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Mutations in the rice liguleless gene result in a complete loss of the auricle, ligule, and laminar joint

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Abstract The area between the upper part of the leaf sheath and the basal portion of the leaf blade contains several specialized organs, such as the laminar joint, auricle and ligule. Here we report the identification of T-DNA insertional mutant lines that lack all of these organs. The gene knocked out in the mutant lines encodes a protein that contains a SBP (*SQUAMOSA* promoter Binding Protein)-domain and is highly homologous to the maize *LIGULELESS1* (*LG1*) gene. At the amino acid sequence level, the OsLG1 protein is 69% identical to maize LG1 and 78% identical to barley LG1. We named the rice gene *OsLIGULELESS1* (*OsLG1*). Transient expression of an OsLG1:RFP (Red Fluorescent Protein) fusion protein indicated that the protein is localized to the nucleus. Transgenic plants harboring the *OsLG1* promoter:*GUS* (*β-glucuronidase*) reporter gene construct display preferential expression in developing laminar joint regions and meristemic regions. The gene is also weakly expressed in the ligule, auricles, and leaf sheaths at the basal region. These results indicate that *OsLG1* is a transcriptional factor that plays an important role in building the laminar joint between leaf blade and leaf sheath boundary, thereby controlling ligule and auricle development.

Keywords Laminar joint · Liguleless · Rice · SBP · T-DNA insertion mutant

Introduction

A rice leaf consists of a leaf sheath, a leaf blade, and a laminar joint that contains a pair of auricles and the ligules. The auricle is a hairy and sickle-shaped organ that surrounds the culm, thus keeping the leaf sheath and culm together. The ligule is a thin and tongue-like white membrane that keeps the leaf sheath closed tightly against the auricles to prevent rainwater or dust from entering. The laminar joint is mechanical tissue that bends the leaf blade away from the vertical axis toward the abaxial side when the leaf blade and leaf sheath are fully elongated (Hoshikawa 1989). Since the laminar joint angle increases proportionally to brassinosteroid level, a joint bending assay has been used for screening brassinosteroid-related mutants (Wada et al. 1981; Yamamuro et al. 2000; Hong et al. 2004).

Morphological features of the ligule, auricles, and laminar joint, as well as their presence or absence, are used as key identification features of agricultural and horticultural crop varieties. For example, barnyard grasses (*Echinochloa crusgalli*) do not have a ligule and goose grasses (*Eleusine indica*) are absent in auricles (Miller 1999). Although most rice varieties contain all three organs, some rice cultivars (*Oryza sativa* L. Jal lahri) lack the auricles.

Development of the laminar joint region has been characterized in maize at genetic and molecular levels using *liguleless1* (*lg1*) and *liguleless2* (*lg2*) mutants (Becraft et al. 1990; Harper and Freeling 1996; Moreno et al. 1997; Walsh et al. 1998). Loss of function of the *lg1* gene prevented the proper formation of ligules and auricles

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at the blade-sheath boundary. Ligules were completely absent from about the first 10 leaves; however, on the upper leaves a rudimentary ligule developed without accompanying auricles. In contrast, the *lg2* mutant showed an age-dependent phenotype (Harper and Freeling 1996; Walsh and Freeling 1999). The first and sometimes the second leaf completely lacked the ligule and auricle, but the organs were developed at the margin of the third leaf. Successive leaves gradually developed more ligules and auricles, until they looked almost normal. Therefore, the two genes appear to play different roles in ligule–auricle development. *LG1* encodes a putative transcription factor containing an internal domain of 77 amino acids with significant similarity to the conserved domain present in SBP (*SQUAMOSA* Promoter Binding Protein) DNA binding proteins (Moreno et al. 1997). *LG1* mRNA exists predominantly in the ligular region of very young leaves but is also present weakly in the sheath and blade (Moreno et al. 1997). The *LG2* gene encodes a bZIP (basic-leucine zipper) transcription factor and is ubiquitously expressed throughout leaf primordia (Walsh et al. 1998). bZIP transcription factors regulate pathogen defense, light and stress signaling, seed maturation, and the development of various organs such as leaves and flowers (Jakoby et al. 2002). Double mutant analysis suggests that *LG1* and *LG2* act in the same pathway (Harper and Freeling 1996). It was proposed that *LG2* specifies the precise location of ligule and auricle development and interacts with *LG1* to control the development of ligule and auricle. Comparative genetic studies using the *LG1* gene identified putative candidates for barley (Rossini et al. 2006) and sorghum (Zwick et al. 1998).

In rice, little is known about molecular mechanism that controls the development of the laminar joint region and the ligule. Ligule development is started by periclinal division of the adaxial side in leaf primordium. Some genes which expressed in the ligular region were identified. The *GL2*-type homeobox gene, *ROC1 (Rice outermost cell-specific gene1)* is specifically expressed in the epidermis including the outmost cells of the ligule (Ito et al. 2002). The rice *SCARECROW* (*OsSCR*) gene is expressed not only in the endodermal cell layer of the root, but also in ligular cells (Kamiya et al. 2003).

Among the 2,000 traits have been reported in rice (Kinoshita 1998; Kurata et al. 2005), three mutants, *auricleless* (*aur*), *liguleless* (*lg*), and *collarless* (*col*) affect the leaf blade-sheath boundary (Maekawa 1988; Sanchez and Khush 1998). However, the mutants have not been studied at the molecular level. Here, we report the molecular characterization of rice *LIGULELESS1* (*OsLG1*), a gene that plays a key role in development of laminar joint and the organs developed from the region.

Materials and methods

Plant growth

Surface-sterilized T2 seeds of *oslg1* and wild-type (*Oryza sativa* cv. Japonica Dongjin) were germinated in the Murashige and Skoog (MS) medium comprising 3% sucrose, 0.2% phytagel and 0.55 mM myo-inositol. Plants were grown for 7 days at 27°C in the continuous light conditions and then transplanted into soil in the greenhouse until maturity

Screening of T-DNA insertional allele in the *OsLG1* gene from the DNA pools

Systematic reverse genetics screening was performed as described previously (Lee et al. 2003). PCR was performed using the *OsLG1*-specific primers (5′-TCCACCCGAACC CTAGATAGATGA-3′ and 5′-ATGAACACTGACCTGC TGCACTG-3′), and the T-DNA right-border primer (5′-AT CCAGACTGAATGCCCACAGGC-3′). The PCR product from the superpool DNA was separated by agarose gel and the blot was hybridized with the *OsLG1*-specific probe. After identification of an individual line, T-DNA insertion was confirmed by sequencing the PCR product.

Genotyping the mutant plants

PCRs for genotyping were performed in 20 μl of a mixture containing 20 ng of plant DNA, 10× Taq buffer, 0.2× band doctor, 0.2 mM dNTP, 0.2 unit of Taq polymerase (Solgent, Daejeon, Korea), and 1 μM of the primers. The protocol included 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 120 s. Primers for *oslg1-1* genotyping were *OsLG1–1* primers a (5′-ATGCGTGCATGTTTCTAG GT-3′), *OsLG1-1* primers b (5′-ACTGTTCCATCCCA GAGACA-3′, and for a T-DNA right-border primer c (5′-ATCCAGACTGAATGCCCACAGGC-3′). Primers for *oslg1-2* genotyping were *OsLG1-2* primers a (5′-TCCACC CGAACCCTAGATAGATGA-3′), *OsLG1-2* primer b (5′-CTCAAACATGCGAAGGAGAAGTCA-3′), and for a T-DNA right-border primer c (5′-ATCCAGACTGAATG CCCACAGGC-3′).

RT-PCR analysis

Total RNA was extracted from various organs using Tri-reagent (MRC Inc., Cincinnati, Ohio, USA) according to the manufacturer's instruction manual. For first-strand cDNA synthesis, 2 μg of total RNA was reverse-transcribed in a volume of 25 μl containing 10 ng of oligo (dT) 12–18 primer, 10 mM dNTPs, and 1 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA). The

PCR reaction was performed in a 20 μl solution containing a 2 μl aliquot of the cDNA reaction, 0.2 μM gene specific primers, 10 mM dNTPs, 1 unit of Taq DNA polymerase (Solgent, Daejeon, Korea), 5× band doctor, and 10× reaction buffer. The reaction included an initial 5-min denaturation at 94°C, followed by 23–32 cycles of PCR (94°C, 30 s; 58–60°C, 30 s; and 72°C, 1–1.5 min), and a final 10 min at 72°C. Afterward, 10 μl of the reaction mixture was analyzed on a 1.2% (w/v) agarose gel. *OsLG1* specific primers (forward: 5′-TCCACCCGAACCCTAGATAGATGA-3′ and reverse: 5′-GCAGCTCTTCTTGGCATCGT-3′) and *OsActin* primers (forward: 5′-CCTCTTCCAGCCTTCCTTCAT-3′ and reverse: 5′-ACGGCGATAACAGCTCCTCTT-3′) were used.

Quantitative real-time RT-PCR

Real-time PCR was performed using a Roche Lightcyler with cDNA prepared from the 4th (early emerged) and 5th (newly emerged) leaves of 30-day-old plants. cDNA was synthesized using total RNA isolated from the region of the sheath 3–8 mm directly below the laminar joint, 2 mm of the laminar joint region containing sheath and blade, and 3–8 mm of the leaf blade directly above the laminar joint. Real-time RT-PCR procedure was performed as described previously (Han et al. 2006). The mRNA level of *ubiquitin* was used to normalize the expression ratio of each gene. The primers for *OsLG1* were 5′-CCAAGAAGAGCTGCAGGAAG-3′ and 5′-CCATGGCTTGGTCTTGATCT-3′. The primers for the rice *ubiquitin* gene were 5′-TGAAGACCCTGACTGGGAAG-3′ and 5′-CACGGTTCAACAACATCCAG-3′. The changes in gene expression were calculated using the ΔCt method.

Transient expression of the *OsLG1-RFP* fusion

To investigate the cellular localization, we constructed an *OsLG1-RFP* fusion molecule placed under the control of the CaMV *35S* promoter. We replaced the stop codon of *OsLG1* with a *Sal*I restriction site via PCR, using the forward primer (5′-TCTAGAGCGAGGATGATGAACGTTCCAT-3′) and the reverse primer (5′-GTCGACCAGTGATCGAAGTCGAGATCAA-3′). The amplified fragment was inserted between *Xba*I and *Sal*I of the pCaMV35S-RFP vector (Sohn et al. 2003; Jang et al. 2004), generating an in-frame fusion between the *OsLG1* and *RFP* genes. As a positive control, the *OsSNB* coding region was ligated in-frame to the N terminus of the soluble modified green fluorescent protein (*smGFP*) under the control of the CaMV *35S* promoter (Jung et al. 2002; Jang et al. 2004; Lee et al. 2006). Protoplast isolation and the electroporation procedure are as described previously (Kyozuka and Shimamoto 1991) with some modifications. Rice seeds were germinated on MS medium under continuous light at 26°C. Leaves of 10-day-old seedlings were cut into approximately 0.5 mm strips and placed in 20 ml enzyme solution containing 0.6 M mannitol, 10 mM MES, 1 mM $CaCl_2$, 0.1% BSA, 1.5% cellulase R-10 (Yakult Honsha), 0.1% pectolyase, and 0.5% macerozyme R-10 (Yakult Honsha). After vacuum-infiltration for 10 min, samples were digested at 25°C with gentle shaking at 40 rpm for 5 h. The same volume of KMC solution (117 mM KCl, 82 mM $MgCl_2$, and 85 mM $CaCl_2$, pH 5.6) was added to the enzyme solution and filtered by using a 20 μm nylon mesh. Protoplasts were collected by centrifuging at 1,500 rpm for 90 s at 4°C. Pellets were resuspended in EP3 solution containing 70 mM KCl, 5 mM $MgCl_2$, 0.1% MES, and 0.4 mM mannitol. Protoplasts were counted and 2×10^6 protoplasts were mixed with 20 μg of the plasmid DNA. Electroporation was performed in ice using the Gene Pulser Xcell Electroporation System (Bio-Rad) at 300 V cm^{-1} with a 450 μF capacitor. The protoplasts were transferred to incubation solution (0.6 mM mannitol, 4 mM MES, and 4 mM KCl, pH 5.6) and kept in dark for 12–18 h at 28°C. Expression of the fusion construct was monitored by a fluorescence microscopy (Zeiss, Jena, Germany) and the images were captured with a cooled charge-coupled device camera (Zeiss, Jena, Germany).

Construction of the *OsLG1* promoter:*GUS* fusion

The 1.8-kb promoter region of *OsLG1* was obtained by PCR using forward primer (5′-AAGCTTTTGCCCAAATAATGACACGATATA-3′) and reverse primer (5′-GGATCCCCTCGCGGCCTGTCTCCTCCTC-3′). The PCR product was inserted into the pGA1230 vector containing the *GUS* reporter gene (Jeon et al. 2000b). Transgenic plants carrying the construct were obtained by the stable transformation method as previously reported (Lee et al. 1999). Histochemical GUS staining was performed as described by Jefferson et al. (1987) and Dai et al. (1996). For light microscope analysis, the tissues were fixed in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde, and then embedded in Technovit 8100 Resin (Kulzer & Co., Wehrheim, Germany). The samples were sectioned to 4- to 9-μm thickness with a microtome (Leica, Nussloch, Germany), and observed under a microscope (Nikon, Kanagawa, Japan) using bright- and dark-field illumination.

Sequences alignments of SBP domain protein

Multiple sequence alignments were performed using the CLUSTAL X program (Thompson et al. 1997) and BioEdit program (Version 7.0.5, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The following SBP-containing proteins

were used as queries: AtSPL3 (At2 g33810), AtSPL4 (At1 g53160), AtSPL5 (At3 g15270), AtSPL8 (At1 g02065), and AtSPL14 (At1 g20980) from Arabidopsis, ZmLG1 (U89496) from maize, HvLG1 (AM117950) from barley and OsLG1 (Os04 g56170) from rice.

Scanning electron microscope analysis

The laminar joint region of 10-day-old seedling shoots and 6th leaves (newly emerged) from 70-day-old plants was used for SEM analysis. Samples were pre-fixed with 3% glutaraldehyde-sodium phosphate buffer (0.1 M) for 3 h at room temperature and rinsed with 0.1 M sodium phosphate buffer. Post-fixation was performed with 2% osmium tetroxide (OsO_4), followed by rinsing with 0.1 M sodium phosphate buffer. After fixation, the samples were dehydrated through an ethanol series, and infiltrated through an isoamyl acetate series. The samples were critical point-dried, then sputter-coated with palladium. The mounted specimens were observed with a scanning electron microscope (Leo S1450VP, Carl Zeiss, Germany).

Results

Isolation of a liguleless mutant from rice T-DNA tagging population

We have previously reported the generation of T-DNA tagging lines in rice (*Oryza sativa* var. japonica) using the *Agrobacterium*-mediated co-cultivation method (Jeon et al. 2000a; Jeong et al. 2002). From the population, we have identified a liguleless mutant line, 3A-12312.

Wild-type rice plant leaves consist of three parts: the blade, sheath, and laminar joint (Fig. 1A–E). The leaf blade is a broad, flat structure located at the upper part of the leaf. The sheath is located between leaf blade and a node, and warps around the stem internode above this node. The laminar joint region is a belt-like structure present in the boundary between blade and sheath (Fig. 1A, C–E). A pair of auricles and a ligule are present at the sides of the lamina joint region (Fig. 1B–E). The ligule is a thin, white, tongue-like organ and is said to be the degenerated tip of the leaf sheath (Fig. 1B–F) (Hoshikawa 1989). Auricles are horn-like tissues that surround the culm, thus keeping the leaf sheath and the culm together (Fig. 1C–E).

In the liguleless mutant that we have isolated, leaf sheaths were directly connected to blades without a laminar joint region (Fig. 1A). In addition, the ligule and auricles were not present at the region (Fig. 1B). At maturity, homozygotic mutant plants showed upright attitudes (Fig. 1G), and the ligules and auricles were absent in all the leaves including the flag leaves (Fig. 1C–F). We named this mutant allele *Oryza saliva liguleless1-1* (*Oslg1-1*).

Scanning electron microscopy analyses

From SEM analyses, the epidermal tissue on the abaxial side of the wild-type leaf blades was found to consist of stomata, silica cells with a wart-like protuberances, and trichomes (Fig. 2B). The epidermis of the sheath was basically similar to that of the leaf blade, containing stomata, dumbbell-shaped silica cells, and trichomes (Fig. 2D). The epidermis of the laminar joint region had smooth surfaces lacking silica cells or trichomes (Fig. 2C). The laminar joint region exhibited progressive thickening from the margin toward the midrib (Fig. 2A). In *oslg1-1* mutant plants, the epidermis of the leaf blade and sheath was identical to that of wild-type plants, containing stomata and silica cells (Fig. 2F and H). However, cellular structures characteristic of the laminar joint region were absent from of *oslg1-1* mutant plants (Fig. 2E and G).

In the adaxial view, the leaf blade (Fig. 3B and F) and the sheath (Fig. 3D and H) of 70-day-old *oslg1-1* and segregating wild-type plants showed no difference in the surface structure. The epidermis of laminar joint in the wild-type consisted of wrinkled, unshaped cells containing the no trichomes and silica cell (Fig. 3C). In *oslg1-1* mutants, the epidermis of laminar joint region had no obvious boundary (Fig. 3G, Supplementary Fig. 1). Histological analysis showed the difference in the cellular structure between wild-type and *oslg1-1* plants did not observe in the 10-day-old plants (Supplementary Fig. 2).

Isolation of the T-DNA-tagged gene

The DNA sequence flanking the inserted T-DNA was isolated from the *oslg1-1* mutant line using the inverse PCR method (Jeong et al. 2006). T-DNA was found inserted in the first intron of the gene LOC_Os04g56170 (The Institute for Genomic Research, http://www.tigr.org/tdb/e2k1/osa1/LocusNameSearch.shtml), located on chromosome 4 (Fig. 4A, Supplementary Fig. 3). The full-length cDNA sequence of the gene is present in KOME (AK068104), and it encodes a predicted protein of 416 amino acids (Fig. 4B).

Analysis of the OsLG1 protein sequences using the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) identified a conserved SBP DNA binding domain, which is present only in plants (Fig. 4B). The SBP domain consists of 76 highly conserved amino acids that overlap with a nuclear localization signal (NLS). The SBP proteins bind specifically to the GTAC core motif in the *Antirrhinum majus SQUAMOSA* promoter and *Arabidopsis thaliana*

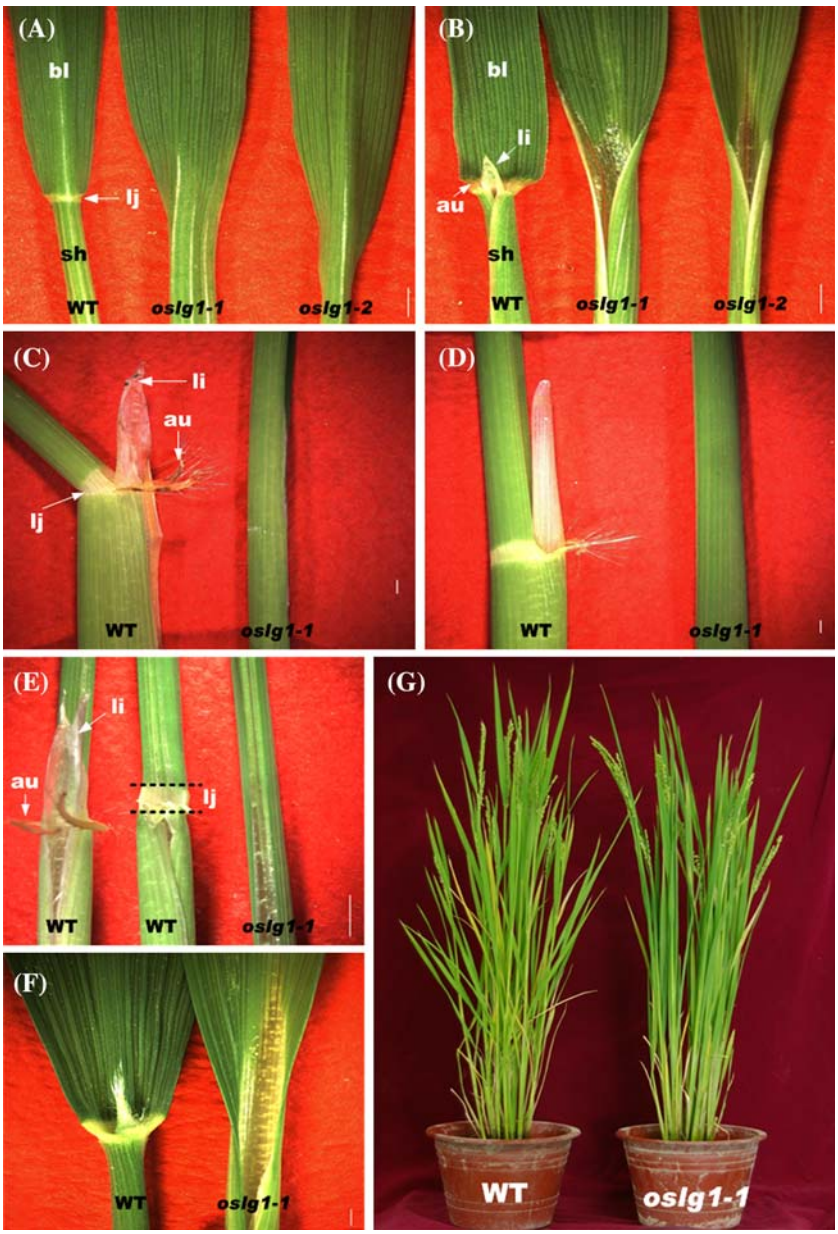

Fig. 1 Phenotypes of the wild-type and *oslg1* plants. Comparison of leaves in the abaxial side (**A**) and adaxial side (**B**) among wild-type plant (*left*) and *OsLG1* knockout plants (10-day-old seedlings). (**C**) Comparison of 2nd leaves in the wild-type plant (*left*) and *oslg1-1* mutant plant (*right*) at 70-day-old plants. Comparison of 5th mature leaves in the abaxidal side (**D**) and in the adaxial side (**E**) at 70-day-old plants. (**F**) Comparison of flag leaves in the wild-type plant (*left*) and *oslg1-1* mutant plant (*right*) at flowering stage, and (**G**) Comparison of wild-type plant (*left*) and *oslg1-1* mutant plant (right) at maturity. li, ligule; au, auricle; lj, laminar joint; bl, leaf blade; sh, leaf sheath. Bar, 100 μm

APETALA1 promoter (Klein et al. 1996; Birkenbihl et al. 2005). Structural analysis of the SBP domain showed two zinc finger-like structures (Zn-1 and Zn-2) formed by eight conserved cysteine and histidine residues, a Cys_3His-$Cys_2HisCys$ or $Cys_6HisCys$ sequence motif (Fig. 4B) (Yamasaki et al. 2004). A NLS sequence (KKSCRKRLAD HNRRRRK) is found within the highly conserved SBP DNA binding domain (aa 242–259) (Fig. 4B). Cardon et al. (1999) have shown that the *Arabidopsis* SBP box consists of two exons interrupted by an intron, and that the position of this intron is conserved. *OsLG1* also has a conserved intron within the SBP box (Fig. 4A)

A homology search using the BLASTP algorithm (BLOSUM 62 matrix) showed that the predicted protein is most homologous to maize LG1 (ZmLG1) with 69% identity (Supplementary Fig. 4). The next homologous protein among the maize SBP proteins is ZmSBP3, which is 27% identical to OsLG1 (Supplementary Fig. 5). In barley, OsLG1 showed 56% identity with HvLG1. The most homologous protein in *Arabidopsis* is AtSPL8 (encoded by At1G02065), which is 51% identical to OsLG1.

Comparative genetics approaches showed that the region on rice chromosome 4L, where the *OsLG1* gene is located, is syntenous to maize chromosome 2S, where *LG1* is present (Becraft et al. 1990; Kaplinsky et al. 2002). Likewise, the rice chromosomal region is syntenous to barley chromosome 2H, where the barley *HvLG1* gene is

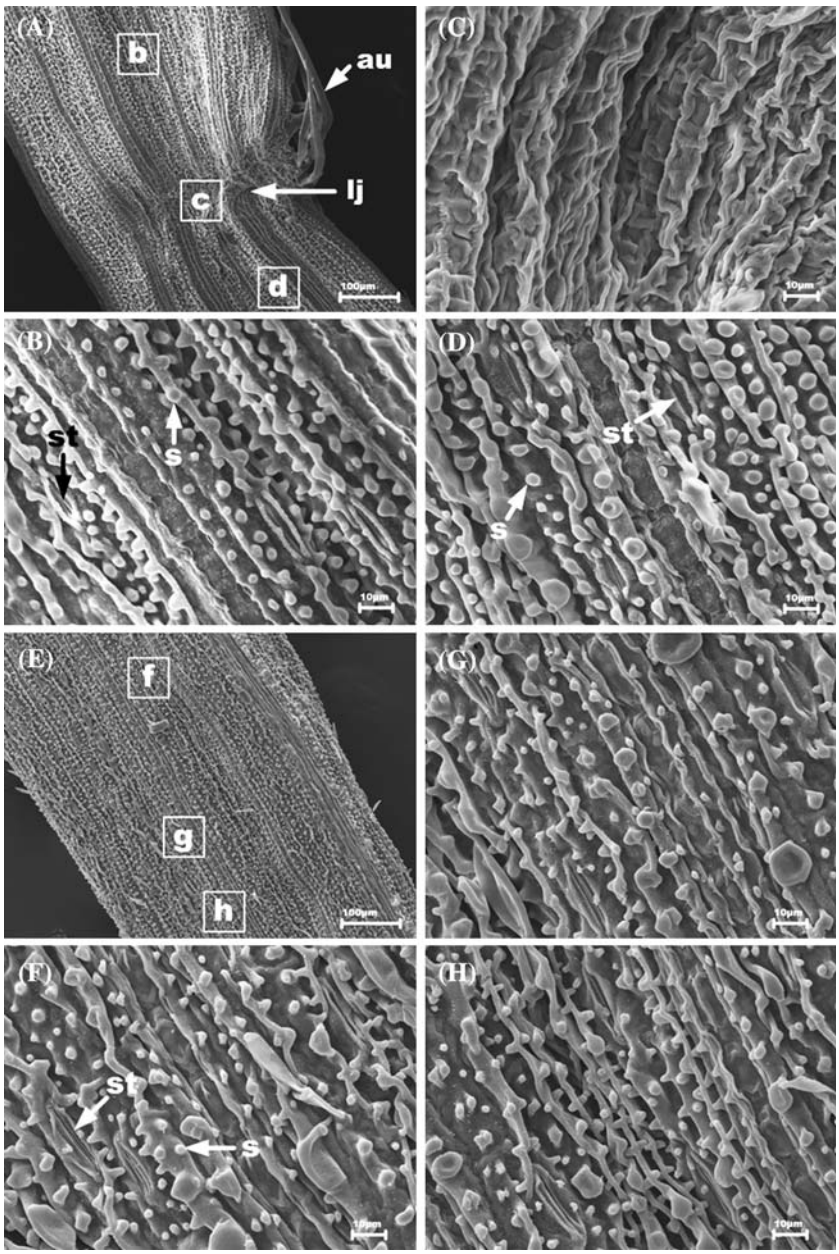

Fig. 2 Scanning electron micrographs of the abaxial surfaces of wild-type and *oslg1-1* leaves. The epidermis structures of the laminar joint region of 10-day-old wild-type plant (**A**) were compared to those of the *oslg1-1* plant at the same stage (**E**). The boxed areas b, c, d, f, g, and h were enlarged to Figs. **B**, **C**, **D**, **F**, **G**, and **H**, respectively. **B** and **F**, leaf blade; **C** and **G**, laminar joint; **D** and **H**, leaf sheath. s, silical cell; st, stomata

located (Rossini et al. 2006). These results indicate that the *OsLG1* gene identified by our T-DNA insertional mutagenesis is an ortholog of the maize *LG1* gene and barley *HvLG1*.

Identification of an additional allele by systematic reverse genetics screening

To verify that the liguleless phenotype observed from the *oslg1-1* mutant was indeed due to T-DNA insertion, we conducted co-segregation analysis between the phenotype and genotype. We obtained T_2 progeny from the original mutant plant and their genotypes were analyzed. Among the 80 plants analyzed, 14 were liguleless mutants. Genotyping of these plants showed that all of the mutants were homozygous (*oslg1*/*oslg1*) to the T-DNA insertion, whereas the remaining 66 were either wild-type or heterozygous (*OsLG1*/*oslg1*) plants (Supplementary Fig. 6). In T_3 generation, among the 40 plants examined, 13 were liguleless mutants and all of them were *oslg1*/*oslg1* (data not shown). Genomic DNA gel-blot analysis using the 5′ region of *GUS* probe, which is located in T-DNA, showed that one copy of T-DNA was inserted in Line 3A-12312 (Supplementary Fig. 7).

To further confirm that the liguleless mutant phenotype is due to the mutation in the *OsLG1* gene, we obtained an

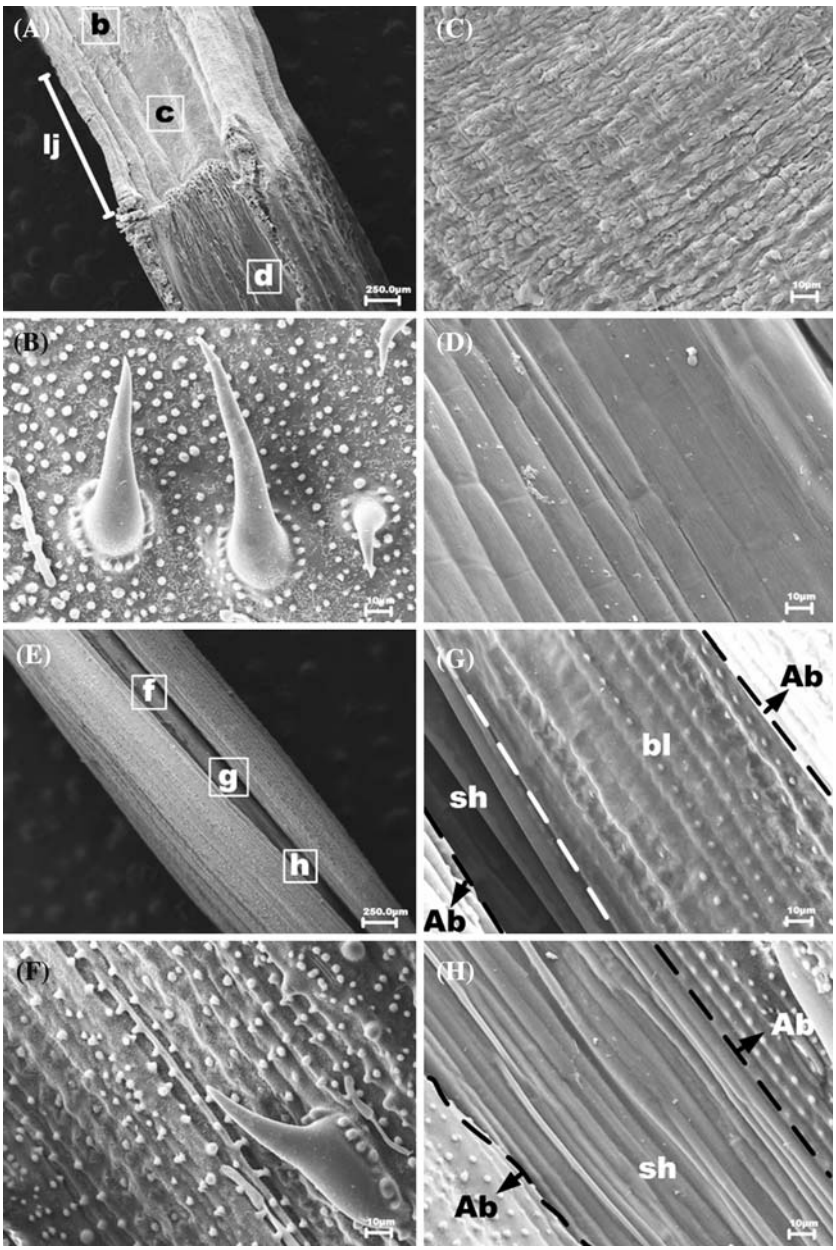

Fig. 3 Scanning electron micrographs of the adaxial surfaces of wild-type and *oslg1-1* leaves. The epidermis structures of the laminar joint regions of 70-day-old wild-type plant (**A**) were compared to those of the *oslg1-1* plant at the same stage (**E**). The boxed areas b, c, d, f, g, and h were enlarged to Figs. **B**, **C**, **D**, **F**, **G**, and **H**, respectively. **B** and **F**, leaf blade; **C** and **G**, laminar joint; **D** and **H**, leaf sheath. lj, laminar joint; sh, leaf sheath; bl, leaf blade. Ab, abaxial side view

additional insertional allele (*oslg1-2*) from the T-DNA insertional populations via the systematic reverse genetics screening method (Lee et al. 2003). In Line 1B-15010, T-DNA was inserted 5′ UTR region (–1 bp) from the translation initiation site (+1) of the *OsLG1* gene (Fig. 4A, Supplementary Fig. 3). The *oslg1-2* mutant plants showed the same mutant phenotypes as observed from the *oslg1-1* plants (Fig. 1A and B), confirming that the mutant phenotypes were caused by a loss-of-function mutation of the *OsLG1* gene. Semi-quantitative PCR experiments showed that the *OsLG1* mRNA is absent in both o*slg1-1* and *oslg1-2* mutants (Fig. 4C).

OsLG1 protein is targeted to the nucleus

Previous studies indicate that the ZmLG1 protein and SBP domain-containing proteins are localized into the nucleus (Moreno et al. 1997; Birkenbihl et al. 2005). The presence of the NLS sequence in the OsLG1 protein and a high similarity among these proteins suggest that the OsLG1 protein would be also localized to the nucleus. In order to determine its cellular location, the *OsLG1* coding region was fused to the *red fluorescent protein* (*RFP*) gene under control of the 35S cauliflower mosaic virus (CaMV) promoter (Fig. 5A) (Sohn et al. 2003; Jang et al. 2004), and

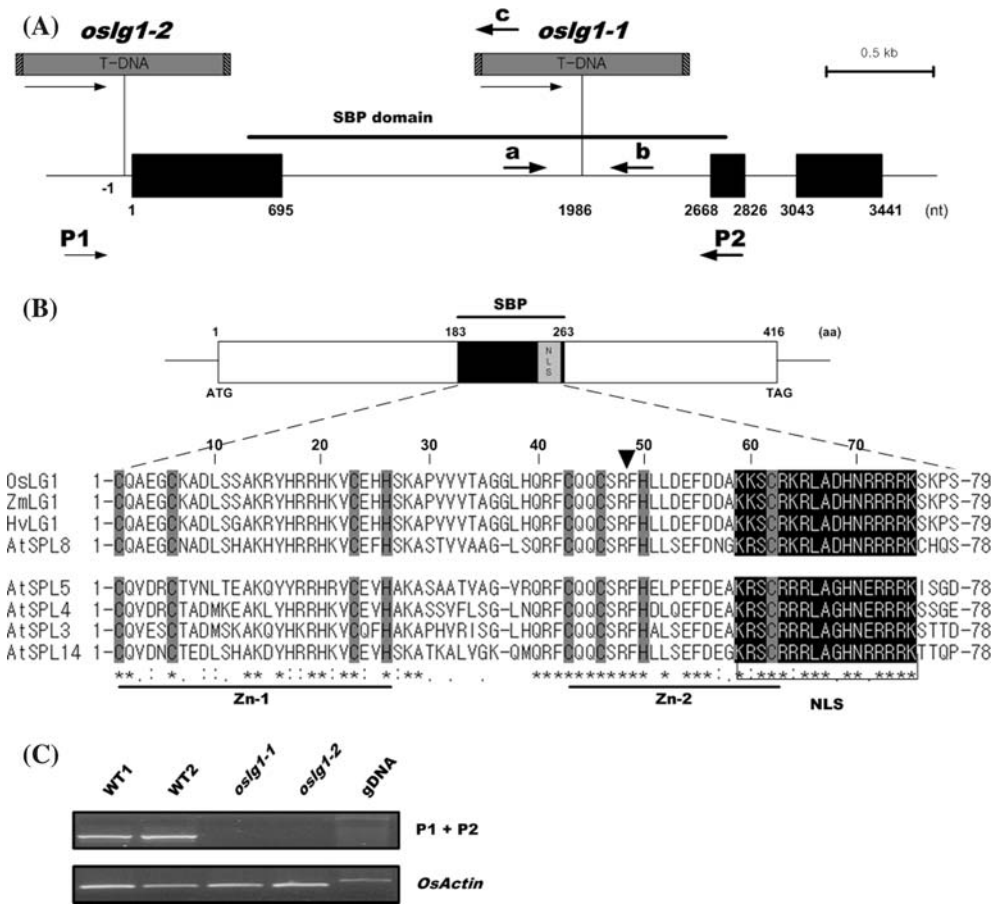

Fig. 4 Genomic structure of the *OsLG1* gene and insertion positions of T-DNA. (**A**) Scheme of the *OsLG1* gene and relative insertion positions of T-DNA. Black boxes represent exons and intervening lines represent introns. Insertion positions of T-DNA are indicated at top. Number 1 indicates the translational initiation site and 3441 indicates the end of translation. An arrow below the T-DNA indicates the direction of the GUS reporter gene in the tagging vector. a, b, and c are primers used for genotyping. SBP, *SQUAMOSA* promoter binding protein domain. NLS, nuclear localization signal. Bar, 0.5 kb. (**B**) Alignment of SBP domains. Shade box indicates the eight conserved cysteine and histidine residues. Zn-1 and Zn-2 indicate two Zn-coordinating structures. The triangle (▼) above the sequences refers to the position of an intron found in the SBP domain. * complete conservation; : indicates strong conservation; ., weak conservation. The accession numbers of the SBP genes are listed in "Materials and methods" section. (**C**) RT-PCR analysis of the *OsLG1* transcripts in wild-type, *oslg1-1*, and *oslg1-2*. RT-PCR was performed using the P1 and P2 primers shown in Fig. 4A. *OsActin* was used as a control to demonstrate equal RNA loading

the construct was transiently expressed in mesophyll protoplasts isolated from rice seedling leaves. As a positive control, a previously characterized nuclear targeted protein, OsSNB:GFP, was co-expressed in the protoplasts (Lee et al. 2006). Figure 5B illustrates that the RFP signal was found in the nucleus and coincided with the GFP signal, indicating that OsLG1 is a nuclear protein and may function as a transcriptional regulator.

OsLG1 is preferentially expressed in the laminar region

To study the expression pattern of *OsLG1*, we constructed a binary vector carrying a fusion molecule between the *OsLG1* promoter and the *GUS* reporter gene. The vector was introduced into rice by the *Agrobacterium*-mediated transformation method (Lee et al. 1999). Among five independently transformed T_1 transgenic plants, four showed GUS positive in the same expression pattern. We observed an intensive GUS signal in the laminar joint regions and in the vein of the sheath but not in roots (Fig. 6A–C) (Xie et al. 2006). GUS expression was detected in the laminar joint region of the newly emerged leaves (Fig. 6A and D). GUS expression was preferentially detected in the initiation site of the laminar joint region from the edge to midrib in leaves at younger stages of development (Fig. 6G, H, and I). Cross sections of the tissues showed preferential GUS staining in the vascular bundle tissues of the sub-apical region (Fig. 6J and K), laminar joints (Fig 6O), leaf sheaths (Fig. 6K–M, Supplementary Fig. 8) especially in the region directly below the

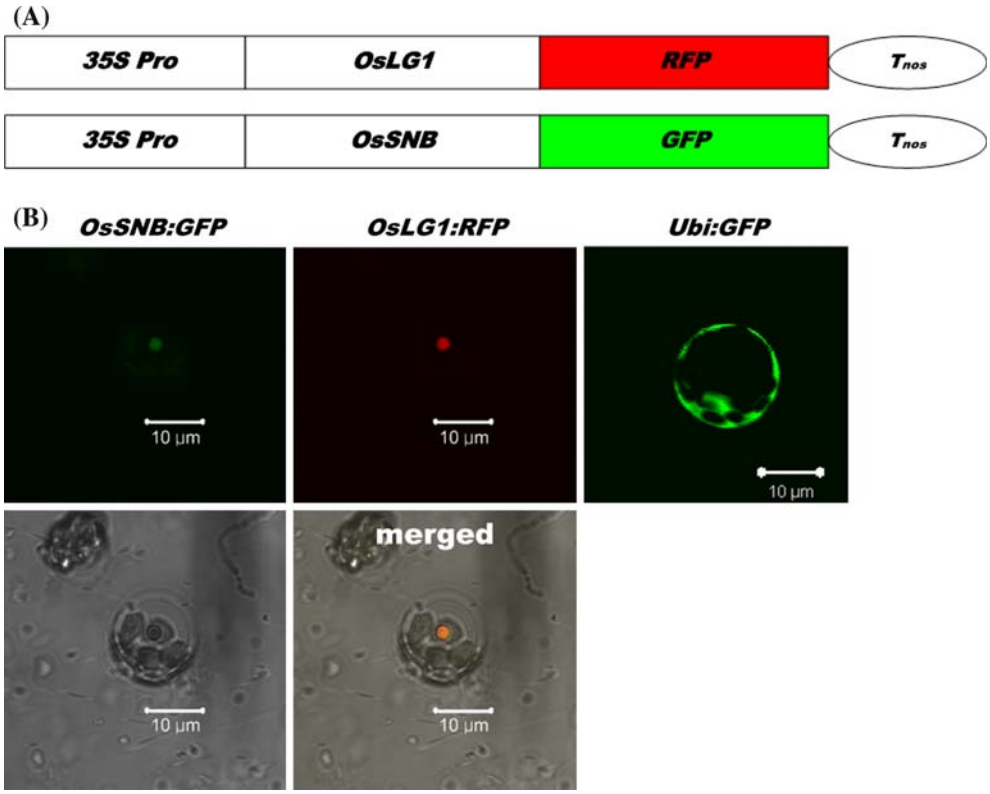

Fig. 5 Nuclear localization of OsLG1:RFP protein. (**A**) Schematic diagrams of fusion constructs. *35S Pro*, CaMV *35S* promoter; *Tnos*, *nopaline synthase* gene terminator. (**B**) Rice mesophyll protoplasts were co-transformed with *35S pro:OsLG1:RFP* and *35S pro:OsSNB:GFP*. Images were obtained by a confocal laser scanning microscope (Zeiss) at either GFP channel (upper left) or RFP channel (*upper middle*). The merged image between two channels is presented (*lower middle*). The protoplast visualized by a bright microscope is also shown (*lower right*). As a control, the *Ubi:GFP* construct was independently transformed to protoplasts (*upper right*)

laminar joint (Fig. 6N), and ligules and auricles (Fig. 6P). Whereas a low level of expression was observed in newly emerging leaves (Fig. 6L and M), strong expression was detected in the laminar joints (Fig. 6O).

To examine whether the GUS assay accurately represented the expression pattern of *OsLG1*, we conducted a semi-quantitative RT-PCR analysis using total RNA prepared from leaf blades directly above the laminar joint, laminar joint regions, and sheaths directly below the joint. The result in Fig. 7A shows that the level of *OsLG1* transcript expression is higher in the laminar joint regions (Samples 2 and 5) and a low level of the transcript was detected in sheaths (Samples 1 and 4). A similar preferential expression pattern was observed from both the 5th and 4th leaves. Real-time PCR analyses also support the observation that the *OsLG1* transcript level was higher in the laminar joint regions (Fig. 7B). Semi-quantitative RT-PCR analysis showed *OsLG1* expressed in the ligule and auricles (Supplementary Fig. 9). These results are consistent with the GUS expression pattern, which shows preferential activity at the laminar joint and sub-apical regions and a low activity in sheaths, ligules and auricles. Therefore, we could conclude that the GUS assay showed a good correlation with the *OsLG1* expression pattern.

Discussion

In this study, we identified T-DNA insertional mutants in the *OsLG1* gene. The knockout mutants were defective in the ligule, auricles, and lamina joint. We have characterized the mutants in detail and studied expression patterns of the gene using the GUS reporter.

The *OsLG1* encodes a SBP DNA binding protein containing a highly conserved region of 76 amino acids that overlaps with a NLS. SBP domain proteins were originally identified from *Antirrhinum majus* and bind to the promoter region of *SQUAMOSA* (Klein et al. 1996). Recently, 19 SBP (*OsSPL*) genes were identified in the rice genome. They can be classified into six subgroups (S1–S6), and *OsLG1* is *OsSPL8*, which a member of the subgroup S5 containing the maize *LIGULELESS1*. Among the rice *SBP* genes, *OsSPL2, OsSPL12, OsSPL13, OsSPL14, OsSPL16, and OsSPL18* are likely regulated by *OsmiR156* (Xiong et al. 2005; Xie et al. 2006). To date, there is no report on functional characterization of the *OsSPL* genes. *OsLG1* (*OsSPL8*) is the first characterized SBP gene in rice.

The *oslg1* mutants showed defects in the laminar joint during leaf development. In particular, *OsLG1* was required for the proper development of the laminar joint at the leaf blade-sheath boundary. In addition, the mutations

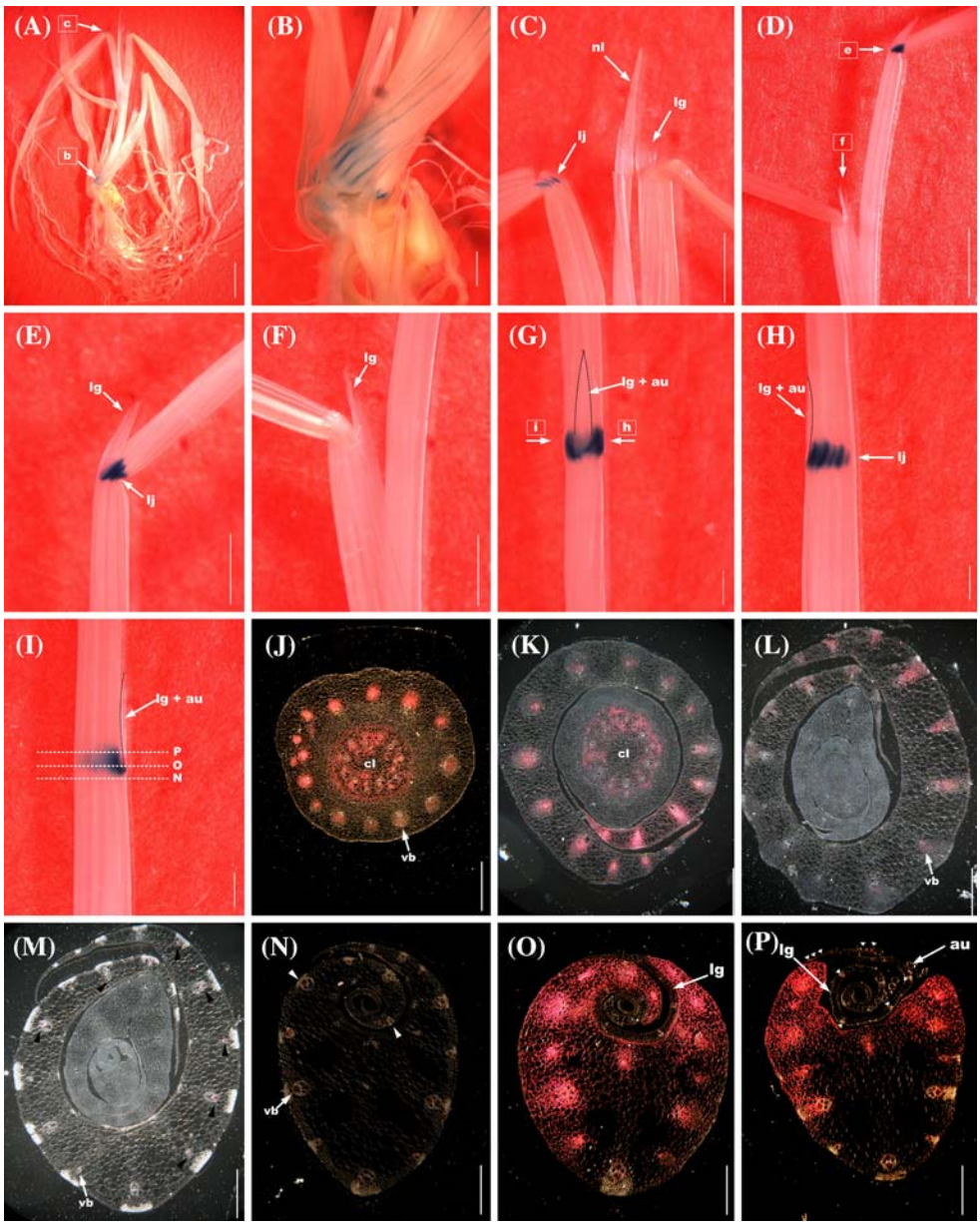

Fig. 6 Histochemical localization of GUS activity directed by the *OsLG1* promoter:*GUS* fusion in transgenic rice plants. (**A**–**C**) Expression pattern of the *OsLG1:GUS* transgenic plants at six-leaf stage. GUS images at the laminar joint regions at four-leaf stage (**D**–**F**) and six-leaf stage (**G**–**I**). Bar, 1 mm. (**J**–**P**) Cross sections of *OsLG1:GUS* T_1 transgenic plants. Photographs were taken with a dark-field microscope, where GUS activity appeared as pink color. (**J**–**M**) Cross section of sub-apical region. (**N**) Cross section of the lower part of the laminar joint region. (O) Laminar joint region. (P) Upper part of the laminar joint region. Pink color can be seen in the vascular bundle of the ligule and auricles (*white arrowhead*). au, auricle; cl, central lacuna; lg, ligule; vb, vascular bundle; lj, laminar joint; nl, newly emerged leaf. The boxed areas b, c, e, f, h, and i were enlarged to Figs. **B**, **C**, **E**, **F**, **H**, and **I**, respectively

also affected auricle and ligule formation. In maize, two recessive mutations, *lg1* and *lg2*, have been reported to affect ligular region development (Becraft et al. 1990; Harper and Freeling 1996). A *lg1* mutation did not influence the overall growth pattern in leaf development but altered the anticlinal division pattern in the preligular region, causing the removal of the ligule and auricle. However, the presence of some rudimentary ligules in upper leaves suggests that the *lg1* mutation is leaky (Becraft et al. 1990; Sylvester et al. 1990; Moreno et al. 1997). The second mutant *lg2* also affected correct initiation and positioning of the ligule and auricle (Harper and Freeling. 1996; Walsh et al. 1998). A synergistic phenotype was observed in the upper leaves of double mutants of *lg1* and *lg2*, indicating that *ZmLG1* and *ZmLG2* function in the same pathway (Harper and Freeling. 1996). Another recessive mutation, *extended auricle1* (*eta1*) caused the outgrowth of auricles and diffusion of the leaf blade-sheath boundary. In double mutants of *eta1* and *zmlg1* or *zmlg2*, synergistic phenotypes were also found as observed from the *zmlg1 zmlg2* double mutant. The *eta1 zmlg1* mutants do not form the ligule and auricles in both the lower and upper leaves, suggesting that *eta1* is also involved in the formation of ligules and auricles (Osmont et al. 2003).

Phylogenetic and comparative genetic analysis indicated that the *OsLG1* gene is orthologous to *ZmLG1* (Kaplinsky et al. 2002; Xie et al. 2006). However, while a single mutation of *OsLG1* resulted in complete loss of the ligules,

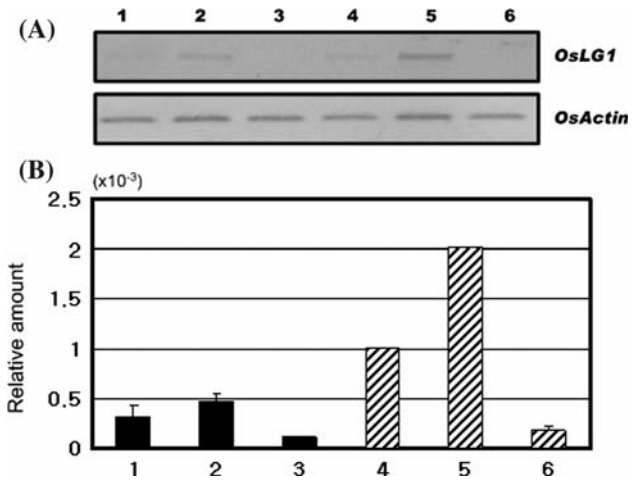

Fig. 7 RT-PCR analysis of the *OsLG1* expression pattern. PCR was performed using the *OsLG1*-specific primers the P1 and P2 primers shown in Fig. 4A. *Actin* was used as a control to demonstrate equal RNA loading. (**A**) Samples were obtained from 4th (1–3) and 5th leaves (4–6) of 30-day-old plants. 1 and 4, 3–8 mm of sheath directly below the laminar joint; 2 and 5, laminar joint region containing the 2 mm of sheath and blade region; 3 and 6, 3–8 mm of leaf blade directly above the laminar joint. (**B**) Quantitative real-time RT-PCR analysis with the RNA samples prepared from the three independent samples shown in Fig. 7A. Error bars indicate the propagation of the standard deviation of three biological replicates. *Ubiquitin* served as a reference for normalization

auricles, and laminar joints throughout the plant, the maize *lg1* mutant displayed a leaky phenotype and required an additional mutant to suppress organ formation in the upper leaves. Harper and Freeling (1996) have proposed two possible explanations for the production of rudimentary ligules in adult leaves. One proposal was "adult ligule elaboration program", in which rudimentary ligule is produced in an age-dependent manner, and the other proposed explanation was gene duplication. A single *OsLG1* mutation caused a complete loss of the ligule development in rice. Similarly, a mutation in barley *Liguleless (HvLG1)* gene, which is collinear with the *Oslg1* gene and maize *lg1* gene also generated complete liguleless phenotype on all leaves (Pratchett and Laurie 1994; Rossini et al. 2006). Therefore, the first possibility can be eliminated if the same molecular mechanism is under operation in both rice and maize. The reason that maize *lg1* and *lg2* mutants are leaky is probably due to the recent genome duplication (Ahn et al. 1993; Swigonova et al. 2004). A homologue (LOC_Os01g64020) of *ZmLG2* is present in the rice genome. We did not observe the liguleless phenotypes in the T-DNA knockout mutants in the *oslg2* homologous gene, perhaps due to genome duplication in rice as well (unpublished data).

Prior to this study, three mutants that affect the leaf sheath-blade boundary have been reported in rice (Kurata et al. 2005). The *auricleless* (*aur*) mutant lacks the auricle and rudimentary ligule, and the *lg* mutant is defective in the ligule, auricle, and laminar joint (Maekawa 1988). The *collarless* (*col*) mutant lacks the laminar joint region (Sanchez and Khush 1998). Among these three mutants, the *lg* mutant phenotypes bear the strongest resemblance to our *oslg1* mutant. Sanchez and Khush (1998) have proposed that the *lg* mutant phenotypes were caused by double mutations in two independent genes: *collarless* and *liguleless*. Maekawa (1988) has isolated another *liguleless* mutant that showed defective development of all three organs. He demonstrated that the *liguleless* mutant is allelic to *lg* examined by Sanchez and Khush (1998). The results from our study support Maekawa's observation that a single mutation in the *LG* gene is the source of alteration in all three organs. One possible explanation for this discrepancy is that the *lg* mutation examined by Sanchez and Khush could be leaky, therefore resulting in occasional development of the organs in mutant plants. It is also possible that the *OsLG1* gene we studied is different from the *LG* gene previously reported, although they are on the same chromosome 4.

In *Arabidopsis*, the most homologous gene to *OsLG1* is *AtSPL8* (51% identity at protein level). However, its function is quite diverged from that of rice. Mutation in the *Arabidopsis* gene affects pollen sac development and causes the reduction of fertility (Unte et al. 2003). Since auricles and ligules are not present in Arabidopsis, the divergence is expected.

In addition to *LG1* and *LG2*, other genes are also involved in ligular region development in maize. The *kn1 (knotted 1)* mutations cause the formation of ectopic knots (Vollbrecht et al. 1991), and the *lg3 (Liguleless 3)* mutation transforms blades to sheaths (Muehlbauer et al. 1997). *RS1 (Rough sheath 1)* is a dominant mutation that causes rough sheath (Schneeberger et al. 1995), and *gnarley1 (gn1)* produces altered ligules and sheaths (Foster et al. 1999). Therefore, these genes likely affect ligular region development.

Although the expression pattern of the maize *LG1* gene had been investigated by the RT-PCR method (Moreno et al. 1997), its precise expression pattern has not been investigated. They reported that the gene was expressed in the ligular region of newly emerged leaves and meristems containing primordia regions in young seedlings. To study the expression pattern of the *OsLG1* gene in detail, we generated a fusion molecule between the *OsLG1* promoter and the *GUS* reporter gene. When the molecule was introduced to rice, the reporter gene expression was preferentially detected in the laminar joint region of newly emerged leaves. In the laminar joint region, GUS was expressed more strongly in the edge. The gene was preferentially expressed in the vascular bundle. This result indicates that the lack of the ligule and auricle in the *oslg1* mutant is likely due to the failure of laminar joint development.

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