African Journal of Biotechnology Vol. 10(10), pp. 1802-1811, 7 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1885
ISSN 1684–5315 ©2011 Academic Journals

Full Length Research Paper

Molecular and genetic characterization of *OSH6* (*Oryza sativa Homeobox* 6) using dissociation (*Ds*) insertion mutant rice

Sung-Han Park[1], Doh-Won Yun[1], Gang-Seob Lee[1], Ung-Han[1] Yoon[1], Hyeon-So Ji[1], Byoung-Ohg Ahn[1], Soo-Chul Park[1], Yong-Hwan Kim[2], Yong-Kab Kim[3] and Chang-Kug Kim[1]*

[1]Genomics Division, National Academy of Agricultural Science (NAAS), Rural Development Administration (RDA), Suwon 441-707, Korea.
[2]Policy Development Office, Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Anyang 431-060, Korea.
[3]School of Electrical Information Communication Engineering, Wonkwang University, Korea.

Accepted 6 December, 2010

Genetic studies of dissociation (*Ds*) insertion mutant rice plants indicated that ectopic expression of truncated *OSH6 (Oryza sativa Homeobox 6)* mRNA may be responsible for the mutant phenotype of *knotted* leaf formation at the peduncle. Additionally, ectopic expression of truncated *OSH6* mRNA in the *OSH6-Ds* mutant plant led to alteration of other homeobox genes including *OSH15* in leaf tissues. The *OSH6-Ds* mutant plant exhibited altered expression of more than 118 genes on a 22K rice microarray in comparison with wild type plants. Of these genes, 20 were up- or down-regulated in both *OSH6-Ds* and *OSH6*-overexpressing (*OSH6-35S*) plants. Especially, *OsDof3* was not expressed in floral organs, but was present in the panicles of both *OSH6-Ds* and *OSH6-35S* plants. It is assumed that truncated *OSH6* transcript might be actively involved in the gene expression during organ development. The genetic relationship between *OSH6-Ds* and *OSH15* suggested that the formation of the extra leaf is independent of *OSH6-Ds* or *OSH15* expression. These results suggest that truncated *OSH6* mRNA influences lateral organ growth and development by regulating the expression of specific gene groups.

Key words: *Oryza sativa Homeobox 6* (*OSH6*) genes, *Ds* insertion lines, *OSH15* mutant.

INTRODUCTION

Plant formation and maintenance require the activity of self-organizing groups of cells called meristems found at shoot and root apices. The *knotted*-like *homeobox* (*KNOX*) transcription factors are essential for the formation of the shoot apical meristem and for proper meristem function and maintenance (Byrne et al., 2001; Kerstetter et al., 1997). Furthermore, ectopic expression of *KNOX* genes confers meristematic characteristics to the *KNOX*-expressing cells (Reiser et al., 2000) that can respond to an integrated signal which exclusively identifies meristem cells (Horigome et al., 2009). *KNOX* genes were initially identified as dominant or semi-dominant mutants where ectopic expression results in mutant phenotypes (Smith et al., 1992; Muehlbauer et al., 1999). The *KNOX* genes (*OSH1, OSH6, OSH15* and *OSH71* in rice) were ectopically expressed in all mutant leaves. These genes indicate the presence of multiple loci involved in *KNOX* repression in rice leaves (Katsutoshi et al., 2009). *OSH6*, *OSH15* and *OSH71* exhibited similar expression patterns in shoot apical meristems (SAM) even though the onset of their expression differed during embryogenesis (Sentoku et al., 1999).

Rice *OSH6* (*Oryza sativa Homeobox 6*) is an ortholog of *Lg3* (Liguleless3) in maize. Ectopic expression of *OSH6* results in a 'blade to sheath transformation' phenotype at the midrib regions of leaves, similar to the phenotype of dominant *Lg3* mutants (Park et al., 2007). Maize *Lg3* shares amino acid identity with rice *OSH6* and over-

*Corresponding author. E-mail: chang@korea.kr.

expression of *OSH6* results in a similar phenotype that includes the loss of the ligule and auricles, as well as disorder in the boundary between the blade and sheath (Sentoku et al., 2000). Transgenic analysis of *OSH15* using a series of domain deletions showed that certain types of morphological abnormalities could be attributed to a specific domain (Nagasaki et al., 2001). *OSH6* shares 79% amino acid identity with maize *Lg3* and *OSH15* shares 50% amino acid identity with *OSH6* and *OSH71* (Sentoku et al., 1999). Promoter sequences of rice *KNOX* genes were not sufficient for proper expression of the *KNOX* genes, as promoter activities were detected in various organs including leaves (Ito and Kurata, 2008).

The *KNOX* proteins are comprised of four conserved domains, the *MEINOX, SE, ELK* and *HD* (homeodomain) domains (Kerstetter et al., 1994). These proteins contain the *KNOX* and BEL1-like homeodomain (*BELL*) members that function as heterodimers (Hamant and Pautot, 2010). These proteins have been previously examined in biochemical and yeast systems (Muller et al., 2001; Nagasaki et al., 2001; Smith et al., 2002). The *HD* domain is essential for interactions between *KNOX* proteins and target sequences. The *MEINOX* and *GSE* domains exhibit several functions including transrepression and dimerization.

The dissociation (*Ds*) insertion mutants were generated through the *Ac/Ds* gene trap system (Park et al., 2007). Genetic analysis of *Ds* insertion mutants is one of the most important methods for understanding the rice genome and breeding rice (Zhu et al., 2007; Kim et al., 2010). Several studies have successfully used microarrays to analyze gene expression changes in a number of crop species, including rice (Kim et al., 2009a, b). Here, we identified *OSH6*-related genes using microarray analysis of *Ds* insertion mutants.

MATERIALS AND METHODS

Plants

The *Ds* insertion mutant plant (*OSH6-Ds*), *OSH6*-over-expressing plant (*OSH6-35S*), *OSH15* mutant plant and wild-type rice (*DongJin*) were used. The *OSH6-Ds* mutant was generated through the *Ac/Ds* gene trap system using the *Dongjin* cultivar (Park et al., 2009) and was obtained from a population of *Ds* transposants (Chin et al., 1999; Kim et al., 2004). The insertion allele was identified by cloning the region flanking the *Ds* element using inverted polymerase chain reaction (iPCR). Sequencing revealed that a defective *Ds* element was located near the 3' end of the third exon of *OSH6* located on chromosome 1 (Park et al., 2007).

The *OSH15* mutant, another rice *KNOX* class I homeobox gene, was obtained from the National Institute of Agrobiological Sciences (NIAS) in Japan. A recessive *OSH15* mutation arose from insertion of *TOS17*. Plants homozygous for the single T-DNA insertion in the *OSH15* gene as confirmed by PCR, exhibited internode aberrations and internode elongation defects. These phenotypic aberrations of the *OSH15* mutant were described previously (Sato et al., 1999).

In situ hybridization

To obtain sections of shoot apical meristem and floral organ samples, a standard tissue-embedding method was employed. Two rice organ samples were fixed in 0.25% glutaraldehyde, 4% paraformaldehyde and 100 mM Na-phosphate (pH 7.5) for one day at 4°C (Kouchi and Hata, 1993). Samples were vacuum-infiltrated in the fixative until tissues sank. For paraffin embedding, tissues were dehydrated through a graded EtOH series followed by a *tert*-butanol series. Samples were embedded in paraffin for 3 days in a 58°C oven. For *in situ* RNA hybridization, *OSH6*-specific DNA was prepared from the coding and 3'-untranslated regions of *OSH6*. PCR fragments were cloned into pGEM vectors. Antisense RNA probes were generated using DIG RNA labeling kits (Roche Applied Science, Laval, QC, Canada). Sections of embedded tissues, 8 µm thick, were mounted on slides pretreated with polylysine. The mixture of three RNA probes was added to a final concentration of 0.4 - 4 µg/ml and incubated for 12 to 16 h at 50°C. Hybridization signals were visualized with an alkaline-phosphatase-coupled antidigoxigenin antibody (Roche Applied Science).

RNA extraction and analysis

Total RNA was isolated from eight different tissues from various developmental stages of wild type plants. The tissues examined included callus, SAM, root, stem sections containing SAM and panicles of three different flowering stages. SAM, root and stem sections were isolated from 2-week-old plants. Panicles were collected from young and mature plants in the booting and heading floral stage. Tissue samples were frozen in liquid nitrogen and ground to powder with a mortar and pestle. Total RNA was extracted with easy-BLUE reagents (iNtRON Biotechnology, Seoul, Korea) or RNeasy Kit (Qiagen, Valencia, CA, USA). For reverse transcription-PCR (RT-PCR), 2 g of total RNA was treated with RNase-free DNase and first-strand cDNA was synthesized using an oligo (dT) primer (Invitrogen, Carlsbad, CA, USA) and ReverTra Ace (Toyobo, Tokyo, Japan). cDNA was used as a template for PCR amplification with gene-specific primers. To distinguish cDNA from genomic DNA, all primer-amplified DNA contained an intron. PCR primers were designed to amplify the conserved coding region and the 5'- or 3'- untranslated regions of each gene. PCR reactions consisted of denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. PCR products were separated on agarose gels and stained using ethidium bromide. PCR products were then transferred to Hybond N^+ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and hybridized with a ^{32}P-labeled *OSH* probe.

For northern hybridization, 1.3% formaldehyde gels were prepared in MOPS/EDTA buffer {0.5 M MOPS (pH 7.0) and 0.01 M Na_2EDTA (pH 7.5)}. Vacuum-dried RNA samples (30 µg) were dissolved and heat-denatured in formaldehyde/formamide buffer and subjected to gel electrophoresis. Gels were washed with water and 10 x SSC, and transferred to pre-wetted Hybond N^+ (Amersham Pharmacia Biotech). Hybridization was performed at 65°C in Church buffer (1% BSA, 200 µM EDTA, 0.5 M sodium phosphate and 7% SDS) containing the ^{32}P-labeled probe.

Microarray preparation, hybridization, data extraction and analysis

The 22K rice microarray (Agilent Technologies, Tokyo, Japan) comprisng of 21,938 oligonucleotide probes was synthesized from 28,469 full-length rice cDNAs (Yazaki et al., 2004). Total cellular RNA was isolated from shoot apical meristem regions of homozygotic *OSH6-Ds*, *OSH6-35S* and wild-type shoots from rice seedlings, 5 days after germination (DAG) using an RNeasy Plant Mini Kit (Qiagen). RNA sample integrity was estimated using a Bioanalyzer 2100 RNA 6000 nanochip (Agilent Technologies) and denaturing formaldehyde agarose gel electrophoresis. High-quality

purified mRNA was amplified, labeled and hybridized to the oligonucleotide array following the manufacturer's protocols (Agilent Technologies). Data scanning, quantification and processing were performed as described previously (Yazaki et al., 2004). Briefly, washed slides were scanned with an Agilent DNA microarray scanner (model G2565BA). Feature extraction and image analysis software (v. A.6.1.1, Agilent) were used to locate and delineate each spot in the array. Gene-specific probes were used for hybridization. To integrate each spot's intensity, filtering and normalization via the LOWESS method was used. All of the expression profiles are available upon request from the Rice Expression Database (RED, http://cdna02.dna.affrc.go.jp/RED/) at the National Institute of Agrobiological Sciences (NIAS). To validate data from the microarray experiments, the expression of selected genes was examined using northern hybridization and RT-PCR. Actin mRNA was used as a loading control for mRNA abundance and linearization of amplified DNA from various numbers of PCR cycles.

Yeast two hybrid system

The *Arabidopsis* cDNAs encoding *KNAT5, BEL1* and *BHL2* (1151, 1834 and 1900 bp, respectively; Dr. Kim Jae Yeon, Gyeongsang National University, Korea) were cloned into a *pGAD424* vector (Bartel et al., 1993). The *OSH6* and full-length *OSH6* cDNAs were amplified via PCR and a *Bgl*II site was added, after which fragments were digested with *Bgl*II and ligated into a *pGBD-C2* vector (James et al., 1996). Plasmids were introduced into the yeast two hybrid host strain *PJ69-4A* using the lithium acetate (LiAc) method. Transformants were selected by growth on Leu-/Trp-/His-/Ade- media. Growth of colonies indicated the activation of three marker genes, *ADE2* and *HIS3* (conferring histidine prototrophy and resistance to 3-AT), as well as *lacZ*.

RESULTS

In situ localization of truncated *OSH6* mRNA in the SAM and inflorescence meristem

To examine the correlation between the mutant phenotypes and the ectopic accumulation of truncated *OSH6* transcripts (*OSH6-Ds*), we conducted *in situ* hybridization analyses. As seen in a previous study, RT-PCR analysis revealed ectopic accumulation of *OSH6-Ds* transcripts in *OSH6-Ds* mutant leaves. However, *OSH6* mRNA is not normally expressed in lateral leaves (Park et al., 2007). The detailed patterns of *OSH6-Ds* expression in the mutant plants was assessed through RNA *in situ* hybridization with *OSH6-Ds* truncated antisense RNA as a probe. The expression pattern of *OSH6-Ds* mRNA was different from that of the wild type *OSH6* transcripts. In a longitudinal section of the shoot meristem, wild type *OSH6* mRNA was detected in the shoot apical cell and boundary region of the SAM (Sentoku et al., 1999), whereas no expression was detected in the lateral organ. In homozygous mutant plants, *OSH6-Ds* mRNA was detected at the marginal part of the leaf that developed at the tip of each leaflet. Additionally, *OSH6-Ds* mRNA expression was down-regulated in the SAM region. After floral transition, mRNA expression of both wild type *OSH6* and *OSH6-Ds* was detected in floral organs. In developing floral organs, transcripts were expressed in the palea, lemma and stamen (Figure 1).

Microarray analysis of *OSH6-Ds* and *OSH6-35S* plants

The *OSH6* gene containing the *Ds* transposon insertion (*OSH6-Ds)* produced mRNA that was both terminated prematurely and expressed ectopically. The heritable and qualitative changes in expression of genes containing transposable element insertions have been well documented (Martienssen et al., 2004). In addition, the *OSH6-Ds* transcripts were initiated from the same site as the wild-type sequences, but were terminated before the insertion site. Therefore, it is probable that the *Ds* element caused this premature termination (Park et al., 2007). Microarray technology can be used to examine changes in genome-wide gene expression in response to specific stimuli or to identify differences in genome-wide gene expression between sample populations (Kim et al., 2009; Kim et al., 2010). To identify genes with altered expression in *OSH6-Ds* mutant plants, microarray analyses were performed on wild type, *OSH6-Ds* mutant and *OSH6-35S* (*OSH6*-overexpressing) plants using a 22K rice microarray. All expression comparisons were determined with respect to wild-type expression. As compared with wild type plants, 69 genes were up-regulated and 49 genes were down-regulated 2-fold or greater in *OSH6-Ds* and/or *OSH6-35S* plants. Specifically, 43 and 41 genes in *OSH6-Ds* were expressed up- and down-regulated, and 43 and 11 genes in *OSH6-35S* were up- and down-regulated, respectively (Figure 2A). Of these genes, those with known functions were predominantly involved in transcriptional regulation, carbohydrate and lipid transport/metabolism, cell wall/membrane biogenesis and defense mechanisms. Among the genes with putative functions, those belonging to classes involved in transcriptional regulation and carbohydrate transport/metabolism were more frequently up-regulated (52%), whereas many genes involved in lipid transport/metabolism, cell wall/membrane biogenesis, and defense mechanisms were down-regulated (70%). Approximately, 40% of the up-regulated genes were identical in both *OSH6-Ds* and *OSH6-35S* plants. Three of the 11 down-regulated genes in *OSH6-35S* were also down-regulated in *OSH6-Ds* plants (Figure 2A). Therefore, out of the 54 genes with altered expression in *OSH6-35S*, 20 showed similar expression patterns in *OSH6-Ds* plants. Table 1 shows the genes that were regulated similarly in both lines as compared to wild type plants. The *AK101744*, *AK073177* and *AK066488* genes showed the oppositely regulated expression between the two lines.

To validate the data from the microarray experiments, the expression of 10 select genes was examined with RT-PCR using the same RNA samples as used for the microarray experiments (Figure 2B). Among them,

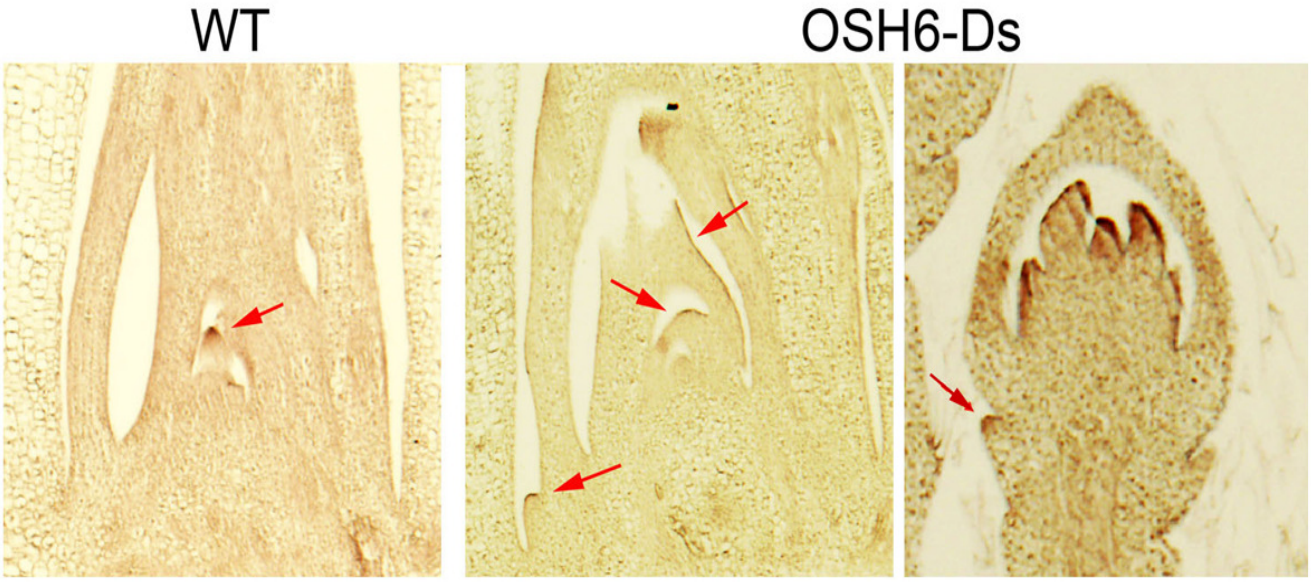

Figure 1. *In situ* examination of truncated *OSH6* mRNA expression in vegetative and reproductive apexes of *OSH6-Ds* rice mutant. Arrows indicate *OSH6* and *OSH6-Ds* expression in wild type (WT) and *OSH6-Ds* mutant plants, respectively. *In situ* hybridization with a shoot apical meristem region in wild type (left), *OSH6-Ds* mutant (middle) plant and floral organ in mutant (right). Sections of 7-days-old shoot apical meristem from wild-type and mutant, and floral organ were probed with digoxigenin labeled antisense RNA and viewed under a light microscope. Signal-positive cells are marked with red arrows.

AK107294 gene shows the expression pattern of a gene encoding *OsDOF3* protein. In wild-type plants, the gene *OsDOF3* is expressed only in seedlings and not floral organs before fertilization and this gene is suggested to have a role in protein-protein associations in the gibberellin-mediated expression of the *RAmy1A* gene in the rice alleurone (Washio, 2003). Strikingly, *AK107294 (OsDOF3)* gene expression was strongly up-regulated in both *OSH6-Ds* and the *OSH6* over-expressing lines (Table 1 and Figure 2C). Additionally, ectopic activation of *OsDOF3* was observed in *OSH6-35S* transformants. This result further supported the fact that truncated *OSH6* transcript might be actively involved in gene expression during organ development. Taken together, these data indicate that the expression of some genes might be influenced by both the truncated and over-expressed forms of the *OSH6* peptide.

OSH6-Ds protein interacted with different HD proteins

To understand the function of *OSH6-Ds* protein in plant development, it is necessary to characterize the biochemical properties of this protein. The three-amino-acid-loop-extension (TALE) class of homeoproteins: class I and II *KNOX* and *BEL* genes, play a central role in plant developmental processes, control meristem formation and/or maintenance, organ morphogenesis, organ position and several aspects of the reproductive phase (Hamant and Pautot, 2010). Different interaction affinities have been reported among the TALE HD proteins and select interactions enhance binding affinities to target DNA sequences (Smith et al., 2002). Consequently, similar to PREP/MEINOX proteins in animals, *KNOX* and BELL proteins form heterodimers that are thought to constitute the functional entities regulating plant development (Bellaoui et al., 2001; Smith et al., 2002). Also, *KNOX*/BELL heterodimerization is implicated in nuclear import of plant TALE proteins and has been shown to increase DNA-binding affinity and specificity. Every member of the *KNOX* family has at least one interaction partner amongst the BELL proteins and vice versa, supporting the notion that BELL/*KNOX* heterodimerization plays a general role in TALE protein function.

A yeast two hybrid system was used to examine the dimerization capacity of *OSH6-Ds* with TALE HD proteins and relation was tested to know whether truncated *OSH6-Ds* could interact with other homeodomain proteins. Based on previous studies (Reiser et al., 2000; Smith et al., 2002), candidate proteins for interactions with the various forms of *OSH6* included *BEL1*-like or other TALE HD proteins. Therefore, *Arabidopsis KNAT5*, *BEL1* and *BLH2* (BEL1-like homeodomain protein 2) were coexpressed with either *OSH6-Ds* or wild type *OSH6*. *BEL1* is a TALE homeodomain transcription factor of which the N-terminal sequence can form an amphipathic-helix. BEL domains in *BEL1* and *BLH2* are responsive for the interaction between the TALE/BELL proteins and the TALE/*KNOX* proteins (Reiser et al., 2000).

In this yeast two hybrid system, the N-terminal domains of *OSH6* and *OSH6-Ds*, including the MEINOX domain,

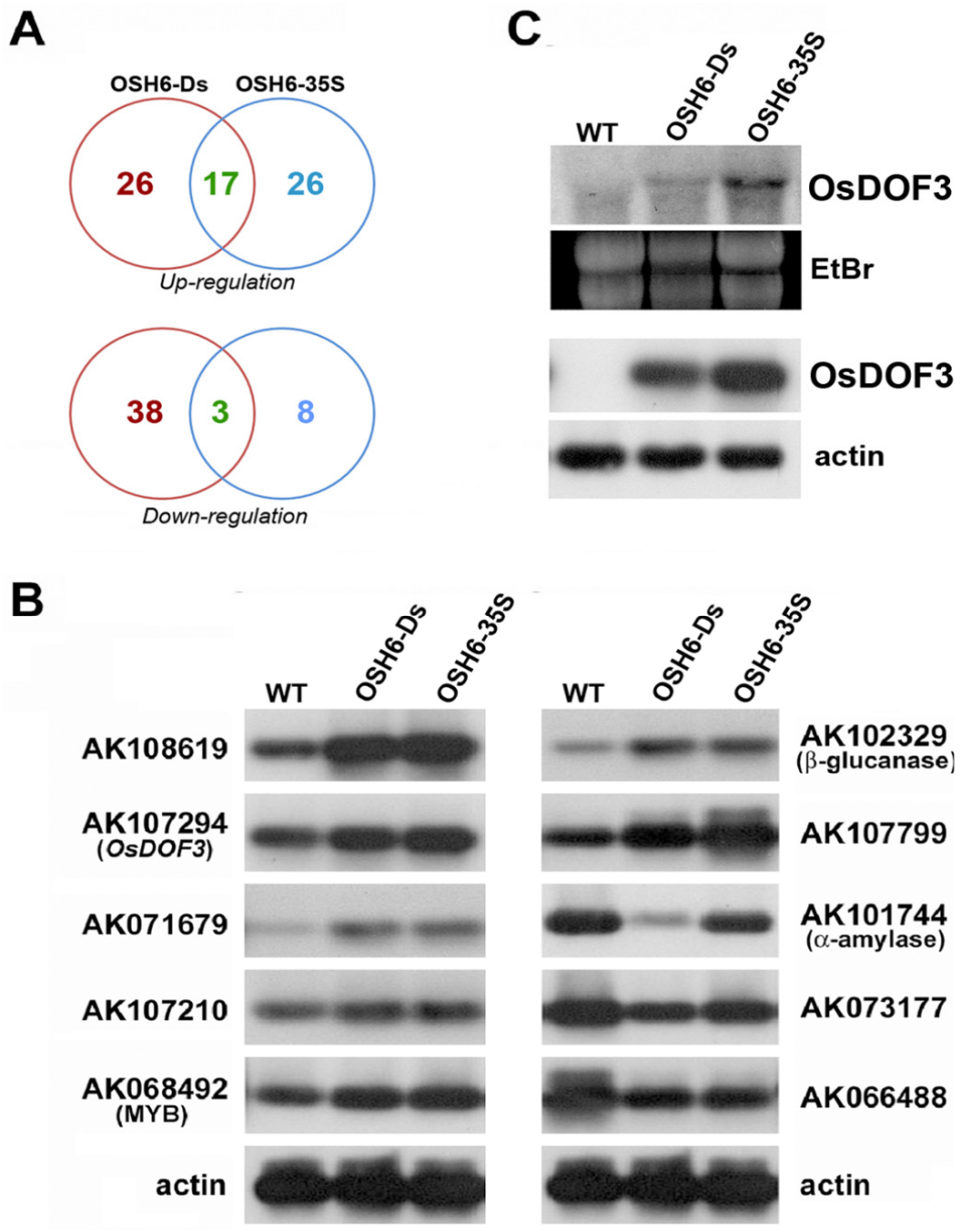

Figure 2. Gene expression profiles in *OSH6-Ds* and *OSH6-35S* mutants as compared to wild type plants. (A) Venn diagrams grouping genes that were greater than 2-fold up- or down-regulated in *OSH6-Ds* and/or *OSH6-35S* plants as compared to wild type plants. (B) Microarray experiments were confirmed by RT-PCR analyses or northern hybridization. *Actin* mRNA was used as a loading control. (C) *OsDOF3* expression was detected using RT-PCR (top) and northern hybridization (bottom) in the panicles from wild type, *OSH6-Ds* and *OSH6-35S* rice mutants.

mediated heterodimerization with *BEL1* (Bellaoui et al., 2001). Even though full-length *OSH6* showed comparable affinities for *BEL1*, *BLH2* and *KNAT5*, truncated *OSH6* exhibited weak interactions with *BEL1*, *BLH2* and *OSH6*, but an increased affinity for *KNAT5*. Currently, the molecular basis of how *KNAT5* has a stronger affinity for *OSH6-Ds* than the other proteins is unknown. Nonetheless, the N-terminal MEINOX domain alone likely has different dimerization affinities from the full-length protein. Therefore, it is likely that altered *OSH6* affinity for TALE

Table 1. Genes up- or down-regulated more than 2-fold in both *OSH6-Ds* and *OSH6-35S* plants as compared to wild type plants in the 22K rice microarray.

Accession No.	Putative function	Group	Expression ratio	
			OSH6-Ds	*OSH6-35S*
Carbohydrate transport/metabolism				
AK101744	Rice *α*-amylase	Down	-26.32	-2.13
Lipid transport/metabolism				
AK071679	Lecithin-cholesterol acyl transferase	Up	3.02	2.33
AK107210	Fatty acid elongase	Up	2.15	2.69
Cell wall/membrane/defense response				
AK108619	Glycine-rich cell wall protein	Up	23.33	19.14
AK102329	*β*-lucanase	Up	2.03	2.03
AK073177	Protein phosphorylation	Down	-2.43	-2.67
Transcriptional regulation				
AK068492	MYB transcription factor	Up	3.42	3.95
AK107294	Dof zinc finger protein (*OsDOF3*)	Up	2.44	2.75
Secondary metabolite/protein turnover				
AK107799	C2 superfamily, membrane trafficking	Up	2.44	3.73
AK066488	Subtilase, peptidase activity	Down	-2.82	-3.49
Poorly characterized				
AK102606	Function unknown	Up	15.37	12.03
AK111053	Function unknown	Up	5.04	5.13
AK110644	Function unknown	Up	4.35	2.61
AK064111	Function unknown	Up	3.85	3.58
AK072148	Function unknown	Up	2.03	6.28

HD proteins is a factor that led to the differences in transcriptional profiles and phenotypes between wild type, *OSH6-Ds* and *OSH6-35S* plants (Figure 3).

Expression patterns of *OSH6* and *OSH15*

To examine whether phenotypic expression of *OSH6-Ds* is influenced by *OSH6* itself, or by other *KNOX* genes, we analyzed another rice *KNOX* gene, a recessive *OSH15* mutation arose from insertion of *TOS17* (Sato et al., 1999) and the *osh15* mutant exhibited a semi-dwarf phenotype due to short internodes. This possibility is supported by previous observations that animal and plant TALE HD proteins often interact with other homeodomain proteins to regulate their function. Also, *OSH15* interacted with all of the OSH proteins and dimerization with wild type of *OSH6* protein (Nagasaki et al., 2001). To reveal the expression pattern of *OSH6,* we performed RT-PCR expression profiles of *OSH6* and *OSH15* in various tissues from 2-week-old plant. As a control, *OSH1* mRNA was similarly examined. As previously reported (Sato et al., 1999; Sentoku et al., 1999), *OSH1* expression was restricted mainly to SAM and inflorescence meristems. *OSH6* transcripts were detected at high levels in SAM, callus, root and floral tissues of both booting and heading stages (Figure 4A). Expression was not restricted to the meristematic organs. *OSH6* mRNA was detected in the florets of panicles, but was not detected in the leaves (Figure 4B). The low transcript levels in the stem sections containing SAM were likely due to the absence of transcripts in the leaf tissues of the stem samples. In conclusion, the expression pattern of *OSH15* was similar to that of *OSH6* and two *OSH* proteins (*OSH6* and *OSH15)* were much more ubiquitous than that of *OSH1.*

Genetic relationship between *OSH6-Ds* and *OSH15*

The *OSH15* and *OSH6-Ds X OSH15* mutants were examined on the *OSH6-Ds* genetic background. Since an *OSH15* mutant was available, we examined whether the phenotypic expression of *OSH6-Ds* was influenced by *OSH6* itself or by *OSH15*. The *OSH15* mutant exhibited a

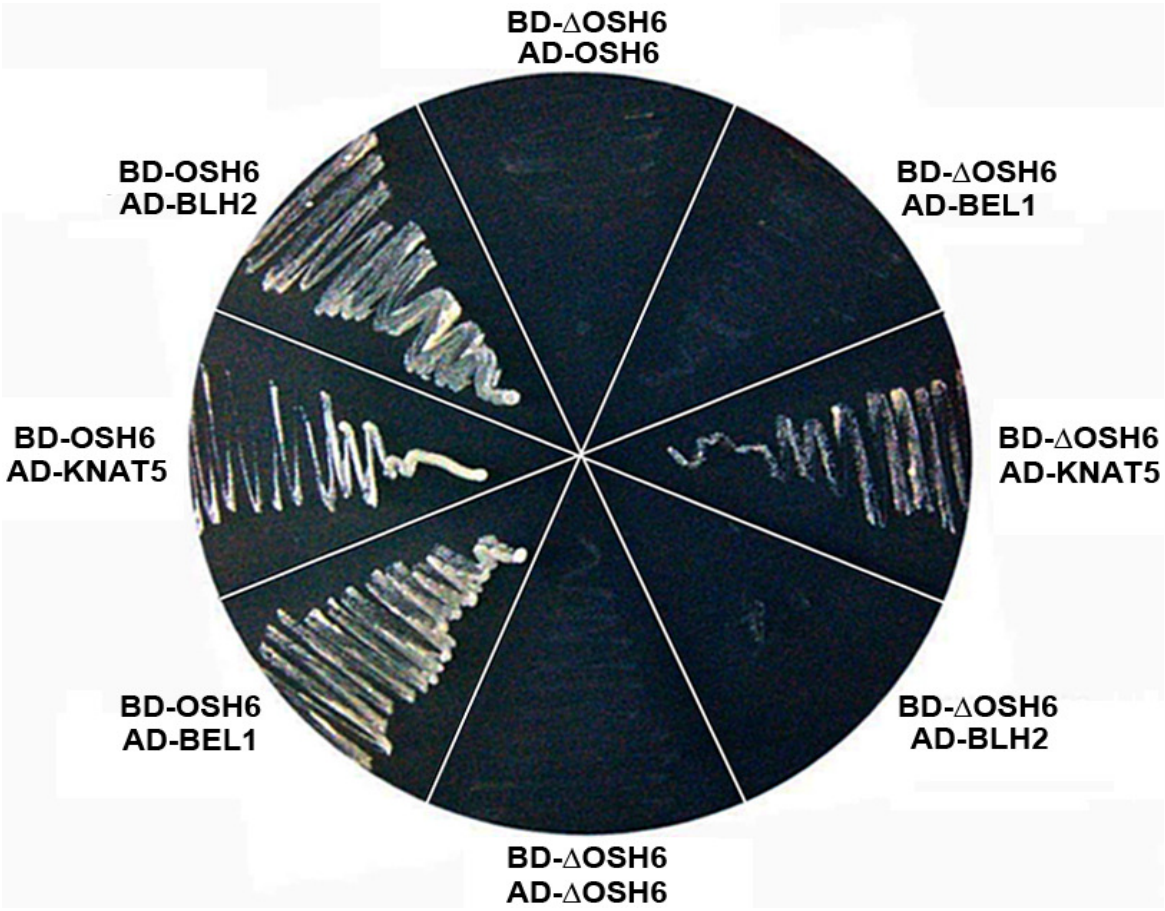

Figure 3. Yeast two hybrid analysis of interactions of *OSH6-Ds* and *OSH6* with *KNAT5, BEL1* and *BLH2*. Yeast was incubated for 3 days on plates containing Leu-/Trp-/His-/Ade- selection media, and the binding affinities of *OSH6-Ds* or *OSH6* with *KNAT5*, *BEL1* and *BLH2* were determined from their growth. pBD and pAD indicate the vectors carrying the DNA-binding or activation domains, respectively.

semi-dwarf phenotype due to short internodes. A double mutant (*OSH6-Ds X OSH15)* of *OSH15* and *OSH6-Ds* was generated by genetic crosses. Figure 5 compares the phenotypes of the single mutants and the double mutant. The *OSH6-Ds* single mutant and double mutant *OSH6-Ds X OSH15* still produced the extra bract-leaf in the floral peduncle (Figure 5A, left). However, in the F2 generations, the *OSH15* and *OSH6-Ds X OSH15* mutants did not exhibit any extra structure in the peduncles (Figure 5A, right). As expected, RT-PCR analysis showed that the truncated mRNA was present in the leaves and flower tissue of only lines carrying the *OSH6-Ds* allele (Figure 5B). These data suggest that the formation of the extra leaf is independent of the expression of *OSH15* or *OSH6-Ds*.

DISCUSSION

We performed a computational analysis using a 22K rice microarray to identify *OSH6* (*O. sativa* Homeobox 6) in *Ds* insertion mutant rice. Using this microarray, 118 genes were screened to identify those that were up- or down-regulated greater than 2.0-fold. Of these genes, 20 were up- or down-regulated in both the *OSH6-Ds* and *OSH6*-over-expressing (*OSH6-35S*) plants. Strikingly, *AK107294 (OsDOF3)* gene expression was strongly up-regulated in both *OSH6-Ds* and the *OSH6-35S* mutant and ectopic activation of *OsDOF3* was observed in *OSH6-35S* mutants. Dof proteins are members of a major family of plant transcription factors, associated with diverse phenomena including light, phytohormone and defense responses, seed development and germination unique to plant. This results indicates that the novel function of *OsDOF3* is responsible for a *KNOX* class I homeobox mediation of plant later organ development. Therefore, this truncated *OSH6* transcript might be actively involved in the gene expression during organ development and expression of some genes might be influenced by both the truncated and over-expressed forms of the *OSH6* peptide. The expression pattern of *OSH15* was similar to that of *OSH6*, and two *OSH*

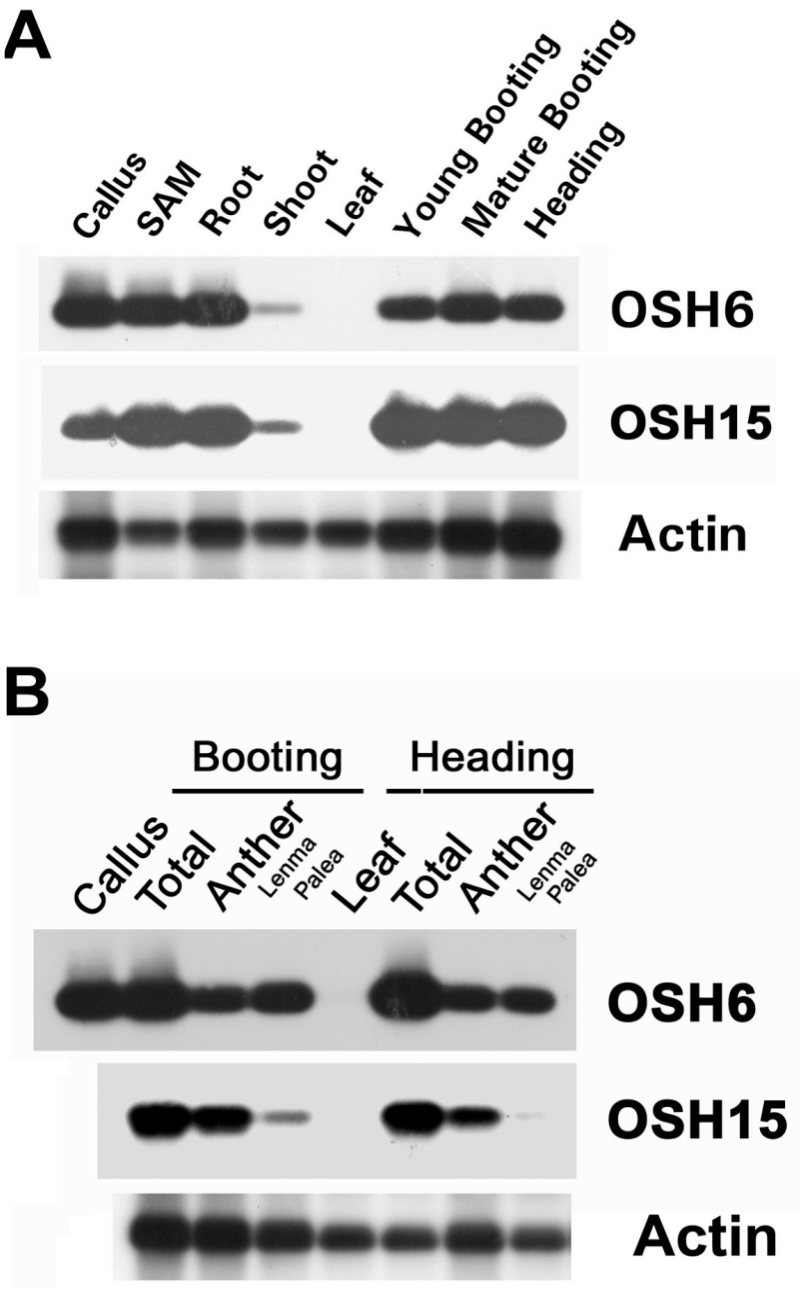

Figure 4. Expression patterns of the *OSH6* gene in rice tissues. Various tissues, including young shoots (A) and floral tissues (B) of wild-type plants were examined by RT-PCR to examine *OSH6, OSH15* and actin transcript levels. PCR products were hybridized with cDNA from each corresponding gene for northern blot analysis.

proteins (*OSH6* and *OSH15)* were much more ubiquitous than that of *OSH1.* The genetic relationship between *OSH6-Ds* and *OSH15* suggested that the formation of the extra leaf was independent of the expression of *OSH6-Ds* or *OSH15.* This study provides valuable insight in that, truncated *OSH6-Ds* mRNA might likely influence lateral organ growth and development by regulating the expression of 20 candidate gene groups which were commonly regulated in both the *OSH6-Ds* and *OSH6-35S* rice mutants.

ACKNOWLEDGEMENTS

This study also was supported by 2010 Postdoctoral Fellowship Program of National Academy of Agricultural

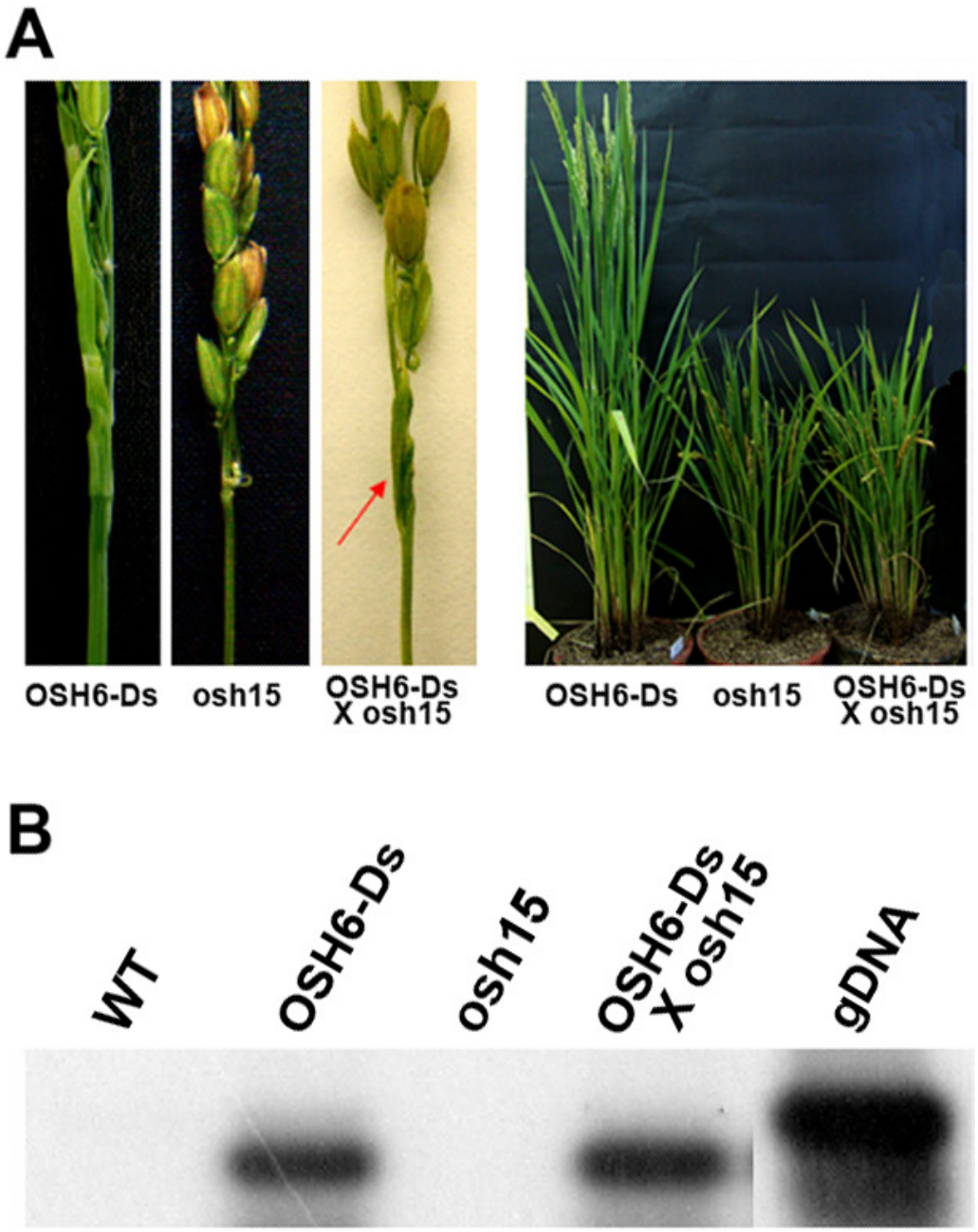

Figure 5. Phenotype and RT-PCR comparisons of single- or double-mutants of *OSH6-Ds* and *osh15*. (A) Phenotypic comparisons of panicle nodes. Arrows indicate 'bract-leaf.' (B) RT-PCR analysis of truncated mRNA. gDNA indicates genomic DNA.

Science, Rural Development Administration, Republic of Korea, by a grant (code #CG3310) from Crop Functional Genomics Center of the 21st Century Frontier Research Program and was supported by the National Academy of Agricultural Science (No. PJ006651).

REFERENCES

Bartel PL, Chien R, Sternglanz, Fields S (1993). Using the two- hybrid system to detect protein-protein interactions, Cellular Interactions in Development: A Practical Approach, edited by Hartley DA. Oxford University Press, Oxford pp. 153-179.

Bellaoui M, Pidkowich A, Samach K, Kushalappa SE, Kohalmi et al. (2001). The Arabidopsis *Bell1* and *Knox* Tale homeodomain proteins interact through a domain conserved between plants and animals. Plant Cell, 13: 2455-2470.

Byrne M, Timmermans M, Kidner C, Martienssen RA (2001). Development of leaf shape. Curr. Opin. Plnat Biol. 4: 38-43.

Chin HG, Choe MS, Lee SH, Park SH, Koo JC, Kim NY, Lee JJ, Oh BG, Yi GH, Kim SC, Choi HC, Cho MJ, Han CD (1999). Molecular analysis of rice plants harboring an *Ac/Ds* transposable element-

mediated gene trapping system. Plant J. 19:615-624.
Hamant O, Pautot V (2010). Plant development: a TALE story. Comptes Rendus Biologies, 333(4): 371-81.
Horigome A, Nagasawa N, Ikeda K, Ito M, Itoh JI, Nagato Y (2009). *OPEN BEAK* is a negative regulator of class 1 *knox* genes and a positive regulator of class B floral homeotic gene. Plant J. 58: 724-736.
Ito Y, Kurata N (2008). Disruption of *KNOX* gene suppression in leaf by introducing its cDNA in rice. Plant Sci. 174:357-365.
James P, Halladay J, Craig EA (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics, 144: 1425-1436.
Katsutoshi T, Yukihiro I, Shinichiro Y, Akio M, Hirohiko H, Nori K (2009). Isolation and mapping of three rice mutants that showed ectopic expression of *KNOX* genes in leaves. Plant Sci. 177: 131-135.
Kerstetter R, Vollbrecht E, Lowe B, Veit B, Yamaguchi J, Hake S (1994). Sequence analysis and expression patterns divide the maize *knotted1*-like homeobox genes into two classes. Plant Cell, 6: 1877-1887.
Kerstetter RA, Laudencia-Chingcuanco D, Smith LG, Hake S (1997). Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. Development, 124: 3045-3054.
Kim CK, Han JH, Shin YH, Park SH, Yun DW, Ahn BO, Kim DH, Park BS, Hahn JH (2009). A genome browser database for rice (*Oryza sativa*) and Chinese cabbage (*Brassica rapa*). Afr. J. Biotechnol. 8(20): 5253-5259.
Kim CK, Kikuchi S, Kim YK, Park SH, Yoon YH, Lee GS, Choi JW, Kim YH, Park SC (2010). Computational identification of seed-specific transcription factors involved in anthocyanin production in black rice. BioChip J. 4(3): 247-255.
Kim CK, Kikuchi K, Satoh K, Kim JA, Kim DH, Kim YH, Park SH, Yoon YH (2009). Genetic analysis of seed-specific gene expression for pigmentation in colored rice. BioChip J. 3: 125-129.
Kim CK, Park SH, Kikuchi S, Kwon SJ, Park SY, Yoon UH, Park DS, Seol YJ, Hahn JH, Park SC, Kim DH (2010). Genetic analysis of gene expression for pigmentation in Chinese cabbage (*Brassica rapa*). BioChip J. 4(2): 123-128.
Kim CK, Yoon UH, Lee GS, Lee HK, Kim YH, Hahn JH (2009). Rice genetic marker database: An identification of single nucleotide polymorphism (SNP) and quantitative trait loci (QTL) markers. Afr. J. Biotechnol. 8(13): 2963-2967.
Kim CM, Piao HL, Park SJ, Chon NS, Je BI, Sun BY, Park SH et al. (2004). Rapid, large-scale generation of *Ds* transposant lines and analysis of the *Ds* insertion sites in rice. Plant J. 39: 252-263.
Kouchi H, Hata S (1993). Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. 238: 106-119.
Martienssen R, Lippman Z, May B, Ronemus M, Vaughn M (2004). Transposons, tandem repeats, and the silencing of imprinted genes. Cold Spring Harbor Symposia on Quantitative Bio. Symp. 69:371-380.
Muehlbauer GJ, Fowler JE, Girard L, Tyers R, Harper L, Freeling M (1999). Ectopic expression of the maize homeobox gene *liguleless3* alters cell fates in the leaf. Plant Physiol. 119: 651-662.
Muller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W (2001). *In vitro* interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of *Knox* gene function. Plant J. 27: 13-23.
Nagasaki H, Sakamoto T, Sato Y, Matsuoka M (2001). Functional analysis of the conserved domains of a rice *KNOX* homeodomain protein, *OSH15*. Plant Cell, 13: 2085-2098.
Park DS, Park SK, Han SI, Wang HJ, Jun NS, Manigbas NL, Ahn BO, Yun DW, Yoon YH, Kim YH, Lee MC, Kim DH, Nam MH, Han CD, Kang HW, Yi GH (2009). Genetic variation through Dissociation (*Ds*) insertional mutagenesis system for rice in Korea: progress and current status. Mol. Breed. 24: 1-15.
Park SH, Jun NS, Kim CM, Oh TY, Huang J, Xuan YH, Park SJ, Je BI, Piao HL, Park SH, Cha YS, Ahn BO, Ji HS, Lee MC, Suh SC, Nam MH, Eun MY, Yi G, Yun DW, Han CD (2007). Analysis of gene-trap *Ds* rice populations in Korea. Plant Mol. Biol. 65: 373-384.
Park SH, Kim CM, Je BI, Park SH, Park SJ, Piao HL, Xuan YH, Choe MS, Satoh K, Kikuchi S, Lee KH, Cha YS, Ahn BO, Ji HS, Yun DW, Lee MC, Suh SC, Eun MY, Han CD (2007). A *Ds*-insertion mutant of *OSH6* (*Oryza sativa* Homeobox 6) exhibits outgrowth of vestigial leaf-like structures, bracts, in rice. Planta, 227: 1-12
Reiser L, Sanchez-Baracaldo P, Hake S (2000). Knots in the family tree: evolutionary relationships and functions of *knox* homeobox genes. Plant Mol. Biol. 42: 151-166.
Sato Y, Sentoku N, Miura Y, Hirochika H, Kitano H, Matsuoka M (1999). Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. EMBO J. 18: 992-1002.
Sentoku N, Sato Y, Kurata N, Ito Y, Kitano H, Matsuoka M (1999). Regional expression of the rice *KN1*-type homeobox gene family during embryo, shoot, and flower development. Plant Cell, 11: 1651-1664.
Sentoku N, Sato Y, Matsuoka M (2000). Overexpression of rice OSH genes induces ectopic shoots on leaf sheaths of transgenic rice plants. Dev. Biol. 220: 358-364.
Smith HMS, Boschke I, Hake S (2002). Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. Proc. Natl. Acad. Sci. USA, 99: 9579-9584.
Smith LG, Greene B, Veit B, Hake S (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development, 116: 21-30.
Washio K (2003). Functional dissections between GAMYB and DOF transcription factors suggest a role for protein-protein associations in the gibberellins-mediated expression of the *RAmy1A* gene in the rice aleurone. Plant Physiol. 133: 850-863.
Yazaki J, Shimatani Z, Hashimoto A, Nagata Y, Fujii F, Kojima K, Suzuki K, Taya T, Tonouchi M, Nelson C, Nakagawa A, Otomo Y, Murakami K, Matsubara K, Kawai J, Carninci P, Hayashizaki Y, Kikuchi S (2004). Transcriptional profiling of genes responsive to abscisic acid and gibberellin in rice: phenotyping and comparative analysis between rice and Arabidopsis. Physiol. Genomics, 17: 87-100.
Zhu QH, Eun MY, Han CD, Kumar CS, Pereira A, Ramachandran S, Sundaresan V, Eamens AL, Upadhyaya NM, Wu R (2007). Transposon insertional mutants: a resource for rice functional genomics. In: Upadhyaya NM (ed) Rice functional genomics challenges progress and prospects. Springer Press pp. 224-262.