

# Isolation and Characterization of a *Ds*-tagged *liguleless* Mutant in Rice (*Oryza sativa*. L)

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## Abstract

A *liguleless* mutant, which showed complete loss of lamina joint region at the junction between leaf blade and leaf sheath, was isolated from a *Ds* insertional mutants derived from the source cultivar, Dongjin. This mutant could not affect other developmental patterns like phyllotaxis. Southern blot analysis, using GUS as a probe, revealed that the *liguleless* mutant contained three *Ds* copies transposed in the rice genome. Among the four genomic sequences flanking the *Ds*, one was mapped in the intergenic region (31661640 - 31661759), and the other two predicted a protein kinase domain (12098980 - 12098667) as an original insertion site within a starter line used for massive production of *Ds* insertional mutant lines. Another predicted and inserted in first exon of *liguleless* 1 protein (*OsLGI*) that was mapped in coding region (LOC\_Os04g56170) of chromosome 4. RT-PCR revealed that the *OsLGI* gene was not expressed *liguleless* mutants. Structure analysis of *OsLGI* protein revealed that it predicted transcription factor with a highly conserved SBP domain consisting of 79 amino acids that overlapped a nuclear localization signal (NLS). RT-PCR revealed that *OsLGI* is mainly expressed in vegetative organs.

Key words: rice, AclOs, *liguleless*

## Introduction

The rice leaf consists of a leaf sheath and a leaf blade, and at their junction, are a pair of auricles and the ligule. A development pattern of the lamina joint region containing auricles or ligule is present in most monocots (Mauseth 1988). The leaf sheath encloses and supports the internodes.

The ligule is a thin, tongue-like white membrane. It is also known to keep the tip of the leaf sheath surrounding the culm and prevents rain water from entering. In lower leaves, it is triangular with an acute tip, while in higher leaves, it is usually split into two sharp tips.

The auricle is shaped like a horn with long hairs on its surface. It surrounds the culm, keeping the leaf sheath and the culm together. The lamina joint, called the collar, is a white belt-like portion at the leaf sheath and blade boundary (Hoshikawa 1989).

Since the lamina joint inclination is known to be sensitive to the concentration of brassinosteroids (BRs), several BR-related mutants have been isolated in rice by exogenous application of BRs (Wada et al. 1981; Yamamuro et al. 2000).

Some mutants with abnormal development of the lamina joint region have been characterized in maize and rice (Becraft et al. 1990; Harper and Freeling 1996; Kurata et al. 2005; Lee et al. 2007; Maekawa 1988; Sanchez and Khush 1998; Walsh et al. 1998).

*Liguleless 1* (*LG 1*) in maize did not form the ligule and auricle, and it failed to develop the boundary between the leaf sheath and leaf blade (Becraft et al. 1990). Maize *LG1* encodes a putative transcription factor that contained the SBP domain (Moreno et al. 1997), while Maize *LG2* encodes a bZIP transcription factor (Walsh et al. 1998). Some mutants showing abnormal development pattern between the leaf sheath and leaf blade were also reported previously in rice, including short auricle (*lg*), auricleless mutant (*aul*) and liguleless (*lg*) (Maekawa 1988; Sanchez and Khush 1998). Recently, a *liguleless* rice mutant (*oslg1*), showing complete loss of the auricle, ligule and lamina joint, has

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been isolated and characterized from T-DNA insertional population (Lee et al. 2007). *OsLG1* encodes a protein that contains an SBP (SQUAMOSA promoter binding protein) domain as a transcription factor (Lee et al. 2007).

We isolated a *liguleless* rice mutant produced by *Ds* insertional mutagenesis, and the mutant is allelic with *OsLG1* which was previously reported (Lee et al. 2007). Hence, we designated the new mutant as *oslg1-3* and reported the isolation and characterization of the *liguleless* rice mutant that may play an important role in the development of the lamina joint region.

## Materials and Methods

### Plant Materials

Severe mutants related to abnormal leaf development were isolated from over 20,000 *Ds* insertional mutant lines. Among them, a *liguleless* mutant which lacks the lamina joint region at the junction between leaf blade and leaf sheath was isolated. It was transplanted into a pot and grown in a greenhouse for fur-

ther observation.

### DNA extraction and Southern blot analysis

The cetyl-methyl-ammonium bromide (CTAB) method was used with minor modifications for extracting total genomic DNA from rice leaves (Murray et al. 1980). The extracted DNA was digested with appropriate restriction enzymes. Five micrograms of each digested DNA were electrophoresed in 0.8% agarose gel for 16 h at 25V and then blotted onto a nylon membrane. The blots were hybridized with <sup>32</sup>P-labeled probes, then further incubated with probes at 65 °C for 14 h in hybridization buffer containing 6 × SSC, 5 × Denhardt's, 0.5% SDS, 50 mM Tris (pH 8.0), 10 mM EDTA, 0.1 mg/ml heat-denatured salmon sperm DNA, and 5% dextran sulfate. Membrane filters were washed for 30 min at 65 °C in 0.2 × SSC containing 0.1% SDS.

### Amplification and cloning of *Ds* flanking sequences

*Ds* flanking sequences were isolated using DNA SpeedUp™ premix kit (Seegene) according to the manufacturer's instruction. The primers used to amplify 3' end of *Ds* were DsN2; GTCC-

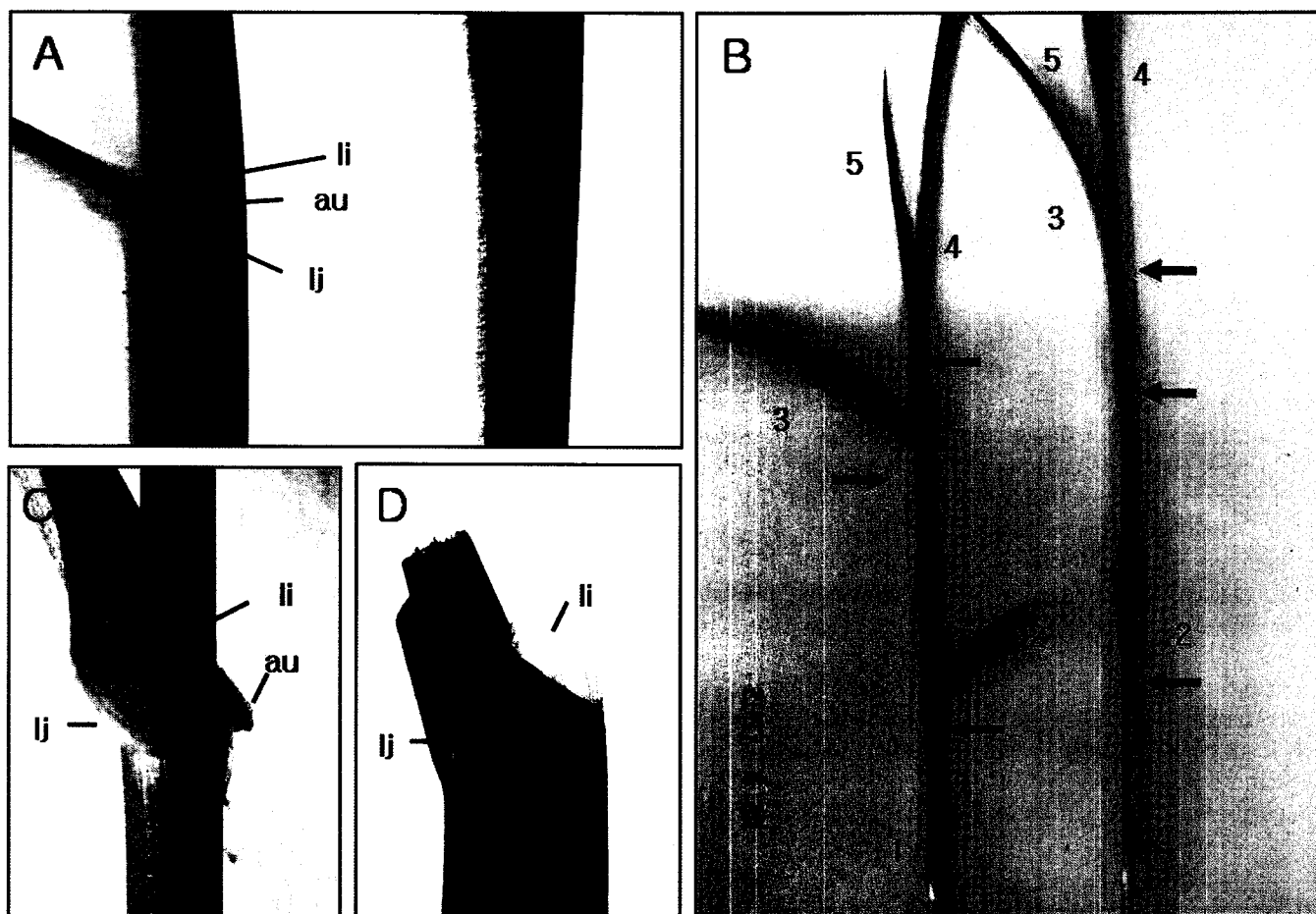


Fig. 1. Phenotypes of the wild type and *liguleless* mutants. A, *liguleless* mutants in 3<sup>rd</sup> leaf stage. *liguleless* mutant shows complete loss of lamina joint region with auricles and ligules: Left, wild type; Right, *liguleless*. B, *liguleless* mutant could not affect phyllotaxis: Left, wild type; Right, *liguleless*. C, 7<sup>th</sup> leaf stage of wheat cultivar, "Chokjung". D, maize in 5<sup>th</sup> leaf stage. Li: ligule; au: auricle; lj: lamina joint.

GATTTCCGACTTTAACCC, TAIL3-2; CCCGTTTCGTTTTTCG-TTACCGGTATATCCCGTTTTTCG, *Ds* 3-4; GTTACCGA-CCGTTTTTCATCCC. The first PCR reaction was performed independently in four individual tubes using primer pairs that were combinations of the *Ds*N2 and the four DW-ACP (DNA walking annealing control primer). The first PCR reactions were performed and followed by 1 cycle at 94 °C for 5 min, 42 °C for 1 min, 72 °C for 1 min and then 30 cycles at 94 °C for 30 s, 60 °C for 30 min, 72 °C for 100 s. The second PCR with a combination of TAIL3-2 and DW-ACPN primers were performed by denaturation at 94 °C for 30 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 100 s. Products amplified by PCR using continuous nested primers were separated on agarose gels and eluted. Purified PCR products were cloned using pGEM-T Easy Vector System (Promega, Madison, WI, USA) and further sequenced. Inverse PCR was also conducted to clone the *Ds* flanking sequence.

### RT-PCR

The total RNA from rice organs were extracted using RNeasy Plant Kit (QIAGEN) according to the manufacturer's instruction. Then, cDNAs were synthesized using Superscript III Reverse Transcriptase System (Invitrogen) for RT-PCR. The first-strand cDNA synthesis was performed in 20 ul reaction mixture containing 5 ug total RNA, 0.25 mM each of the four dNTPs, and 1 uM oligo dT. After being preheated at 65 °C for 5 min, the cDNA synthesis mixture was added with 10X RT buffer, 0.1 M DTT, 40 units RNaseOUT, and 200 units Superscript III reverse transcriptase. A 1 ul sample of synthesized cDNA product was used as template DNA for RT-PCR. The primers were used to survey the expression level of *OsLG1* gene in mutants as follows: F 5'-CCACATGACCTCCTCCAGTT, 1R5'- GTCTC-CAATGCCTGCGTTCT, and for a 3' end of *Ds* 3-4 5'-GTTACCGACCGTTTTTCATCCC.

### Alignments of SBP domain

Multiple sequence alignments were performed using the CLUSTAL X program. The following SBP-containing sequences were used as queries: *ZmLG1* (U89496) from maize and *OsLG1* (Os04g56170) from rice.

## Results and Discussion

### Isolation and characteristics of the *liguleless* (*oslg1-3*) mutant

The rice leaf consists of a leaf sheath and a leaf blade with a pair of auricles and the ligule at their junction. The development pattern of the lamina joint region, including auricle or ligule, is a common event in most monocot plants like wheat, barley, or maize (Fig. 1C, D; Mauseth 1988). An *liguleless* mutant, which showed complete loss of the lamina joint region at the junction between leaf blade and leaf sheath, was previously isolated from a *Ds* insertional mutant (Fig. 1A, B; Ahn et al. 2008).

We could not observe the morphological difference between

the wild type and the mutant in terms of the coleoptiles and the lamina joint region of the first leaf at juvenile stage. The difference was first observed in the second leaves and also in mature leaves during the reproductive stage. Since leaves of the mutant showed complete loss of the lamina joint region, thus, they have erect stature with weak roll pattern. The *liguleless* mutation gene did not affect other developmental features such as phyllotaxis (Fig. 1B). This mutant was promoted for flowering one week earlier than the wild type. Two *liguleless* mutants (*oslg1-1* and *oslg1-2*) showing a complete loss of the auricle, ligule, and lamina joint was previously reported (Lee et al. 2007). *oslg1-1* and *oslg1-2*, identified from T-DNA tagging lines, were mutated in both promoter and first intron of *OsLG1* gene, respectively. So we designated *Ds*-tagged *liguleless* as *oslg1-3*. Several mutants affecting the development of organs at the lamina joint region, *decussate* (*dec*), *liguleless* (*lg*), *auricleless* (*aul*), and *collarless* (*col*), have been reported in rice but they have not been studied at the molecular level (Itoh and Nagato 1998; Maekawa 1988; Sanchez and Khush 1998). The *lg* mutant had very short ligules and undeveloped auricles (Maekawa 1988). The *dec* rice mutant showed defects in the leaf blade-sheath boundary (Itoh and Nagato 1998). The *aul* mutant has no auricles, and the ligule is rudimentary (Sanchez and Khush 1998). Two recessive mutants, *lg1* and *lg2*, have been reported to affect ligular region development in maize (Becraft et al. 1990; Harper and Freeling 1996). The *lg1* prevented the formation of ligules and auricles during

Dongjin  
*liguleless*

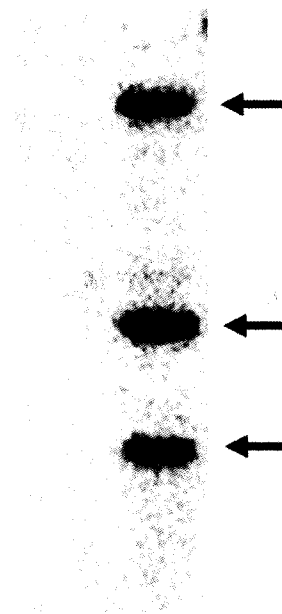
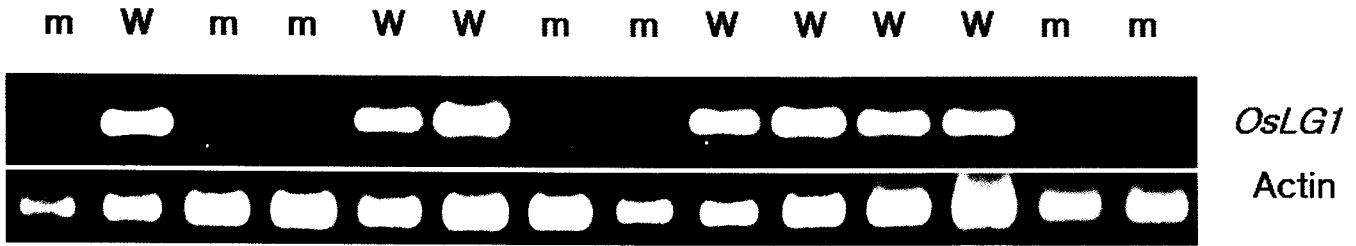


Fig. 2. Southern blot analysis using GUS as a probe. Genomic DNA was digested with *EcoRI*, fractionated on a 0.8% agarose gel and hybridized with the GUS probe. Arrow indicates three *Ds* element transposed in *liguleless* mutant.



**Fig. 3.** Expression levels of *OsLG1* in *liguleless* mutants. RT-PCR indicated that the *OsLG1* transcript was not expressed in all *liguleless* mutants. Actin was used as an internal control. WT: wild type; m: *liguleless* mutant.

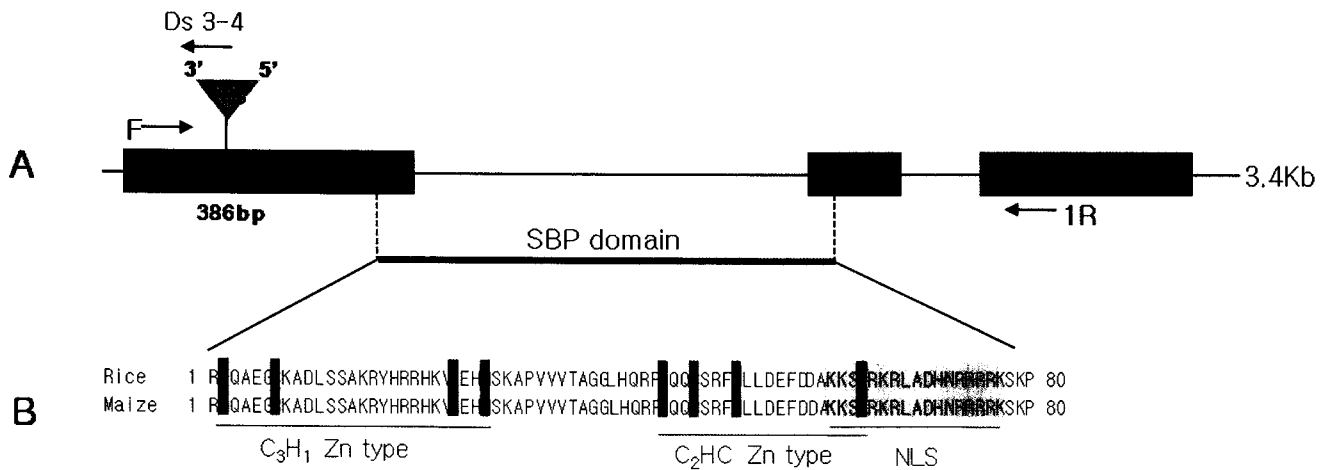
leaf development, and *lg2* affected the correct initiation and position of the ligule and auricle (Harper and Freeling 1996). Maize *LG1* encodes a putative transcription factor that contained SBP domain (Moreno et al. 1997), and *LG2* encodes a bZIP transcription factor (Walsh et al. 1998). Mutation in the *OsLG1* gene showed complete loss of the lamina joint region including auricles and ligules, while the maize *lg* mutant indicated weak phenotype, resulting in loss of partial organs. This suggests that *LG1* have a different role in establishing the ligule region between maize and rice. Therefore, there is a need to obtain additional allelic mutants related to *OsLG1* or loss of the auricle, lamina joint and the ligule to know the mechanism of leaf development in rice.

**Cloning and structure of *OsLG1***

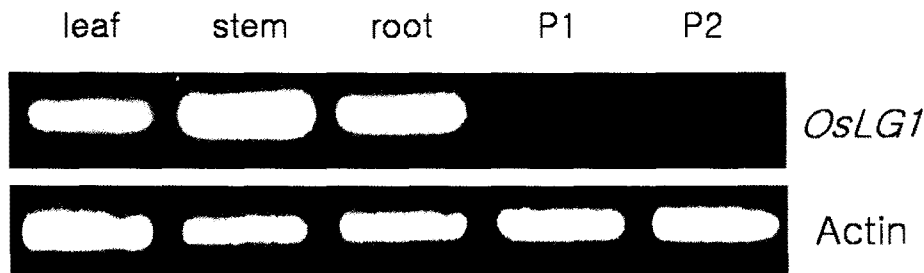
Southern blot analysis, using GUS as a probe, revealed that a *liguleless* mutant contained three *Ds* copies transposed in the rice genome (Fig. 2). To identify the interrupted gene in the *liguleless* mutant, genomic sequences flanking the *Ds* were isolated using DNA SpeedUp™ premix kit (Seegene) or inverse PCR. Six PCR products amplified by continuous nested primers specific to *Ds* sequences were cloned using pGEM-T Easy

Vector System (Promega), and then were further sequenced. We performed blast search with six FSTs rescued using TIGR database (www.tigr.org/). One of the *Ds* flanking sequences was mapped in the intergenic region (31661640 - 31661759) of chromosome 3. The other two flanking sequences predicted the same protein kinase (12098980 - 12098667) of chromosome 3. The protein kinase flanking *Ds* was originally mapped in chromosome 3 of the starter line used for massive production of *Ds* insertional mutant population. Therefore, we excluded intergenic and protein kinase flanking sequences in this study. Another flanking sequence predicted the same *liguleless 1* protein (*OsLG1*) and was mapped in coding region (LOC\_Os04g56170, *OsLG1*) of chromosome 4 (Fig. 4A). Southern blot analysis indicated that *OsLG1* was encoded by a single gene (data not shown).

Since *OsLG1* is an ortholog with protein of maize, we further focused on a *liguleless* gene. RT-PCR was conducted to survey the expression level of *liguleless* gene in wild type and mutant. Among the 24 plants sowed, six were *liguleless* mutants. RT-PCR revealed that the *OsLG1* transcript was not expressed in four *liguleless* mutants, while two showed a very weak expression pattern (Fig. 3). These suggested that mutation in the *oslg1-3* phenotype was caused by transposition of *Ds* elements in the



**Fig. 4.** Structure of *OsLG1* gene and *Ds* position inserted. A, relative insertion positions of *Ds*. *Ds* inserted at 386bp of first exon in *OsLG1*. F and 1R primers were used to survey expression level of *OsLG1*. B, alignment of SBP domain. Shade box indicates the eight conserved cysteine and histidine residues. SBP domain overlapped DNA binding sequences and nuclear localization domains. NLS: nuclear localization signal.



**Fig. 5.** Structure of *OsLG1* gene and *Ds* position inserted. A, relative insertion positions of *Ds*. *Ds* inserted at 386bp of first exon in *OsLG1*. F and 1R primers were used to survey expression level of *OsLG1*. B, alignment of SBP domain. Shade box indicates the eight conserved cysteine and histidine residues. SBP domain overlapped DNA binding sequences and nuclear localization domains. NLS: nuclear localization signal.

*OsLG1* gene. A full-length cDNA of *OsLG1* gene was cloned using RT-PCR, and then was further sequenced. Sequencing analysis revealed that the *OsLG1* gene consisted of three exons and two introns of a 1,248 bp (Fig. 4A). Structure analysis of the *OsLG1* protein indicated that it contained a highly conserved SBP domain consisting of 79 amino acids that overlapped a nuclear localization signal (NLS) (Yang et al. 2008; Fig. 4). The SBP box gene encodes putative plant-specific transcription factors (Klein et al. 1996; Yang et al. 2008). The *OsLG1* contained both the Zn-finger-like structure, Cys<sub>3</sub>His-type, and the Cys<sub>2</sub>HisCys-type (Birkenbihl et al. 2005; Lee et al. 2007; Fig. 4B; Yang et al. 2008). These results indicated that *OsLG1* may play an important role in constructing the lamina joint region, including auricle and ligules, as well as a transcription factor. Nineteen redundant SBP box genes were identified in rice using the known SBP domain sequences and *OsLG1* designated as *OsSPL8* (Os04g56170) (Yang et al. 2008).

Recently, we isolated a new mutant interrupted in other SBP domain and designated it as *OsSPL7* on chromosome 4 (Os04g46580). However, this mutant showed normal leaf development unlike the *oslg1-3* mutant, indicating its other role in plant development or life cycle. This shows that 19 redundant SBP box genes may conduct various functions in plant development.

### Expression pattern of the *OsLG1* gene

To survey the expression pattern of *OsLG1* gene in wild type rice, RT-PCR was performed in various organs. RT-PCR showed that *OsLG1* was mainly expressed in leaf, stem, and root corresponding to the vegetative organ or stage, whereas expression level in P1 stage (within 10 mm of panicle) was rapidly decreased and that of P2 stage (50 - 60 mm of panicles) was almost not expressed (Fig. 5). A previous study showed that the *OsLG1* protein was localized in the nucleus using an *OsLG1*:RFP (Red Fluorescent Protein) fusion protein (Lee et al. 2007). These expression patterns may play an important role as a transcription factors in leaf development, particularly the lamina joint region including the ligule and auricle during the vegetative phase.

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### References

- Ahn BO, Kang KH, Eun MY, Jeon YH, Yun DW, Ji HS, Park SH, Nam MH, Suh SC, Lee MC. 2008. Trait Variation and Molecular Characteristics of 1,870 *Ds* insertional rice lines. Korean J. Breed. Sci. 40: 39-47
- Becraft PW, Bongard-Pierce DK, Sylvester AW, Poethig RS, Freeling M. 1990. The *liguleless-1* gene acts tissue specifically in maize leaf development. Dev. Biol. 141: 220-232
- Birkenbihl RP, Jach G, Saedler H, Huijser P. 2005. Functional dissection of the plant-specific SBP domain: overlap of the DNA binding and nuclear localization domains. J. Mol. Biol. 352: 585-596
- Harper L, Freeling M. 1996. Interactions of *liguleless 1* and *liguleless 2* function during ligule induction in maize. Genetics 144:1871-1882
- Hoshikawa K. 1989. The growing rice plant, 1st ed. Nobunkyo, Tokyo, pp 87-101
- Itoh J-I and Nagato Y. 1998. A mutation associated with phyllotaxy and leaf blade-sheath boundary in rice. Rice Genetics Newsl. 15: 90-92
- Klein J, Saedler H, Huijser P. 1996. A new family of DNA binding proteins includes putative transcriptional regulators of the *Antirrhinum majus* floral meristem identify gene SQUAMOSA. Mol. Gen. Genet. 250: 7-16
- Kurata N, Miyoshi K, Nonomura KI, Yamazaki Y, Ito Y. 2005. Rice mutants and genes related to organ development, morphogenesis and physiological traits. Plant Cell Physiol. 46: 48-62
- Lee JW, Park JJ, Kim SL, Yim JU, An JH. 2007. Mutations in the rice *liguleless* gene result in a complete loss of the auricle, ligule, and lamina joint. Plant Mol. Biol. 65: 487-499
- Maekawa M. 1988. A new allele at the *lg* locus conferring short ligules. Rice Genet. Newsl. 5: 87-88
- Mauseth JD. 1988. Plant anatomy. The Benjamin/Cummings Pub. Co., California.
- Moreno MA, Harper LC, Krueger RW, Dellaporta SL, Freeling M. 1997. Liguleless1 encodes a nuclear localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes Dev. 11: 616-628

- Murray, M.G., and Thompson, W.F.** 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 19: 4321-4325
- Sanchez AC, Khush GS.** 1998. A gene for collarless phenotype in rice. *Rice Genet. Newsl.* 15: 99-100
- Wada K, Marumo S, Ikekawa N, Morisaki M, Mori K.** 1981. Brassinolide and homobrassinolide promotion of lamina inclination of rice seedlings. *Plant Cell Physiol.* 22: 323-325
- Walsh J, Waters CA, Freeling M.** 1998. The maize gene *liguleless 2* encodes a basic leucine zipper protein involved in the establishment of the leaf blade-sheath boundary. *Genes Dev.* 12: 208-218
- Yang ZF, Wang XF, Gu SL, Hu ZQ, Xu H, Xu CW.** 2008. Comparative study of SBP box gene family in Arabidopsis and rice. *Gene* 407: 1-11
- Yamamuro C, Ihara Y, Wu X, Noguchi T, Fujioka S, Takatsuto S, Ashikari M, Kitano H, Matsuoka M.** 2000. Loss of function of rice brassinosteroid insensitive 1 homolog prevents internode elongation and bending of the lamina joint. *Plant Cell* 12: 1591-1605