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ORIGINAL RESEARCH

Rice *OsAS2* Gene, a Member of LOB Domain Family, Functions in the Regulation of Shoot Differentiation and Leaf Development

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Abstract *LATERALORGAN BOUNDARIES DOMAIN* (*LBD*) genes, a novel plant-specific family, play specific roles in plant development. Although function of *ASYMMETRIC LEAVES2* (*AS2*), a *LBD* gene, was extensively studied in *Arabidopsis* of dicots, little is known on the role of its ortholog in rice of monocots. In this study, a *LBD* gene that shares higher homology with *Arabidopsis AS2* gene was identified in rice and it was designated as *OsAS2*. Its transcripts were detected throughout predicted leaf primordia in shoot apical meristem (SAM), leaf primordia, and young leaves. Overexpression of the *OsAS2* gene inhibited shoot differentiation, promoted cell division, and delayed cell differentiation in rice calli. Transgenic plants with *OsAS2* gene showed the aberrant twisted leaves, which lack auricle, and leaf structure was abnormal. Furthermore, a few genes involved in shoot meristem development were upregulated in transgenic plants. Our results suggest that proper expression of the *OsAS2* gene is required for shoot differentiation and leaf development in rice.

Keywords *OsAS2* gene · Shoot differentiation · Leaf development · Rice

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Establishment of plant body mainly depends on the activity of shoot apical meristem (SAM), which gives rise to leaves, stems, and axial buds. Histologically, the SAM is divided into three zones: the central zone harbors the stem cells which continually make new cells, whereas initiation of lateral organs occurs in the surrounding peripheral zone, and central stem tissue is produced by underlying rib zone (Groß-Hardt and Laux 2003).

Mutations of *Arabidopsis CUC1*/2, *ATAF1/2*, and petunia *NAM*, which are expressed in boundaries between floral organ primordia and in the boundary between the cotyledons, cause defects in the establishment of several boundaries such as organ fusions (Souer et al. 1996; Aida et al. 1997; Takada et al. 2001). These suggest that the establishment and maintenance of boundary between SAM and lateral organs are important developmental processes. *LATERALORGAN BOUNDARIES DOMAIN* (*LBD*) genes, a novel plant-specific family, also have important functions in the maintenance of lateral organ boundaries (Souer et al. 1996; Aida et al. 1997; Shuai et al. 2002). They are characterized by the LOB domain and the conserved $CX_2CX_6CX_3C$ motif (Iwakawa et al. 2002; Shuai et al. 2002; Yang et al. 2006). The *LBD* gene family contains 43 members and has been classified into two classes (classes I and II), based on sequence similarity in the LOB domain in *Arabidopsis* (Iwakawa et al. 2002; Shuai et al. 2002). Classes I AtLBD proteins are predicted to form a predicted coiled-coil structure with four leucines in $LX_6LX_3LX_6L$ spacing that is reminiscent of a Leu zipper, and they may function in protein–protein interaction. Lacking this structure, the classes II AtLBD proteins may have distinct functions from class I members (Landschultz et al. 1988; Shuai et al. 2002).

LBD genes are expressed especially at the base of lateral organs, implying that it possibly defines a boundary between the initiating organ primordia and stem cells

(Shuai et al. 2002). Function analysis has demonstrated that *LBD* genes have specific roles in control of plant development. *ASL1/LBD36* controls proximal-distal patterning in *Arabidopsis* petals (Chalfun-Junior et al. 2005). The cytokinin-induced *ASL9/LBD3* plays a role around SAM in the regulation of plant development (Naito et al. 2007). *Crown rootless1* (*Crl1*) encodes an AS2/LOB domain transcription factor that is a direct target of an ARF protein and is essential for crown root formation in rice (Inukai et al. 2005). ARL1, a protein with LOB domain in rice, is an auxin-responsive factor involved in auxin-mediated cell dedifferentiation and controls the initiation of adventitious root primordia (Liu et al. 2005), whereas, *ramosa2*, one of *LBD* genes in maize, has functions in the patterning of stem cells in axillary meristems (Bortiri et al. 2006). Recently, it was found that *DH1* is required for the glume formation in rice, indicating the LOB domain gene participating in the development of floral organs in rice (Li et al. 2008).

The *ASYMMETRIC LEAVES2* (*AS2*) in *Arabidopsis* encodes a LOB domain protein and functions in the specification of adaxial/abaxial organ polarity (Byrne et al. 2000; Iwakawa et al. 2002; Sun et al. 2002; Lin et al. 2003). Although much data on *AS2* function have been accumulated in *Arabidopsis*, little is known on the role of an *AS2* ortholog in rice of monocots. In this study, we identified a LOB domain gene *OsAS2* from rice (*Oryza sativa*). In situ hybridization revealed that *OsAS2* transcripts were detected throughout the predicted leaf primordia in SAM, leaf primordia, and young leaves. Overexpression of the *OsAS2* gene affects shoot differentiation, cell division, and cell differentiation in transgenic calli. Overexpressed *OsAS2* gene also induces aberrant twisted leaves with the abnormal leaf structure. Our results might provide the important information to understand *OsAS2* functions in rice.

Materials and Methods

Plant Materials

Rice (*O. sativa* L. *cv.* Zhong Hua 11, obtained from Institute of Plant Physiology and Ecology, Shanghai, China) plants were grown in a field at the Agricultural Experiment Station of Shandong Agricultural University under natural growth conditions.

Gene Cloning

The cDNA fragments of *OsAS2* were amplified by RT-PCR. The PCR primers were designed based on the cDNA clone BAD88526. The sequences of the primers for *OsAS2* were OsAS2-1 (5′-GGATGGCGTCATCGTCAGCG-3′) and OsAS2-2 (5′-ATACTGGCCGGCGCCTTG-3′). The amplified *OsAS2* fragment was inserted into the pGEM-T easy vector (Invitrogen) for sequencing.

Construct Preparation and Plant Transformation

The binary vector used for rice transformation was constructed (Mason et al. 1992; Kulakova et al. 1995). A *Bam*HI and *Kpn*I fragment with an ubi enhancer/promoter and the NOS A terminator were inserted into pCAMBIA1301. To obtain the P_{ubi}::$OsAS2^{OE}$ construct, full-length coding sequence of the *OsAS2* genes were inserted into pCAMBIA1301 digested with *Bam*HI and *Kpn*I. The gene-specific fragments were amplified from the cDNAs by using the following primer pairs: OsAS2-5 (5′-AA GGATCCGGATGGCGTCATCGTCAGCG-3′) and OsAS2-3 (5′- ATGGTACCATACTGGCCGGCGCCTTG -3′).

Seeds of rice were husked and sterilized with 70% (*v*/*v*) ethanol for 5 min. Then, the seeds were sterilized further with 100 ml of 20% (*v*/*v*) commercial bleach for 1 h with gentle shaking. The sterilized seeds were rinsed with sterile water several times. Callus induction, cocultivation with *Agrobacterium tumefaciens*, and the selection of transformed calli were carried out as previously described by Jang et al. (1999).

RNA Isolation and RT-PCR

For RNA isolation, the plant tissues were collected separately in liquid N_2 and stored at −70°C. Total RNA was extracted using TRIZOL regent (Invitrogen), according to the manufacturer's instructions. For expression analysis of *OSH1* and *OsCYCD3;1*, total RNA was isolated from calli. For semi-quantitative RT-PCR analysis, 2 μg of total RNA was treated with DNase I (RNase-free, Promega) to remove genomic DNA. Reverse transcription was carried out with M-MLV Reverse Transcriptase (Promega). Rice *actin1* gene was used as the internal control. The primers in RT-PCR were used as follows:

OSH1-F (5′ -CTACCTCGACTGCCAGAAGG-3′)
OSH1-R (5′ -CCATGTGCATCAATCTCAGG-3′)
OsCYCD3;1-F (5′-AAAGCCATGCCTATGCTGCT-3′)
OsCYCD3;1-R (5′-CTTGCATTGCGTTAATGGAA-3′)
Actin1-F (5′-GTATCCATGAGACTACATACAACT-3′)
Actin1-R (5′- ACTCAGCCTTGGCAATCCACA-3′)

In Situ Hybridization

Plant materials were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) overnight at 4°C, dehydrated through a concentration grade of ethanol, cleared through a xylene series, then infiltrated through a series of paraffin, and finally embedded in 100% paraffin melted at 60°C. Sections were mounted on RNase-free glass slides.

The hybridization and immunological detection were performed according to DeBlock and Debrouwer (1993). The *OsAS2* probe was amplified with the primers OsAS2-8 (5′-GCCCGCGCCAAGTTCGAGC-3′) and OsAS2-3 (5′-ATACTGGCCGGCGCCTTG-3′). The PCR fragments were inserted into pGEM-T vector (Promega) for sequencing and RNA transcription. The DIG-labeled sense and antisense RNA probes were produced by T7 and Sp6 transcriptase, respectively. The reagents used in the experiments were purchased from Roche.

Light Microscopy

For light microscopy, middle parts of leaves and calli in wild-type and overexpression of *OsAS2* were fixed in 4% (*v*/*v*) glutaraldehyde at 4°C overnight, respectively. Then,

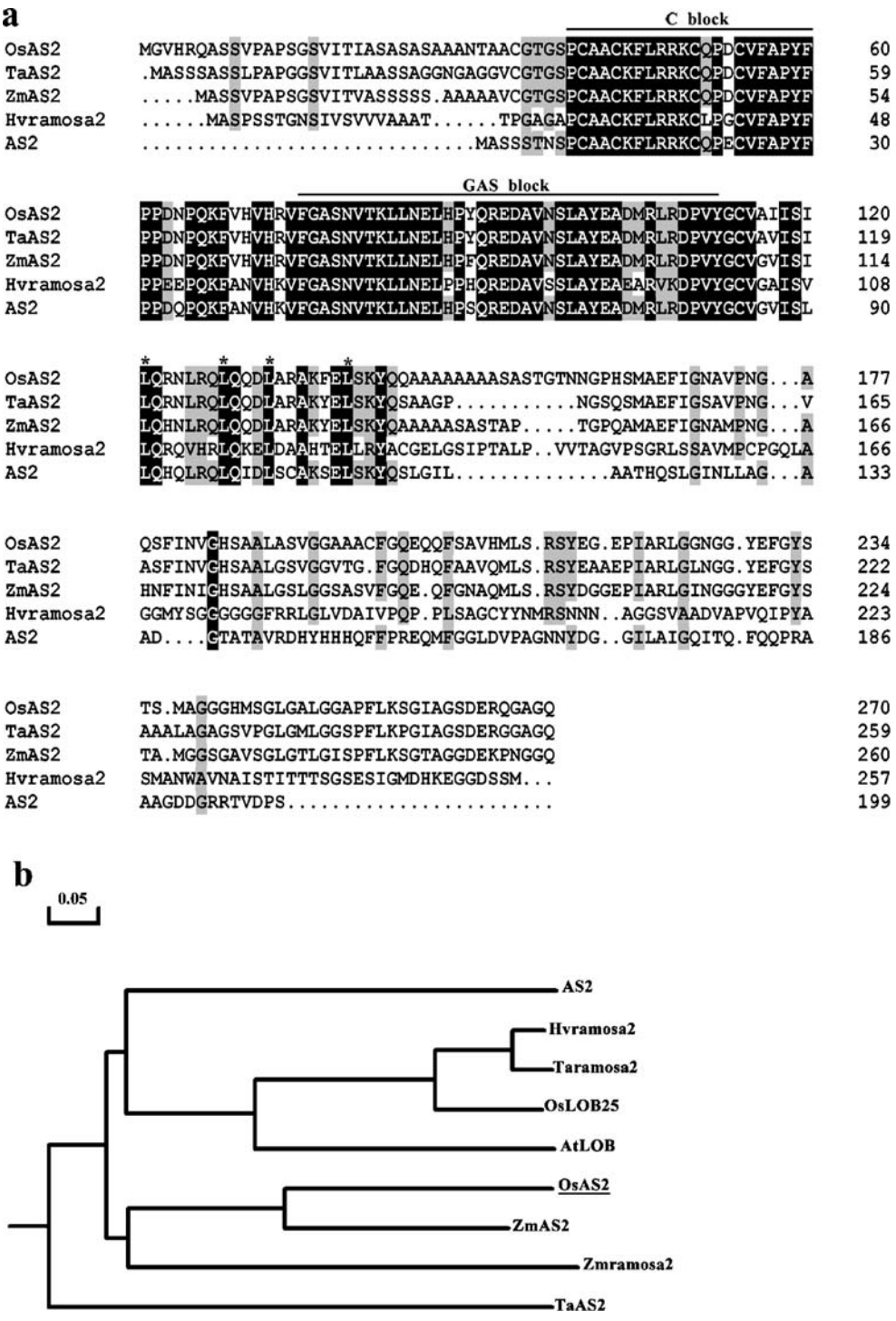

Fig. 1 Sequence analysis of *OsAS2*. **a** Alignment of deduced amino acid sequences of OsAS2 with TaAS2 (*Triticum aestium*), ZmAs2 (*Zea mays*), Hvramosa2 (*Hordeum vulgare*), and AS2 (*Arabidopsis thaliana*). Gaps introduced to improve alignment are indicated by *dashes*. The conserved domains are *bold underlined*. Accession number: OsAS2, EF540766; TaAS2, AY795560; ZmAS2, ABC54560; AS2, AAL38032; Hvramosa2, ABC54561. Dark shading with white letters and gray shading with dark letters reflect 100% and 75% sequence conservation, respectively. **b** Phylogenetic analysis of OsAS2 and LOB domain proteins of other plant species. The tree includes OsAS2, TaAS2, AS2, ZmAS2, AtLOB (NP851253), OsLOB25 (XP462810), Zmramosa2 (ABC54560), Hvramosa2 (ABC54561), and Taramosa2 (ABK79907). The tree is determined using the Vector NTI Advance 10 program

the samples were dehydrated through an ethanol series and embedded in paraffin (Sigma) according to the manufacturer's protocols. The sections (8 μm) were stained with 0.05% (*w*/*v*) toluidine blue O in distilled water and were photographed using an Olympus BH-2 microscope.

Results and Discussion

Isolation and Expression Analysis of *OsAS2* in Rice

To study the roles of an ortholog of *Arabidopsis* AS2 in rice, we identified the candidate gene homologous to *AS2* gene from young leaves of rice by RT-PCR, and it was designated as *OsAS2* (*O. sativa*, *AS2*). Sequencing analysis and dendrogram reveals that OsAS2 is closer to the proteins of the AS2/LOB family. Thus, it is most likely that the *OsAS2* encodes a member of a plant-specific AS2/LOB family in rice (Fig. 1).

To determine expression patterns of *OsAS2*, transcripts of *OsAS2* were analyzed in various tissues of rice. As expected, the transcripts of *OsAS2* were detected in seedlings and most of tissues such as root tips, mature roots, stems, leaves, young florets, carpels, and developing seeds (Fig. 2). Further, localization of *OsAS2* transcripts in young seedlings was examined by in situ hybridization. The signals in seedlings were observed in the predicted leaf primordia in SAM, leaf primordia, young leaves, and a root tip; but, the signals were not detected in a root tip hybridized with a sense probe as control (Fig. 3a–e). In SAM, the signals were mainly detected in the zone, which gives rise to leaf primordia (Fig. 3a). Also, the signals accumulate throughout leaf primordia and young leaves (Fig. 3a, b, c). The expression pattern of *OsAS2* is similar to that observed in wheat SAM and leaf primordia, indicating that the expression patterns between rice and wheat are relatively conserved (Ma et al. 2007). However, previous studies showed that the *AS2* gene in *Arabidopsis* is expressed in the adaxial region of the predicted leaf primordia and in early detectable primordia of SAM (Shuai et al. 2002; Lin et al. 2003). Thus, *OsAS2* expression pattern implies that this gene may play divergent role in regulation of leaf development in rice.

The *OsAS2* Expression Inhibits Shoot Differentiation, Promotes Cell Division, and Inhibits Cell Differentiation in Transgenic Calli

To determine *OsAS2* functions, we transferred this gene to rice by sense (P_{ubi}::$OsAS2^{OE}$) strategy with P_{ubi}::*GUS* as control. More than 80 independent transgenic resistant calli were produced for each construct. In the P_{ubi}::$OsAS2^{OE}$ transgenic calli, shoot differentiation was largely inhibited; however in P_{ubi}::*GUS*, transgenic tissues did not show any inhibition of shoot production as control (Table 1). In control, green tissues were produced within 2 weeks after being transferred to regeneration medium; but, in the P_{ubi}::$OsAS2^{OE}$ transgenic resistant calli, regeneration of green tissues required 3–4 weeks (Fig. 4a, b, e, f). The green tissues in control could develop to plantlets in 3–4 weeks, whereas almost all of P_{ubi}::$OsAS2^{OE}$ green tissues were not able to develop further, which could keep the green color for several months (Fig. 4b, c, f, g). In *Arabidopsis*, lower efficiency of regeneration of transgenic shoots was also observed in overexpressed *AS2* plants than in control plants (Iwakawa et al. 2002), suggesting that inhibitory effects on shoot differentiation caused by *AS2* or its ortholog expression are conserved between rice and *Arabidopsis*. Tissue sections showed that the P_{ubi}::$OsAS2^{OE}$ calli were composed of small, crowded, and densely stained cells, whereas many large cells were observed in the control calli, indicating that the expressed *OsAS2* is responsible for promotion of cell division and inhibition of cell differentiation in transgenic calli (Fig. 4d, h). RT-PCR results showed that *OsAS2* transcripts were significantly increased in the P_{ubi}::$OsAS2^{OE}$ transgenic resistant calli (Fig. 4i).

Overexpression of *OsAS2* Upregulates Transcript Levels of a Few Genes Involved in Shoot Meristem Development

Cyclin D3 plays an important role as a regulator of the population of proliferating cells and in determining cell number and size of shoot lateral organs (Riou-Khamlichi et al. 1999; Dewitte et al. 2003). The expression levels of *Orysa; CYCD3;1* was upregulated in the P_{ubi}::$OsAS2^{OE}$ green tissues, indicating that overexpressing *OsAS2* promotes expression of the gene that might be responsible for the promotion of cell division and the inhibition of cell differentiation.

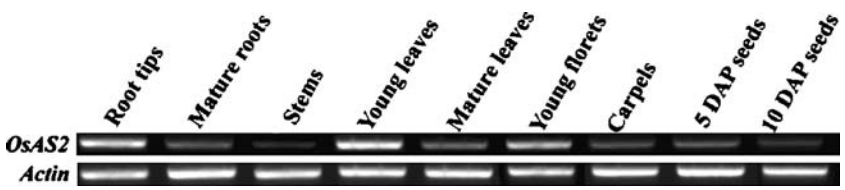

Fig. 2 Expression patterns of *OsAS2*. RT-PCR was carried out after RNA extraction from root tips, mature roots, stems, young leaves, mature leaves, young florets, carpels, and seeds at day 5 after pollination (*DAP*), seeds at day 10 after pollination (*DAP*). *Actin* was used as a control

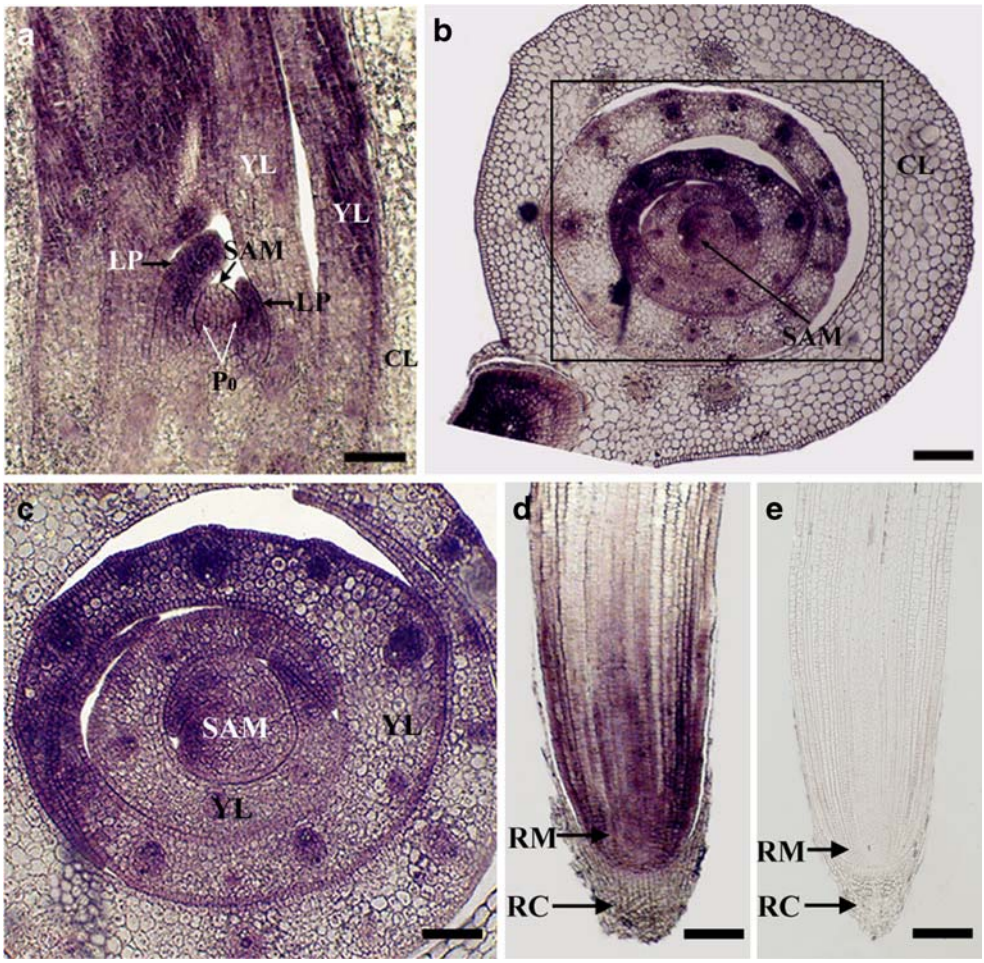

Fig. 3 Localization of *OsAS2* mRNA in rice seedlings by in situ hybridization. An *OsAS2* antisense probe was used in **a**, **b**, **c**, and **d**, and an *OsAS2* sense (control) probe was used in **e**. **a** Longitudinal section of the shoot apex at day 3 after germination. **b** Transverse section of the shoot apex at day 3 after germination. **c** Enlargement of the area marked in **b**. **d** and **e** Longitudinal section of the root tip at day 3 after germination. *CL* coleoptile, *LP* leaf primordium, *RC* root cap, *RM* root meristem, *SAM* shoot apical meristem, *YL* young leaf. Scale bars: 20 μm in **a** and **b**, 15 μm in **c**, and 300 μm in **d** and **e**

O. sativa homeobox1 (*OSH1*) was considered to be required for the establishment and maintenance of SAM (Sato et al. 1996). Most of the *Act1*::*OSH1* rice transformants did not develop any normal leaves but formed clumps of multiple shoots with normal roots (Sentoku et al. 2000). The upregulated expression of *OSH1* was detected in the transgenic tissues of P_{ubi}::*OsAS2*^{OE} (Fig. 5). The increased expression level of *OSH1* suggests that the *OsAS2* gene affects the proper function of SAM via the genes involved in SAM development.

Overexpression of *OsAS2* Results in Abnormal Leaf Development

Although the shoot differentiation was dramatically inhibited in P_{ubi}::*OsAS2*^{OE} transgenic tissues, we obtained four independent transgenic calli, which could produce plantlets (Fig. 6). Most of plantlets grew retardedly and will die after producing a few abnormal leaves compared with control ones (Fig. 6a–c). Among them, it was found that four plantlets could normally grow, but produced twisted leaves (Fig. 6d). Thus, we focused the examination on these leaf morphology and structure. As shown in Fig. 6, transgenic leaves twist upward and have no leaf auricles, which are located at the junction between the leaf blade and the leaf sheath in the control (Fig. 6e, f). All the phenotypic plants showed an increase of *OsAS2* transcripts (Fig. 6g).

Table 1 Frequency of hygromycin (Hm)-resistant plant induction

Constructs	Number of Hm-resistant calli	Number of Hm-resistant plants	Frequency of plant induction (%)
P_{ubi}::*GUS*	94	81	86.2
P_{ubi}::*OsAS2*^{OE}	85	4	4.7

Cellular organization of transgenic and control leaves at the same developmental stage were further examined. The mature leaf 4 of each plant was chosen to be sectioned. Although the examination of cross sections revealed no alteration of the leaf adaxial/abaxial polarity, the transgenic leaf is narrower and thicker than the control one (Fig. 7a, b). The number of ribs is decreased, and the midrib is enlarged in transgenic leaf, compared with the control one (Fig. 7a–f). Most of cells in epidermises, mesophyll, and vascular bundle look abnormal (Fig. 7a–f). In the control vascular bundle of midrib, the phloem and the xylem could be identified clearly; however, in the transgenic leaf, all cells of the

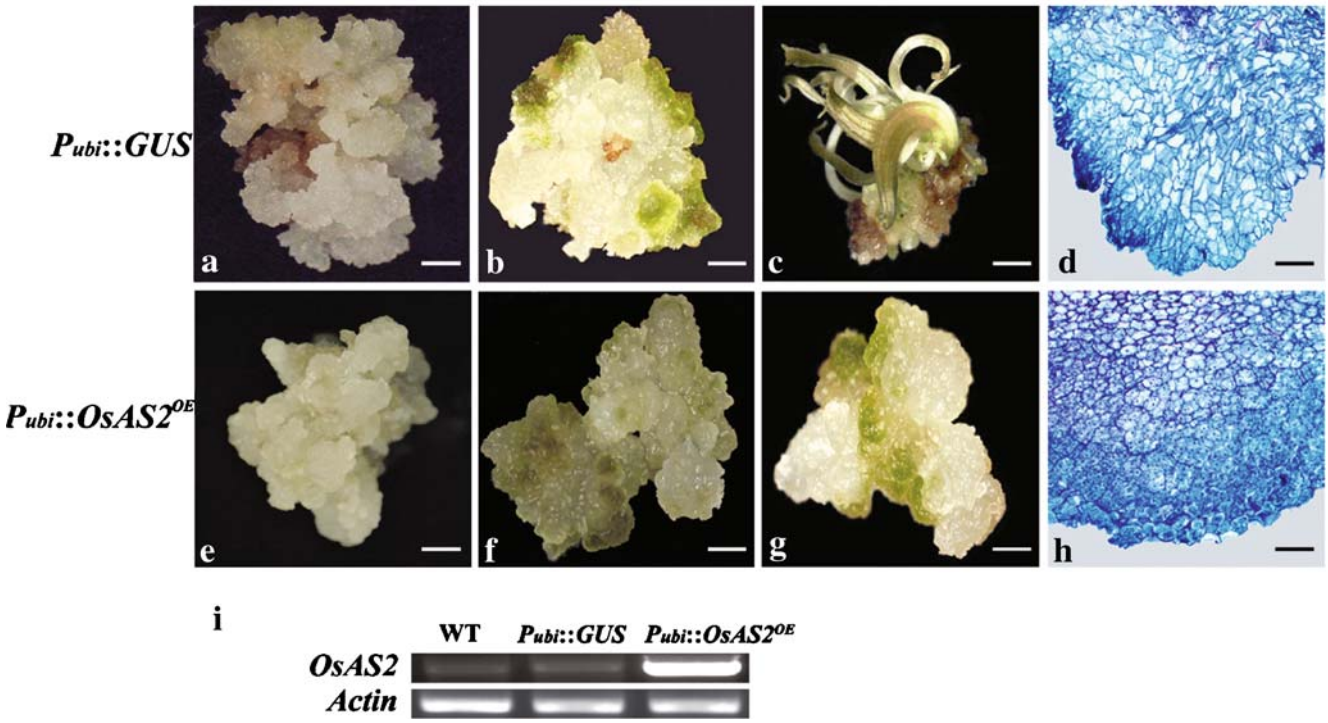

Fig. 4 Morphological analysis of transgenic rice calli. **a** The calli containing P_{ubi}::*GUS* in the media for shoot induction at 1 week. **b** The green calli containing P_{ubi}::*GUS* in the media for shoot induction at 2 weeks. **c** Differentiated seedlings containing P_{ubi}::*GUS* for shoot induction at 4 weeks. **d** Section of the tissue in **a**. **e** The calli containing P_{ubi}::$OsAS2^{OE}$ in the media for shoot induction at 1 week. **f** The green tissues containing P_{ubi}::$OsAS2^{OE}$ for shoot induction at 4 weeks. **g** The green tissues containing P_{ubi}::$OsAS2^{OE}$ for shoot induction at 2 months. **h** Section of the tissue in **e**. **i** RT-PCR analysis of *OsAS2* expression levels in transgenic calli. Scale bars: 750 μm in **a**–**c** and **e**–**g**, 100 μm in **d** and **h**

phloem and some cells in xylem were stained deeply, suggesting that these cells may be less differentiated than those in control vascular bundle (Fig. 7e, f). In other ribs of transgenic leaf, similar phenotypes were observed (data not shown).

In this study, expression of *OsAS2* in rice caused the inhibition of shoot differentiation in transgenic calli and abnormal morphology of transgenic leaf (Figs. 4, 6, and 7; Table 1). Molecular analysis indicated that genes involved in the SAM development were upregulated in transgenic calli (Fig. 5). Therefore, the upregulation of these genes in the overexpressed *OsAS2* plants might disturb establishment and maintenance of SAM and resulted in abnormal SAM and leaf development. In *Arabidopsis*, low efficiency of shoot regeneration and abnormal development of 35S::*AS2* transgenic plants were observed. The genes *SHOOTMRISTELESS* (*STM*) and *WUSHCHEL* transcript levels were increased in 35S::*AS2* transgenic plants (Lin et al. 2003). We suggest that functions of both *OsAS2* in rice and *AS2* in *Arabidopsis* are relatively conserved in shoot differentiation and establishment of leaf morphology.

Overexpressed *AS2* plants in *Arabidopsis* showed a perturbation of normal adaxial–abaxial asymmetry in leaves, resulting in the replacement of abaxial cell types

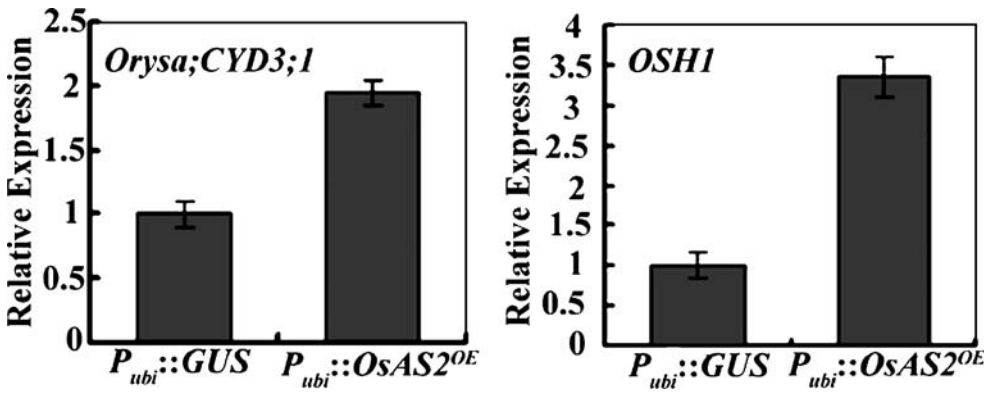

Fig. 5 Relative mRNA accumulations of genes (*Orysa; CYCD3;1* and *OSH1*) in the transgenic rice calli carrying P_{ubi}::$OsAS2^{OE}$. Semi-quantitative RT-PCR was performed with three independent biological replicates. The signals of amplified gene products were calculated with the Gel-Pro analyzer (Media Cybernetics, USA). The difference between them is significant ($P<0.05$)

Fig. 6 Overexpression of *OsAS2* in rice. **a** A P_{ubi}::*GUS* transgenic plant. **b** A P_{ubi}::$OsAS2^{OE}$ transgenic callus with a plantlet. **c** An abnormal P_{ubi}::$OsAS2^{OE}$ transgenic plantlet. **d** The P_{ubi}::$OsAS2^{OE}$ transgenic plants with twisted leaves. **e** The P_{ubi}::*GUS* transgenic leaf has the auricle. **f** The P_{ubi}::$OsAS2^{OE}$transgenic rice leaf without auricle. *Arrowhead* shows auricle. **g** RT-PCR analysis of *OsAS2* expression in transgenic plants and calli. *CK*, P_{ubi}::*GUS* transgenic plant; *L1*, *L2*, P_{ubi}::$OsAS2^{OE}$ transgenic plants in **c** and **d**; *Ca*, P_{ubi}::$OsAS2^{OE}$ transgenic calli; *P*, plantlet. Scale bars: 1 mm in **b** and **c**

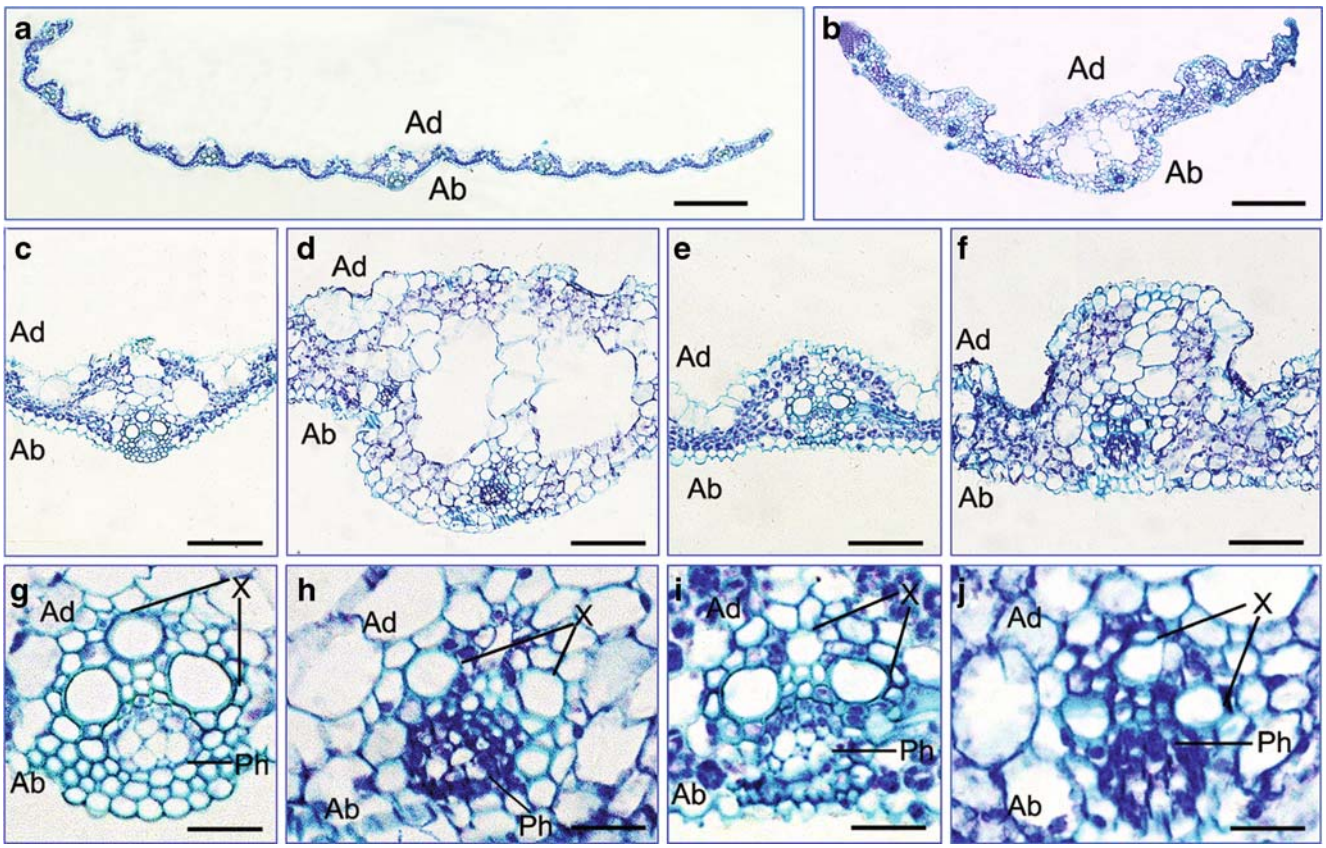

Fig. 7 Transverse section of leaves in P_{ubi}::*GUS* (**a**, **c**, **e**, **g**, **i**) and P_{ubi}::$OsAS2^{OE}$ plants (**b**, **d**, **f**, **h**, **j**). **a** and **b** Transverse sections of young leaf blades. **c** and **d** Enlarged view of the midrib. **e** and **f** Enlarged view of the lateral rib. **g** and **h** Enlarged view of the vascular bundle in the midrib. **i** and **j** Enlarged view of the vascular bundle in the lateral rib. *ad* adaxial, *ab* abaxial, *X* xylem, *Ph* phloem. Scale bars: 200 μm in **a** and **b**, 60 μm in **c** and **d**, 40 μm in **e** and **f**, and 15 μm in **g**–**j**

with adaxial cell types (Lin et al. 2003). Although *OsAS2* overexpression resulted in the abnormal development of cells and thereby the defects of leaf structure, it is likely that the adaxial–abaxial patterning is not affected obviously in transgenic leaf. This fact is consistent with the *OsAS2* expression pattern. The *OsAS2* transcripts were identified throughout leaf primorda, whereas the *AS2* transcripts in *Arabidopsis* accumulated in the adaxial region of either cotyledon or leaf (Iwakawa et al. 2007). In addition, the overexpressed *OsAS2* plants lacked leaf auricles. Thus, either *OsAS2* in rice or *AS2* in *Arabidopsis* may have a divergent role in leaf development.

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