissues, we investigated the potential effects of OsIG1 on rice floral development by constructing an inversely repeated RNAi vector (Supplementary Fig. 2A). Over 20 independent transgenic lines were obtained. Three representative transformants with 60–68% reduction of endogenous OsIG1 expression level were selected for detailed phenotypic analysis (Supplementary Fig. 2B). To determine the specificity of OsIG1 silencing, we searched the rice genomic sequence database Rice BLAST (http://RiceBLAST.dna.afrc.go.jp/) using the 485 bp RNAi fragment, and found that four genomic loci (three loci located on Os02g0318851 and one locus located on Os05g34450) with continuous 21–24 bp short sequence identities to the RNAi fragment. The RNAi fragment showed 27% identity to OsIAL1 (Os05g0417000) and 34% to Os02g0318851. Using qRT-PCR analysis we found that the expression pattern of the OsIAL1 gene, with highest abundance in young inflorescence (Supplementary Fig. 3A), is similar to that of OsIG1 (Fig. 1A). Subsequent measurement of expression levels for Os05g0417000 and Os02g0318851 revealed that their expression was nearly unaffected in the down-regulated OsIG1 lines (Supplementary Fig. 3B, C). These results suggest there was only a low probability of incorrect targeting caused by OsIG1-RNAi and that the observed phenotypic alterations most likely resulted from down-regulation of OsIG1 alone.
Next, we examined the effects of OsIG1 deficiency on plant floral development in detail. Generally, a typical WT rice floret normally consists of a pistil in a central whorl (whorl 4), six stamens in whorl 3, two lodicules adjacent to the lemma in whorl 2 and two interlocking organs, palea and lemma, surrounding the inner floral organs in whorl 1. A floret with two pairs of sterile glumes (rudimentary glumes and empty glumes) at its base, constitutes a spikelet (Fig. 3A, B). Compared with WT, OsIG1-RNAi plants exhibited a wide variation in the number and position of floral organs as well as glumes (Fig. 3C–K). In the RNAi spikelets, empty glumes (also called sterile lemmas) were elongated and widened, resembling lemma/palea in shape and size (Fig. 3C).

In addition to increased numbers of empty glumes (Fig. 3D) and extra glume-like organs (Fig. 3E), it was found that the formation of velum-like organs occurred in RNAi lines (Fig. 3F). In whorl 1, the paleae of the RNAi plants exhibited severe defects, including some smaller paleae (Fig. 3G) and others underdeveloped or distorted (Fig. 3H). In whorl 3, some lodicules were often fused at the base and transformed into carpel-like organs (Fig. 3I), suggesting that OsIG1 may be required for lodicule specification in rice. In whorl 3, the filament-plumed stigma chimeras coupled with reduced stamen numbers were also visualized in approximately 5% spikelets of the RNAi lines (Fig. 3J). The OsIG1-RNAi ovaries (Fig. 3K) were clearly swollen compared to WT (Fig. 3B).
Compared with WT, besides the abnormal morphology of floral organs, OsIG1-RNAi plants displayed altered inflorescence shape with elongated and curved peduncles (Fig. 3G, L). To characterize the observed morphological defect in more details, histological analyses were performed. In WT plants after the carpel primordium arises at the floral meristem near the lemma side, a lateral region of the floral meristem adjacent to the carpel initiation transformed into an ovule primordium (Fig. 4A) in which megaspore mother cell (Fig. 4B) occurs to form an embryo sac, the female gametophyte (Fig. 4C). By contrast, the size of the OsIG1-RNAi floral meristem at the early development stage of the pistil was larger than that of the WT (Fig. 4D–F). Also, the ovule primordia initiated on both the palea and the lemma side of floral meristem (Fig. 4G, H). Surprisingly, unusual double ovules, fused (Fig. 4G, H) or not fused (Fig. 4I, J), occurred in approximately 40% of the transgenic ovaries (Table 1). In addition, OsIG1-RNAi repression showed pleiotropic effects on female gametophyte differentiation, resulting in a variety of abnormally developed embryo sacs including embryo sac degeneration (Fig. 4K), an embryo sac with abnormal polar nuclei (Fig. 4L1, L2) and an embryo sac with an extra egg cell (Fig. 4M1, M2), as well as other abnormal embryo sacs, such as an embryo sac without a female germ unit (Fig. 4N), an embryo sac with abnormal nuclear migration (Fig. 4O) and an abnormally small embryo sac (Fig. 4P). The frequencies of various types of abnormalities are summarized in Table 1, with the RNAi lines showing 7–11% embryo sacs with abnormal polar nuclei, 27–29% embryo sac degeneration, 6–9% embryo sacs with an extra egg cell and 20–26% other abnormal embryo sacs.

Although the palea is similar in its outer morphology in rice, a few critical differences exist: the lemma has five vascular bundles, whereas the palea has three vascular bundles and consists of two parts [the body of the palea (bop) and two marginal regions of the palea (mrps)]. The bop shares similar cellular morphology with lemma (Prasad et al., 2005), but the mrp has a distinctive smooth epidermis specific to palea (Fig. 4Q, R; Chen et al., 2006). In approximately 12% of the RNAi spikelets the elongated empty glumes had two vascular bundles as opposed to one in the WT (Fig. 4S–U). Interestingly, most of the degenerated palea (80%) exhibited reduced bop in size and nearly normal mrp (Fig. 4V). And about 6% degenerated palea lacked bop and retained only mrp-like structures with nonsilicified upper epidermis and without trichomes and protrusions (Fig. 4W, X), suggesting that the development of the bop of palea was severely affected in the RNAi lines. Additionally, all of RNAi lines displayed semi-sterile phenotype (the seed setting rate was approximately 38.6%).

To determine the nature of the reproductive defect found in the RNAi lines, we conducted reciprocal crosses and I2/KI staining assays for pollen from both WT and the RNAi lines. The results showed that offspring from the control cross (WT♀×WT♂) exhibited 86–91% seed-set fertility, while the pollination of WT pistils with pollen from RNAi plants produced seed-set rates ranging from 78 to 83% fertility. However, when using RNAi plants as the maternal recipient pollinated with WT pollen (RNAi♀×WT♂), the seed setting rates were scored as 46, 51 and 53% in OsIG1-RNAi repression panicles. Also, I2/KI assay showed that pollen from both WT (Supplementary Fig. 4A) and RNAi plants (Supplementary
RNAi plants exhibited defects in meristem maintenance as well as floral organ development

To study the identity of the abnormal organs observed in the RNAi lines, we examined spikelets at the heading and flowering stage by scanning electron microscopy. Compared with the smooth outer epidermal surface of WT empty glumes (Fig. 5A, B), epidermal cells of the elongated empty glumes in the OsIG1-RNAi lines displayed bulges and bristles that are similar to the phenotype of eg1 mutation and ectopic expression of OsMADS1 in rice (Fig. 5C, D; Jeon et al., 2000; Prasad et al., 2001; Li et al., 2009). In OsIG1-RNAi lines, the extra hull-like organs showed a similar upper epidermis, resembling the WT lemma (Fig. 5E–G). In some cases, the palea was degenerated to form mrp-like structures (Fig. 5G). In other cases, an additional lemma-like organ occurred in place of OsIG1-RNAi palea (Fig. 5H), or OsIG1-RNAi spikelets lacked palea (Fig. 5I), suggesting that the development of palea was severely affected.

To further characterize the developmental defects of the OsIG1-RNAi spikelet, we investigated the flower morphology...
Fig. 4. Histological analyses of WT and OsG1-RNAI flowers. (A–C) Pistil development in the WT. A carpel protruded from the lemma side of the floral meristem (A). When the carpel completely enclosed the ovule primordium, a megaspore mother cell became visible and an integument primordium was differentiated in the ovule primordium (B). In a mature ovule, a vacuolated embryo sac was visible (C). (D) When a carpel protruded, the floral meristem in OsG1-RNAI (D) was larger than that of the WT (A). (E, F) Close-up of floral meristem shown in the WT (A) and OsG1-RNAI (D), respectively. (G–J) Unusual double ovules fused (G, H) or not fused (I, J) occurred in the transgenic ovaries. (K) Embryo sac degeneration in OsG1-RNAI ovule. (L1, L2) Serial section showing OsG1-RNAI embryo sac with extra polar nuclei (arrows). (M1, M2) Serial section showing OsG1-RNAI embryo sac with possible multiple egg cells (arrowheads), extra synergids (triangular arrows) and polar nuclei located in an abnormal position close to the chalazal end (arrows). (N) Embryo sac without female germ unit (arrowhead). (O) Embryo sac with abnormal nuclear migration (or abnormal position of egg cell and synergid cells; arrowhead). (P) Abnormally small embryo sac. (Q–S) Cross-sections of a WT spikelet. (R) Magnification of the area within the box on Q showing interlocking of lemma and palea. (S) The WT empty glume with one vascular bundles (asterisk). (T–X) Cross-sections of the OsG1-RNAI spikelets. (T, U) Two vascular bundles in the OsG1-RNAI empty glumes (asterisks in T), as opposed to one in WT (asterisks in S). Edges of lemma and palea are hooked in the WT (Q), but there is no interlock with the lemma in the OsG1-RNAI spikelet (U–X). (V) An abnormal flower with hull-like organs and two vascular bundles of empty glume (asterisks) in an OsG1-RNAI flower. (W) The OsG1-RNAI spikelet has only two full-developed vascular bundles (asterisks) in degenerated palea. (X) The OsG1-RNAI spikelet containing two separate carpels of OsG1-RNAI; arrowheads indicate the degenerated palea. (X) The degenerated palea (arrowhead) lacking a bop and retaining mrp-like structures in an OsG1-RNAI spikelet. ca, primary carpel; dp, degenerated palea; eg, empty glume; fm, floral meristem; le, lemma; lo, lodicule; mrp, marginal region of palea; pa, palea. Scale bars, 100 μm (A–D, Q–X), 50 μm (E–P).
of down-regulated OsIG1 transgenic lines at early stage by scanning electron microscopy. During the spikelet 6 stage (Sp6), the WT flower formed six stamen primordia, and it was apparent that the lemma with bumps at the top was larger than the palea (Fig. 5J). However, some OsIG1-RNAi spikelets developed extra lemma- or palea-like or glume-like organs. (Fig. 5) Scanning electron micrographs of WT and OsIG1-RNAi spikelets. (A) A WT spikelet. (B) Epidermal surface of an empty glume in the WT. (C) An OsIG1-RNAi spikelet with elongated empty glume (arrow). (D) Close-up view of the epidermal surface of the elongated empty glume in (C) showing a empty-glume-like surface in the central region and a lemma-/palea-like surface in the lateral region. (E) Epidermal surface of the lemma, palea and mrp in the WT. (F) An OsIG1-RNAi spikelet with an extra hull-like organ between the lemma and palea. (G) An OsIG1-RNAi spikelet with hull- or velum-like organs. (H) An OsIG1-RNAi spikelet with an extra lemma instead of the palea. (I) An OsIG1-RNAi spikelet with lacking palea. (J) WT flowers with six stamen primordia and a flat floral meristem. (K–P) OsIG1-RNAi flowers. (K) Showing one palea-like and one extra lemma primordium. (L) One abnormal flower with ectopic palea-like and extra lemma-like structures. (M) OsIG1-RNAi flower producing a stamen primordium ectopically in the centre of the floral meristem (triangular arrow), ectopic lodicule (arrow) and palea-like primordium. (N) One abnormal lemma shape. (O) At the late stage of stamen initiation, the floral meristem in OsIG1-RNAi lines remained bulged instead of being flat. (P) The floral meristem is uneven and bifurcated (arrow and arrowhead). eg, empty glume; el, extra lemma-like structure; fm, floral meristem; gl, glume-like organ; hl, hull-like organ; le, lemma; mrp, marginal regions of the palea; pa, palea; pal, palea-like organ; st, stamen; vl, velum-like organ. Asterisks indicate stamen primordia. Scale bars, 1 mm (A, C, F–I), 100 µm (B), 200 µm (E, D), 50 µm (J–P).
structures (Fig. 5K–M). At the stage of stamen initiation, the floral meristem in WT flowers tended to be flat (Fig. 5I; Itoh et al., 2005). By contrast, the floral meristem in RNAi lines still bulged (Fig. 5N, O), suggesting that floral meristem determinacy was severely affected. Occasionally, floral meristem was doubled in one spikelet (Fig. 5P), suggesting defects in floral meristem determinacy. Together these observations indicated that down-regulated OsIG1 triggered abnormal spikelet development at an early stage, including the formation of ectopic floral organs, changes in organ number and alteration of floral meristem determinacy.

Alteration of vegetative development in OsIG1-RNAi plants

In addition to the altered morphology of reproductive organs, we also observed abnormal vegetative development by OsIG1-RNAi repression with reduced plant height and increased leaf inclination angle. Plant height for 20 field-grown individuals from each genotype was determined at the mature stage, and the average plant heights of the WT control, RNAi-1, RNAi-2 and RNAi-3 lines were 99.1, 90.7, 84.0 and 89.8 cm, respectively (Fig. 6A), indicating a slightly reduced internodal elongation conferred by down-regulated OsIG1 expression. Distinctly, an increased flag leaf inclination angle resulting from OsIG1-RNAi repression was visualized at the heading stage. Leaf angle is the angle of inclination between the leaf blade and the vertical culm (Zhao et al., 2010). The lamina joint is a whitish region at the base of the blade, which joins the rice leaf blade and sheath and functions in bending the leaf blade toward the abaxial side. In the RNAi lines, the bending of the leaf blade occurred at the lamina joint. As shown in Fig. 6B, the leaf angle of the RNAi lines was much greater than that of the WT and the angle of leaves was increased and reached a maximum of approximately 44 °, whereas the leaf angle of WT plants appeared to plateau at approximately 16 °.

It has been reported that maize ig1 mutant leaves have disrupted adaxial-abaxial polarity and fail to repress the expression of meristem-specific KNOX genes in leaf primordia, causing a proliferative, stem cell identity to persist in these cells (Evans, 2007). Occasionally, as shown in Fig. 6C, some leaves of OsIG1-RNAi lines exhibited wrinkled blades, resembling the phenotype observed in plants overexpressing the class I KNOX gene OSH1 (Sato et al., 1996), but no alteration of OSH1 expression was detected in the leaves with the mutant phenotype in the OsIG1-RNAi lines (data not shown). Compared with the WT (Fig. 6D), the number of bulliform cells located between the vasculatures was significantly increased in the RNAi lines (Fig. 6E). In addition, these cells did not show a balloon-like form as occurring in the WT (Fig. 6E). No obvious difference in other leaf cell types or alteration in adaxial-abaxial polarity was observed between the RNAi lines and WT (Fig. 6D, E), indicating that the wrinkled blade phenotype may be caused by the increase in bulliform cell number. Together these results suggested that the OsIG1 gene is involved in division and differentiation of bulliform cell and lateral growth during leaf development.

Down-regulation of the OsIG1 gene affects the expression of the EG1, OsMADS6 and OsMADS1 genes

Recently, a number of plant genes involved in floral organ development have been identified. We asked whether the OsIG1 gene mediates floral development by regulating

![Fig. 6. Vegetative phenotypic analyses of OsIG1-RNAi transgenic lines. (A) Plant height (cm). Student’s t test was used to analyse significant differences between WT and OsIG1-RNAi transgenic lines. Values are means ± SE from 20 plants. **P < 0.01 (t test). (B) Showing the increased leaf angle in OsIG1-RNAi transgenic lines. Arrows indicate the leaf lamina joint. (C) Some leaves of OsIG1-RNAi lines showing wrinkled blades, which was partially similar to that of plants overexpressing the class I KNOX gene OSH1. (D, E) Cross-sections of a WT (D) and OsIG1-RNAi (E) leaf blade. Arrows indicate bulliform cells. Scale bars, 50 µm. A colour version of this figure is available at JXB online.](image-url)
Discussion

OsIG1 may function to maintain empty-glume identity

Regarding the origin and evolution of the sterile lemma, there are two controversial hypotheses. One hypothesis considers that an ancestral rice spikelet has three florets, including one fertile floret and the other two degenerated sterile florets with only the lemmas remaining (Kellogg, 2009). The empty glumes thus would seem to be just the remnants of the two lower reduced florets (Yoshida et al., 2009; Kobayashi et al., 2010). Another hypothesis states that the rice spikelet has only one floret, and the sterile lemma and rudimentary glume are generally regarded as severely metamorphic bract structures (Schmidt and Ambrose, 1998; Terrell et al., 2001; Hong et al., 2010). Recently, several mutants and their underlying genes related to the development of glume-like organs have been identified in rice, including eg1 (Li et al., 2009), long sterile lemma (g1; Yoshida et al., 2009) elongated empty glume (ele1; Hong et al., 2010) and osmads34 (Gao et al., 2010). Among these, glele1 and osmads34 mutants displayed the transformation of sterile lemmas into lemma-like structures (Yoshida et al., 2009; Hong et al., 2010; Gao et al., 2010), suggesting that G1/ELE1 and OsMADS34 repress lemma identity at the sterile lemma positions during rice spikelet development (Yoshida et al., 2009; Hong et al., 2010). Unlike glele1 and osmads34 mutants, eg1 mutation severely affects its number as well as sterile lemmas size (Cai et al., 2014). These observations suggest that different genes are probably involved in the pathway of empty-glume development. In our OsIG1-RNAi lines, we observed an increased number of empty glumes, and in some cases the empty glumes were converted into glume-like organs and the increased number of empty glumes occurred (Fig. 3D). Similar to the studies on EGI, OSMADS34 and G1/ELE1, our data for OsIG1 seems supporting the first hypothesis.

Additionally, nuclear localization of IG1 and its expression at the early developmental stage of empty glumes suggests that IG1 may achieve this function by targeting downstream genes needed for empty-glume growth. As in the loss-of-function eg1 mutation, the spikelets of OsIG1-RNAi lines developed extra glume-like structures (Fig. 3E; Li et al., 2009). In addition, in OsIG1-RNAi lines, empty glumes were elongated and widened, resembling the lemma/palea in shape and size, a phenotype similar to that reported by Prasad et al. (2001, 2005), where OSMADS1 over-expression created a lemma-like epidermis in the transformed glumes. Intriguingly, these phenotypic alterations appear to be consistent with EGl down-regulation and OSMADS1 up-regulation observed in OsIG1-RNAi repression lines (Fig. 7A, C). Our data showed that OsIG1-RNAi repression (by 35 S promoter) could greatly enhance expression of OSMADS6 and OSMADS1 (Fig. 7B, C), and we believe that the abnormal organ development occurring in OsIG1-RNAi lines could be ascribed to, at least in part, the constitutive/ectopic expression of these MADS genes (Fig. 3C, I, J). Also, our qRT-PCR analysis showed that the expression level of EGI was remarkably decreased in all of three OsIG1-RNAi repression lines relative to WT (Fig. 7A), indicating that EGI might function downstream of OsIG1. Nevertheless, Li et al. (2009) and Cai et al. (2014)
reported that EG1 positively regulates expression of the floral meristem and organ identity gene OsMADS1, seemingly contradictory to our expression data for EG1 and OsMADS1 in the OsIG1-RNAi lines. However, this discrepancy implicates the complexity of floral organ gene expression and regulation under various contexts of background. Actually, Cai et al. (2014) observed a significant reduction of OsMADS1 expression in 2 mm inflorescence of EG1-defective lines, but a large portion of this reduction disappeared in the 3–4 mm inflorescence, suggesting their interaction is spatially and temporarily regulated. Different from these observations, opposite expression alteration of OsMADS1 and EG1 was visualized in the background of OsIG1-RNAi lines (Fig. 7A, C). We thought it is probably the different background that should be responsible for triggering the unusual activation/repression of gene expression. Although OsMADS1 has been shown as a positive target of EG1, it can not be ruled out that OsMADS1 acts as a target protein directly or indirectly regulated by other regulatory proteins, such as OsIG1 in the current study or other unidentified regulators. Therefore, our results point out that the effects of OsIG1-RNAi repression are probably not limited to EG1 function and that OsMADS1 as well as its unknown upstream genes also could be activated/repressed in an EG1-independent pathway in OsIG1-RNAi lines. It is indeed interesting that down-regulation of OsIG1 results in up-regulation of OsMADS1 expression in these RNAi lines, suggesting that there may be interplay between OsIG1 and the MADS box to mediate certain development cascades in an EG1-independent pathway. Nevertheless, further investigations are required to examine whether this interaction genuinely exists in the regulation of rice floral development. In addition, it is worth mentioning that another characteristic phenotype of the OsIG1-RNAi lines—that is, double ovules—does not occur in loss-of-function eg1 mutant (Li et al., 2009; Cai et al., 2014), suggesting that unidentified downstream genes of OsIG1 other than EG1 could be responsible for the initiation of ovule number control. All these hypotheses need to be further tested in the future.

OsIG1 regulates palea development

At present, several important genes are known to be involved in specifying the identity of the palea. In the mfo1 mutant mrp is absent and recent studies revealed that MFO1/OsMADS6 is expressed in the mrp (Ohmori et al., 2009; Li et al., 2010). Like the mfo1 mutant, in RNAi lines of LHS1/OsMADS1, mrp is absent in the abnormal palea (Prasad et al., 2005). These results imply that OsMADS1 and OsMADS6 specify palea identity by promoting mrp development. In contrast, dp1, sdp1/rep1 and osmds15 mutants all exhibited changes in the bop rather than the mrp (Li et al., 2008; Wang et al., 2010; Yuan et al., 2009). Our findings show that in most of the degenerated palea the development of mrp was normal while the size of bop was reduced in OsIG1-deficient lines in which the E function gene OsMADS1 and the functionally similar E function gene OsMADS6 were up-regulated. Apparently, the altered expression levels of EGI, OsMADS1 and OsMADS6 in OsIG1-RNAi lines contributed to the abnormal formation of the palea. Since the bop tissue alone was affected by OsIG1-RNAi repression, it is possible that the bop and mrp of the palea are controlled by different pathways. Indeed, several genes, including DP1, SDP1/REPI and OsMADS15, have been reported to determine bop identity (Li et al., 2008; Yuan et al., 2009; Wang et al., 2010). Furthermore, LHS1/MADS1 and MFO1/MADS6 have been demonstrated to be involved in the regulation of mrp identity (Prasad et al., 2005; Ohmori et al., 2009). Additionally, DEGENERATED HULL1 (DHI), a LOB family transcription factor gene, was found to affect both palea and lemma formation (Li et al., 2008; Wang et al., 2011). Thus, it is possible that various genes, such as E function-like genes (OsMADS1 and OsMADS6), palea-specific genes (OsMADS15, DP1 and SDP1/REPI), as well as the LBD gene OsIG1 are required for the formation of palea.

OsIG1 is required for patterning and growth during ovule development

In the maize ig1 mutant, embryo sacs undergo extra rounds of free nuclear division that result in extra egg cells, extra central cells and extra polar nuclei, and also the mutation shows disrupted adaxial–abaxial polarity of leaf development (Huck et al., 2003). Our work revealed that the down-regulated OsIG1 had a similar disruption to embryo sac development (Table 1, Fig. 4L–P), but did not obviously influence the leaf adaxial–abaxial patterning as in the maize ig1 mutant (Evans, 2007), in spite of the altered inclination angle observed in the RNAi transgenic flag leaves (Fig. 6B). The phenotypic variations might be associated with unchanced meristem-specific KNOX gene expression between the transgenic rice lines and WT (data not shown), compared to the fact that alteration of KNOX gene expression is associated with adaxial–abaxial polarity in maize (Evans, 2007). Alternatively, it is possible that the rice RNAi lines retaining relative high residual OsIG1 transcript activities compared with the maize ig1 loss-of-function mutant might be the cause of the unaffected leaf adaxial–abaxial patterning. Interestingly, besides glume-like features, other unique characteristics occurring in OsIG1-RNAi lines has not yet been reported in rice eg1 and maize ig1 mutants, including abnormal carpels and unusual fused double ovules, suggesting that IG1 functions have diversified between the two species. Although our experiments suggest there is less possibility of incorrect targeting caused by OsIG1-RNAi, we could not completely exclude the possibility that the potential incorrect targeting might have effects on vegetative and reproductive processes. Hence, specific mutated lines (e.g. T-DNA insertion lines) would be a useful tool to confirm the phenotypic abnormalities triggered by OsIG1-RNAi repression.

Meanwhile, alteration of floral organ number has been demonstrated to be closely associated with a change in meristem size (Suzaki et al., 2004). In this study, silencing OsIG1 increased floral meristem size at the stage of carpel protrusion (Fig. 4D, F), resulting in increased ovule number (Fig. 4F, G). Additionally, ovule development was indistinguishable from that of the WT at a very early developmental stage. As shown in Fig. 4G, the change in ovule primordia size was
accompanied by shape modification, evidenced by the unusual ovule primordia initiation existing on both sides of the palea and lemma of the floral meristem (Fig. 4D, G). This observation raises the possibility that the initiation of ovule primordia is disrupted by OsIG1-RNAi repression. Therefore, the OsIG1 protein may play an important role in inhibiting ovule primordium formation on the palea side in rice. Although it is well known that establishment of adaxial–abaxial polarity is essential for lateral organ development, little is known about the mechanisms underlying the polarity establishment in the carpel and ovule. On the basis of these observations, we speculate that although adaxial–abaxial polarity was less affected in the leaves of OsIG1-RNAi plants, the unusual ovule patterning of transgenic lines is presumably due to defects in the regulation of polarity. The polarity of the gynoecium (the lemma versus paleal side) may be lost and the gynoecium forms two ovules instead of one. We propose that the gynoecium might contain two ovaries if the transformation is complete (Fig. 4H, I); however, incomplete transformation may result in the fused ovule phenotype (Fig. 4G, H).

In addition, given that aberrant shapes of ovule primordia were initiated by down-regulation of OsIGI (Supplementary Fig. 2B), we postulated that OsIGI might have an important role in maintaining the proper organization of the floral meristem, which is essential for the initiation of ovule primordium. In other words, OsIGI may regulate carpel and ovule primordium initiation by maintaining the proper floral meristem organization during the early developmental phase in rice. Despite the fact that an increased size of the floral meristem/ovule primordial occurred in OsIG1-RNAi repression, the key genes which regulate ovule primordial initiation need to be identified. On the other hand, in the maize ig1 mutant, the proliferative phase is prolonged, suggesting that the IG1 protein restricts the proliferative phase of female gametophyte development. According to this opinion, it is possible that OsIG1 regulates carpel and ovule numbers by preventing overproliferation of their primordia in rice.

In this study, our results revealed that OsIG1 acts as a key regulator in controlling rice spikelet development. It was reported that IG1 interacts with ROUGH SHEATH2 (RS2), which is the maize orthologue of A15Sl (Evans, 2009). Furthermore, it was shown that members of the basic helix-loop-helix (bHLH) family of transcription factors are capable of interacting with LOB (Husbands et al., 2007). Thus, it would be interesting to elucidate whether these genes are the targets of OsIG1 that may be involved in ovule development. Future identification of the specific interacting factors will shed new light on the molecular mechanism of ovule development in grasses.

**Supplementary data**

Supplementary material is available at JXB online.

**Supplementary Fig. 1.** Structure of OsIG1 and sequence analysis of OsIG1.

**Supplementary Fig. 2.** Constructs and molecular analysis of normal and transgenic plants.

**Supplementary Fig. 3.** Expression patterns of OsIAL1 and the expression levels of OsIG1 and Os02g0318851 in the young panicles of OsIG1-RNAi transgenic lines and WT plants.

**Supplementary Fig. 4.** The I2/K1 staining of pollen grains of the WT (A) and OsIG1-RNAi transgenic lines (B–D).

**Supplementary Fig. 5.** Expression-level comparison of flower-development-related genes between WT and OsIG1-RNAi lines in 1, 5, 10, 15 and 20 cm inflorescences.

**Supplementary Table 1.** Primers used in this study.

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