OsARID3, an AT-rich Interaction Domain-containing protein, is required for shoot meristem development in rice

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SUMMARY

The shoot apical meristem (SAM) produces all of the plant’s aerial organs. The SAM is established either during embryogenesis or experimentally in in vitro tissue culture. Although several factors including the Class I KNOTTED1-LIKE HOMEOBOX (KNOXI) proteins, auxin, and cytokinin are known to play essential roles in SAM development, the underlying mechanisms of SAM formation and maintenance are still largely not understood. Herein we demonstrate that OsARID3, a member of the rice (Oryza sativa) AT-rich Interaction Domain (ARID) family, is required for SAM development. Disruption of OsARID3 leads to a defective SAM, early seedling lethality, and impaired capacity of in vitro shoot regeneration. We show that the expression levels of several KNOXI genes and the biosynthetic genes for auxin and cytokinin are significantly altered in the Osarid3 mutant calli. Moreover, we determine that auxin concentrations are increased, whereas cytokinin levels are decreased, in Osarid3 calli. Furthermore, chromatin immunoprecipitation results demonstrate that OsARID3 binds directly to the KNOXI gene OSH71, the auxin biosynthetic genes OsYUC1 and OsYUC6, and the cytokinin biosynthetic genes OsIPT2 and OsIPT7. We also show through electrophoretic mobility shift assays that OsARID3 specifically binds to the AT-rich DNA sequences of the identified target genes. We conclude that OsARID3 is an AT-rich specific DNA-binding protein and that it plays a major role in SAM development in rice.

Keywords: Oryza sativa, shoot meristem, shoot regeneration, auxin, cytokinin, YUC, isopentenyltransferase, KNOXI, AT-rich Interaction Domain.

INTRODUCTION

In higher plants, all above-ground organs are generated from the shoot apical meristem (SAM), which is comprised of a group of stem cells. The SAM is established during embryogenesis, and subsequently produces leaves, axillary buds, stems, as well as floral organs during post-embryonic development (Pautler et al., 2013). The formation and maintenance of SAM is the basis of morphogenesis. The Class I KNOTTED1-LIKE HOMEOBOX (KNOXI) transcription factors have been known for a long time to be indispensable for SAM formation and maintenance (Hake et al., 2004). Loss-of-function mutants of the KNOXI genes, such as knotted1 (kn1) in maize (Zea mays) (Vollbrecht et al., 1991), SHOOT MERISTEMLESS (STM) in Arabidopsis thaliana (Long et al., 1996), and ORYZA SATIVA HOMEobox1 (OSH1) in rice (Oryza sativa) (Tsuda et al., 2011), all show defects in SAM development.

Plant hormones also play critical roles in SAM maintenance (Shani et al., 2006). Cytokinin (CK) is well known to be essential for SAM function. A high level of CK relative to auxin is required for maintaining SAM function not only during in planta shoot development but also during in vitro shoot regeneration (Skoog and Miller, 1957; Meng et al., 2010). CK deficiency usually results in reduced SAM size and activity, as well as decreased shoot regeneration frequency (Higuchi et al., 2004; Miyawaki et al., 2006; Kurakawa et al., 2007; Cheng et al., 2013). During shoot meristem development in Arabidopsis and rice, a positive feedback loop between KNOXI proteins and CK is established, in which the KNOXI proteins promote CK biosynthesis by positively regulating the expression of CK biosynthetic genes, and the expression of KNOXI genes can be induced by CK (Yanai et al., 2005; Sakamoto et al.,...
Auxin is also indispensable for shoot meristem development (Teale et al., 2006; Su et al., 2011). In Arabidopsis, auxin suppresses the expression of type-A RESPONSE REGULATOR (RR) genes, which are negative regulators of cytokinin signaling and have important functions in meristem development (To et al., 2004; Leibfried et al., 2005; Zhao et al., 2010). Auxin also has an effect on the expression of KNOXI genes (Heisler et al., 2005). Moreover, during in vitro shoot regeneration, the establishment of auxin gradients in callus is critical for cell patterning (Gordon et al., 2007).

In vitro shoot regeneration is a widely used approach to regenerate entire plants from explants. Previous studies have shown that the establishment of shoot meristem during in vitro shoot regeneration involves gene expression and cell organization patterns similar to that of the in planta SAM development (Gordon et al., 2007). In addition to the general SAM regulators including KNOXI proteins, hormonal homeostasis and hormone interactions are also important factors for determining in vitro shoot meristem organization (Duclercq et al., 2011; Motte et al., 2014).

The AT-rich Interaction Domain (ARID) family is a class of DNA-binding proteins that are widely present in all eukaryotes (Herrschel et al., 1995; Gregory et al., 1996). Previous studies in yeast, fruit flies, and mammals have demonstrated that ARID proteins play vital roles in many important biological processes including embryonic development and cell cycle control (Kortschak et al., 2000; Wilsker et al., 2002). For instance, the mouse Bright protein and its human homologs ARID3A and ARID3B are required for hematopoietic stem-cell development (Weiss et al., 2011; Webb et al., 2014). DRI, the homolog of Bright in Drosophila, is developmentally essential, and inactivation of DRI results in embryonic lethality (Shandala et al., 1999, 2003). Additionally, in recent years, it has become evident that the human ARID proteins are implicated in the regulation of tumorigenesis (Lin et al., 2014). In contrast, knowledge about the functions of plant ARID proteins is very limited. In Lotus japonicas, the ARID protein SymRK-interacting protein 1 (SIP1) is required for nodule development (Zhu et al., 2008). Recently, the Arabidopsis ARID proteins ARID1 and AtHMGB15 were reported to be required for sperm cell formation and pollen tube growth, respectively (Xia et al., 2014; Zheng et al., 2014). However, so far there is no report about the functions of rice ARID proteins.

In this work, we identify six ARID genes in the rice genome and report the important roles of OsARID3 in shoot meristem development. An OsARID3 loss-of-function mutant had defects in SAM development and failed to regenerate shoot from callus. Endogenous auxin levels were increased whereas CK concentrations were reduced in Osarid3 calli. The hormonal changes were resulted from the altered expression of their biosynthetic and catabolic genes. In addition, the expression of the KNOXI genes in the Osarid3 regenerating calli was significantly decreased, indicating the impaired SAM establishment. Furthermore, we demonstrate that OsARID3 binds to the auxin and CK biosynthetic genes and a KNOXI gene. Our findings suggest that OsARID3 is required for SAM development in rice, which further demonstrates the developmental significance of plant ARID proteins and provides insights into the mechanism of shoot meristem development in plants.

RESULTS

Identification and expression analyses of the rice ARID genes

ARID proteins are widely present in the plant kingdom. The Arabidopsis genome contains 10 putative ARID genes, which have been grouped into four subfamilies based on their phylogenetic relationship and the presence of additional domains including the Hsp20 (ACD_sHsps_p23-like) domain, the HMG (High Mobility Group) box, the ELM2 (Egl-27 and MTA1 homology 2) domain, and the PHD (Plant Homeodomain) finger (Zhu et al., 2008). To identify the rice ARID genes, we performed tBLASTn searches against all available rice DNA databases using the predicted Arabidopsis ARID proteins as query sequences. Our search yielded six rice ARID proteins (OsARID1 to OsARID6), all of which contain the conserved ARID DNA-binding domain. Moreover, the Hsp20, HMG, and PHD motifs present in the Arabidopsis ARID proteins are also found in the rice ARID protein sequences (Figure 1).

Phylogenetic analysis based on the full-length protein sequences (Figure S1) grouped OsARID1 (Os02g27060) and OsARID6 (Os09g37250) into the Hsp20 subfamily, OsARID2 (Os02g48370) and OsARID3 (Os06g41730) into the HMG subfamily, and OsARID4 (Os08g35000) and OsARID5 (Os09g26390) into the PHD subfamily (Figure 1).

Next we investigated the expression patterns of the rice ARID genes in various tissues and organs by real-time quantitative RT-PCR (qRT-PCR) analysis. The rice ARID genes we examined (OsARID1 to OsARID5) were expressed at different levels in various tissues and organs and they displayed very similar expression patterns (Figure S2). All of these showed relatively high expression levels in leaves (vegetative leaf and flag leaf). OsARID1, 2, and 3 were expressed at low levels in roots and stems. The expression of OsARID1, 2 was also low in shoot apices and developing panicles. These results suggested that rice ARID genes were expressed with similar patterns.

Disruption of OsARID3 resulted in severe developmental defects

To elucidate the physiological functions of the rice ARID genes, we obtained a T-DNA insertion mutant of OsARID3 from the POSTECH T-DNA Insertion Database (Jeong et al., 2006; Tsuda et al., 2011). Auxin is also indispensable for shoot meristem development (Teale et al., 2006; Su et al., 2011). In Arabidopsis, auxin suppresses the expression of type-A RESPONSE REGULATOR (RR) genes, which are negative regulators of cytokinin signaling and have important functions in meristem development (To et al., 2004; Leibfried et al., 2005; Zhao et al., 2010). Auxin also has an effect on the expression of KNOXI genes (Heisler et al., 2005). Moreover, during in vitro shoot regeneration, the establishment of auxin gradients in callus is critical for cell patterning (Gordon et al., 2007).

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The T-DNA was inserted into the first exon of OsARID3 that consists of 14 exons (Figure 2a). Both real-time qRT-PCR and western blot analyses showed that the expression of OsARID3 was abolished in this mutant (Figure 2b,c).

The developmental abnormality of Osarid3 was initially observed when we germinated the T2 generation seeds on Murashige and Skoog (MS) medium: about a quarter of the seeds (n = 75/312) from a heterozygous parent (Osarid3/+) exhibited abnormal germination. These seeds either could not germinate or just produced a distorted shoot with a clump of coleoptile-like structures (Figure 2d, e). Longitudinal sections of the aberrant shoots showed that the SAM was absent (Figure 2f). Subsequent genotyping using seed DNA indicated that those seeds showing abnormal germination were all homozygous for the T-DNA insertion (Figure S3).

We further analyzed the embryonic defects of Osarid3 mutant. Embryos at 10 days after pollination (DAP) derived from a heterozygous parent (Osarid3+/−) exhibited abnormal germination. These seeds either could not germinate or just produced a distorted shoot with a clump of coleoptile-like structures (Figure 2d, e). Longitudinal sections of the aberrant shoots showed that the SAM was absent (Figure 2f). Subsequent genotyping using seed DNA indicated that those seeds showing abnormal germination were all homozygous for the T-DNA insertion (Figure S3).

Osarid3 failed to regenerate shoot from callus

Because no viable plants could be produced from the Osarid3 seeds, we tried to regenerate Osarid3 plants through in vitro shoot regeneration using scutellum-derived calli. When cultured on the callus-inducing medium (CIM), coleoptiles from the WT seeds were differentiated in the early days, following by the production of a lump of calli at about 30 days after induction (Figure 3a). However, for Osarid3, no differentiation of the plant organs was observed throughout the entire induction process, which was consistent with the observed germination phenotype, although calli were produced at about 45 days after induction (Figure 3b).

Next, both of Osarid3 and WT calli were sub-cultured twice on CIM and then transferred onto shoot-inducing medium (SIM) for in vitro shoot regeneration. After 2 weeks on SIM, WT calli generated many green spots, where shoot meristems were formed. In contrast, the Osarid3 calli rarely turned green (Figure 3c). After 1 month
on SIM, plantlets were produced from WT calli, while Osarid3 calli decayed and became brown, and failed to regenerate plantlets (Figure 3d). Occasionally, small shoot-like structures were produced from the Osarid3 calli after culturing on SIM for 2 months, but these fortuitous shoots were frail and not viable (Figure S5). These observations suggest that Osarid3 calli failed to establish and/or maintain the shoot meristem during in vitro shoot regeneration.

Auxin and CK homeostasis was disturbed in Osarid3 calli

It is known that plant hormones, especially auxin and cytokinin, play vital roles during not only in planta SAM development but also in vitro shoot regeneration (Meng et al., 2010; Motte et al., 2014). The failure in shoot regeneration of Osarid3 calli suggests that endogenous hormonal levels might be altered in Osarid3. Therefore, we measured the concentrations of auxin and CK in Osarid3 and WT calli. To minimize the potential effects of exogenous auxin and CK contained in SIM on our hormone analysis, we used calli cultured on hormone-free MS medium for 20 days. It was clear that free IAA levels were significantly increased in Osarid3, while concentrations of isopentenyl adenine (iP), a major bioactive form of CK in rice (Sakamoto et al., 2006), were significantly reduced, as compared with the levels in WT (Figure 3e,f). The trans-zeatin (tZ) form of CK could not be detected in our calli samples, probably because of its low basal content in callus. These results demonstrate that disruption of OsARID3 function resulted in alteration in hormone homeostasis.

Complementation of Osarid3

To verify that the phenotypes of the Osarid3 mutant were caused by the T-DNA insertion in OsARID3, we performed a complementation assay through transforming a construct
carrying the OsARID3 full-length cDNA driven by the OsARID3 promoter into homozygous Osarid3 calli. The regeneration capacity of Osarid3 calli was rescued during the transformation process (Figure S6a,b). Subsequent germination test of the T1 complementary transgenic seeds showed segregation of the restored phenotype (Figure S6c,d). We further genotyped the rescued plants and confirmed that they were indeed homozygous for the T-DNA insertion. These results demonstrated that loss of OsARID3 function is responsible for the germination and regeneration phenotypes of the mutant.

Expression profile of OsARID3 and sub-cellular localization of OsARID3

The expression profile analysis of the ARID family in rice indicated that OsARID3 is expressed ubiquitously in all of the tissues and organs investigated (Figure S2). To further investigate its tissue expression patterns, transgenic plants carrying the β-glucuronidase (GUS) (uidA) reporter gene under the control of the OsARID3 promoter were generated. During the germination stage, the GUS signal was mainly detected in embryos, young shoots, and root tips (Figure 4a,b). Longitudinal sections of the shoot apexes showed significant GUS expression in the SAM region (Figure 4c). When the plants were full grown, GUS expression was detected in leaves, root tips, and flowers (Figure 4d–f). In addition, GUS expression was also clearly detected in calli (Figure 4g). These results further demonstrate that OsARID3 is ubiquitously expressed.

Plant hormones play important roles in many developmental processes including embryogenesis, shoot meristem development, and in in vitro shoot regeneration. We therefore investigated whether expression of OsARID3 was affected by auxin, CK, and gibberellin (GA). The results of real-time qRT-PCR analysis showed that the expression of OsARID3 is slightly induced by kinetin, a type of synthetic CK, but exhibited no obvious altered response to IAA or GA treatments (Figure S7). Sub-cellular localization analysis of OsARID3 fused with green-fluorescent protein (GFP) revealed that it is a nuclear protein (Figure 4h).

Expression of genes involved in homeostasis of auxin and CK was altered in Osarid3

Because auxin and CK concentrations were changed in Osarid3 calli, we investigated whether expression of hormonal biosynthetic and metabolic genes was altered. Again we used MS-cultured calli to minimize any effects of exogenous hormones. In the tryptophan-dependent IAA biosynthesis pathway, the YUC flavin monooxygenases are the key enzymes (Zhao et al., 2001; Cheng et al., 2006; Dai et al., 2013) Our real-time qRT-PCR results showed that the transcript levels of OsYUC1, OsYUC4, OsYUC5 and OsYUC6
OsIPT2 levels of tenyltransferase (IPT) (Kamada-Nobusada and Sakakibara, 2009). In the CK biosynthetic pathway, the first and rate-limiting step is catalyzed by adenosine phosphate isopen-tenytransferase (IPT) (Kamada-Nobusada and Sakakibara, 2009). Among the eight IPT genes examined, the transcript levels of OsIPT2, OsIPT3, OsIPT4, and OsIPT7 were significantly decreased in Osarid3, while other members showed no obvious changes (Figures 5b and S8b). Our gene expression data are consistent with the observed hormone concentration changes in Osarid3. We conclude that the altered expression of hormone genes can partially account for the increased auxin and decreased CK levels in the mutant calli.

In addition to biosynthesis, hormonal homeostasis is also controlled by metabolism and conjugation. The auxin-conjugating enzymes encoded by GH3 genes convert free IAA to amino acid-conjugated form, which is for storage or degradation (Staswick et al., 2005). Over-expression of the GH3 genes resulted in reduced auxin levels in transgenic plants (Zhang et al., 2009). We therefore examined the transcript levels of the GH3 family genes in Osarid3 and WT calli and found that five (OsGH3.4, OsGH3.5, OsGH3.8, OsGH3.11, and OsGH3.13) of the 10 investigated GH3 genes were down-regulated in Osarid3, while the other members showed no obvious alteration in transcript levels (Figures 5c and S8c). Therefore, in addition to the up-regulation of the YUC genes, the down-regulation of the GH3 genes might also contribute to the increased free IAA levels in Osarid3. Additionally, we investigated the expression of several putative CYTOKININ OXIDASE (CKX) genes that encode the enzymes participating in CK degradation (Schmulling et al., 2003). However, our results indicated that the transcript levels of these genes showed no obvious difference between Osarid3 and WT (Figure S9).

We also investigated the expression patterns of these differentially expressed genes in the regenerating calli of Osarid3 and WT. Calli at 6 days after regeneration (DAR) were analyzed. The results showed that the expression alterations of the YUC, IPT, and GH3 genes in Osarid3 regenerating calli compared with WT were consistent with the results of MS-cultured calli (Figure S10), suggesting that the effects of Osarid3 on the expression of these genes are independent of the culture conditions.

To further confirm that the expression alterations of these hormone-related genes in Osarid3 were caused by the disruption of OsARID3, we compared the expression levels of these genes in the Osarid3 complementation lines with that of WT plants. As shown in Figure S11, the expression levels of all of these genes in Osarid3 complementation lines were restored close to the levels in WT.

### Reduced expression of the KNOXI genes in Osarid3

The KNOXI genes are indispensable for SAM formation and maintenance. In rice, a double-mutant of the two KNOXI genes, osh1 osh15, failed to maintain the SAM after germination and also could not regenerate shoot from the callus (Tsuda et al., 2011). The severe defects of Osarid3 in SAM development and shoot regeneration suggested that the expression of KNOXI genes might be affected. It has been reported that the expression of KNOXI genes in the callus is induced after transfer onto SIM (Tsuda et al., 2011). Therefore, calli at 6 DAR were used to investigate the expression of the KNOXI genes in Osarid3 and WT. Our results from the qRT-PCR revealed that the transcript levels of OSH1, OSH15, OSH43, and OSH71 were significantly decreased in Osarid3 (Figures 5d and S8d), indicating that during shoot regeneration, the establishment of the shoot meristem might be impaired in Osarid3 calli. In Osarid3 complementation lines, which showed normal shoot regeneration and germination, the expression levels of the KNOXI genes were restored (Figure S11).

### OsARID3 directly binds to YUC, IPT and KNOXI genes

Because OsARID3 is a DNA-binding domain-containing protein, we performed chromatin immunoprecipitation...
(ChIP) assays using a specific antibody against OsARID3 to investigate its direct target genes. Using calli at 6 DAR, we examined the enrichment of different DNA fragments within the –2000 to +800 bp region (relative to the transcription start site) of several YUC, IPT, and KNOXI genes in Osarid3 and WT ChIP samples. The results based on real-time qPCR analysis revealed that the enrichment of several DNA fragments of OsYUC1, OsYUC6, OsIPT2, OsIPT7 and OSH71 were significantly reduced in Osarid3, compared with that in WT (Figure 6). These results suggest that OsARID3 could potentially bind to these genes.

Although the ARID domain was initially recognized for its ability to bind to AT-rich DNA sequences (Herrscher et al., 1995; Gregory et al., 1996), subsequent studies have demonstrated that many ARID proteins bind to DNA with no obvious sequence preference (Patsialou et al., 2005). We therefore investigated the DNA-binding property of OsARID3 by analyzing the binding sites of its target genes through electrophoretic mobility shift assays (EMSAs). Recombinant glutathione S transferases (GST)-fusion OsARID3 protein was used and the direct target genes OsIPT2 and OsIPT7 were examined. For OsIPT2, among the three probes investigated, OsARID3 could bind to probe 2, which is significantly A/T rich, but could not bind to probe 1 or probe 3, which is highly GC-rich and equal with A/T and G/C, respectively (Figure 7a,b). The binding of OsARID3 to probe 2 could be efficiently competed out by a 10-fold excess of unlabeled probe 2 (Figure 7c). Similar results were obtained for OsIPT7; the two AT-rich probes (probe 3 and probe 5) bound OsARID3, but three other probes with equal ratios of A/T and G/C were unable to bind (Figure 7d,e). The binding was also confirmed by competition assays (Figure 7f). These results demonstrated that OsARID3 selectively binds to AT-rich DNA sequences of the target genes.

**DISCUSSION**

How the SAM is generated and maintained has been a central question in plant developmental biology. In this work, we discovered that a T-DNA insertion mutant of OsARID3, a member of the rice ARID genes, is defective in SAM formation and maintenance. Consequently the mutant exhibited aberrant germination and early seedling lethality. Moreover, the mutant calli could not regenerate a plant through the in vitro shoot regeneration process. We demonstrated that several auxin biosynthetic/metabolic genes including YUC genes and GH3 genes were expressed at different levels in the Osarid3 mutant compared with the WT, leading to elevated auxin concentrations in the mutant calli. It appears that OsARID3 negatively modulates auxin biosynthesis by directly targeting the YUC genes, as our ChIP results clearly demon-

![Figure 5](https://example.com/figure5.png)

**Figure 5.** OsARID3 affects the expression of genes involved in hormone homeostasis and KNOXI genes. The expression levels of several YUC (a), IPT (b), GH3 (c), and KNOXI (d) genes were altered in Osarid3 calli. The rice ACTIN gene was used as internal control. Data represent the results of one biological replicate with three technical replicates. Error bars indicate standard error (SE). Underlined genes represent those showing consistent alteration among different biological replicates (n ≥ 2). See Figure S8 for the results of other biological replicates.

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It has been reported that over-expression of OsYUC1 in rice resulted in a significantly decreased regeneration frequency of the transgenic calli, accompanied with increased IAA levels (Yamamoto et al., 2007). Thus, the observed elevated IAA content in Osarid3 calli might be at least partly responsible for the failure of shoot regeneration.

In Arabidopsis, multigenic mutations of the IPT genes result in reduced CK levels and defective SAM development and decreased shoot regeneration frequency (Miyawaki et al., 2006; Cheng et al., 2013). We demonstrated that OsARID3 directly targets OsIPT2 and OsIPT7 through binding to its AT-rich DNA sequences. In Osarid3, both the expression of the IPT genes and the CK content were decreased, suggesting that OsARID3 acts as a positive regulator of CK biosynthesis by directly promoting the expression of the IPT genes. The observed reduction of CK levels also contributes to the shoot regeneration defects observed in Osarid3. Taken together, we propose that during shoot meristem development, OsARID3 simultaneously inhibits and promotes the biosynthesis of auxin and CK, respectively, through directly targeting the YUC and IPT genes, thereby generating the low-auxin and high-CK conditions that are required for SAM maintenance.

The KNOXI proteins are critical for shoot meristem function (Scofield and Murray, 2006). In rice, OSH1 and OSH15 are redundantly required for SAM formation during both embryogenesis and during in vitro shoot regeneration, and a double-mutant of these two genes failed to establish SAM (Tsuda et al., 2011). We discovered that the transcript levels of OSH1, OSH15, OSH43, and OSH71 were significantly decreased in the Osarid3 regenerating calli, indicating the impaired shoot meristem formation. During shoot meristem development in Arabidopsis and rice, expression of the KNOXI genes is induced by CK (Tsuda et al., 2011). Given that the CK content is significantly reduced in Osarid3, we propose that, besides the direct effect of OsARID3 on OSH71 expression, down-regulation of the KNOXI genes in Osarid3 might be partly caused by the reduced endogenous CK level. Additionally, it has been reported that a high auxin level in the lateral organ primordium represses the expression of KNOXI genes (Heisler et al., 2005). Therefore, the increased auxin content in Osarid3 may also contribute to the reduced expression of KNOXI genes.

We have clearly demonstrated that OsARID3 plays an essential role in rice SAM development. Our data indicate that OsARID3 affects SAM development by directly targeting KNOXI genes and biosynthetic genes of auxin and CK.
OsARID3 inhibits the production and accumulation of auxin, a high level of which has negative effects on KNOXI gene expression and on shoot regeneration. OsARID3 also promotes the biosynthesis of CK, which is essential for SAM maintenance and can induce the expression of KNOXI genes. Furthermore, OsARID3 directly binds to OSH71 and likely modulates OSH71 expression. OsARID3 serves as a key transcriptional regulator for SAM development.

EXPERIMENTAL PROCEDURES

Plant materials and genotyping of Osarid3

The mutant line 3A-50739 (Oryza sativa ssp. japonica cv. Dongjin) was obtained from POSTECH Rice T-DNA Insertion Sequence Database (Korea) (http://www.postech.ac.kr/life/pfg/risd/). Due to the early seedling lethality of homozygous plants, this mutant was maintained in a heterozygous state. To identify homozygous seeds, seeds from heterozygous plants were cut in half and the half seed containing the endosperm was used for DNA extraction, while the other half seed containing the embryo was reserved for further phenotypic analysis. The seed DNA extraction procedure was described previously (Kang et al., 1998). Genotyping was performed using the OsARID3 genomic primers P1 and P2 and the T-DNA right-border primer (P3) (Table S1).

In vitro shoot regeneration

To analyze the regeneration phenotype, homozygous Osarid3 seeds and WT siblings were sterilized with 0.15% mercuric chloride for 15 min, followed by extensive washing with sterilized water, and then were inoculated onto callus-inducing medium (CIM; N6 medium containing 3% sucrose, 0.06% casein enzymatic hydrolysate (CH, Sigma-Aldrich; http://www.sigmaaldrich.com/), 0.03% proline, 1% Vitamin solution (0.1 g/L Nicotinic acid, 0.1 g/L Thiamine HCL, 0.2 g/L Glycine and 10 g/L Inositol), 1% Fe²⁺-EDTA solution (0.1 mmol/L FeSO₄, 0.1 mmol/L EDTA), and supplemented with 2 mg/L 2,4-D (Sigma-Aldrich)), and incubated at 28°C in dark conditions. When the calli had proliferated to approximately 5 mm in diameter, they were transferred onto new CIM plates for subculture. After two rounds of subculturing, calli were transferred onto shoot-inducing medium (SIM; MS medium containing 3% sucrose, 0.1% CH, 1% Vitamin solution (0.1 g/L Nicotinic acid, 0.1 g/L Pyridoxine HCL, 0.1 g/L Thiamine HCL, 0.2 g/L Glycine and 10 g/L Inositol), 1% Fe²⁺-EDTA solution (0.1 mmol/L FeSO₄, 0.1 mmol/L EDTA), and supplemented with 2 mg/L 6-BA, 2 mg/L KT, 0.2 mg/L IAA, and 0.2 mg/L NAA), and incubated at 28°C under artificial long-day (LD) conditions (14 h light/10 h dark).

Histological assay

Samples were fixed in FAA fixative solution (formaldehyde:acetic acid:50% ethanol = 5:5:90) for 24 h at 4°C and dehydrated through
a graded ethanol series from 50 to 100%. Ethanol was then replaced with xylene. Samples were then embedded in paraffin wax and cut to 8-10-μm thick sections. Sections were stained with Toluidine Blue (Sigma-Aldrich) and observed with a light microscope (LEICA DM4000B; Leica; http://www.leica.com/).

**Complementation test**

The promoter of OsARID3 (1953 bp upstream of the ATG) was amplified from the rice genomic DNA of Zhonghua11 using the primer set ARID3p-F1 and ARID3p-R1 and inserted into pCAMBIA2301 (obtained from the Centre for the Application of Molecular Biology to International Agriculture, CAMBIA, Australia) at the KpnI site. The 1800-bp full-length cDNA of OsARID3 was amplified from Nipponbare, using the primer set ARID3full-F and ARID3full-R, digested with KpnI and BamHI (TaKaRa; http://www.takara-bio.com/), and was inserted downstream of the OsARID3 promoter in pCAMBIA2301. The sequences of the primers used in plasmid construction are listed in Table S1. The resulting construct (OsARID3pro:OsARID3) was transformed into the Osarid3 homozygous calli by the Agrobacterium-mediated transformation procedure. Both the regeneration capacity of the transformed calli and germination viability of T1 transgenic seeds were investigated.

**Histochemical analysis of GUS activity**

The OsARID3 promoter (1953 bp upstream of the ATG) was amplified from the rice genomic DNA of Zhonghua11 using primers ARID3p-F2 and ARID3p-R2 (Table S1), digested by BamHI (TaKaRa), and inserted into the pDX2181 binary vector (Ye et al., 2012) containing the GUS reporter gene. This construct was introduced into Zhonghua11 plants by the Agrobacterium-mediated transformation procedure. GUS activity assays of ARID3p:GUS transgenic plants were performed using a previously described histochemical staining method (Ning et al., 2011). The stained tissues were observed and photographed with a stereomicroscope (Leica M2 FLIII, Leica)

**Sub-cellular localization**

The full-length CDS of OsARID3 was amplified using the primers ARID3yp-F and ARID3yp-R (Table S1) and cloned into the GATEWAY destination binary vector p2YGW7 (Karimi et al., 2005), fused to the C terminus of YFP. The resulting construct (35S:YFP:ARID3) and the nuclear marker 35S:CFP-GHD7 (Xue et al., 2008) were used for transformation of rice protoplast cells prepared from etiolated Zhonghua11 shoots. Rice protoplast transformation was performed according to the method described previously (Zhou et al., 2009). The fluorescence signal was observed with a confocal laser scanning microscope (Leica TCS SP2, Leica) at 20 h after transformation.

**Quantification of gene expression**

For expression pattern analysis of the rice ARID genes, different organs, including shoots, roots, leaves, stems, flag leaves, flowers, etc., of Zhonghua11 plants grown under normal growth conditions were collected for total RNA extraction using TRIzol reagent (Invitrogen; http://www.lifetechnologies.com). To examine gene expression levels in Osarid3 and WT, total RNA was isolated from calli using TRIzol reagent. 3 μg total RNA were then treated with RNase-free DNase I (Invitrogen). The DNase-treated RNA samples were then reverse-transcribed to cDNA using SuperScript reverse transcriptase III (Invitrogen) in a total reaction volume of 20 μl. Real-time quantitative PCR was performed in a total volume of 10 μl with 2 μl of 10-fold diluted cDNA, 0.2 μM gene-specific primers, and 5 μl SYBR Green Master (Roche; http://www.roche.com/), using the Step One Plus Real-Time PCR System (Applied Biosystems; http://www.lifetecnologies.com.cn/zh/home/brands/applied-biosystesms/) according to the manufacturer’s instructions. Expression levels of the examined genes were quantified by a relative quantitation method (ΔΔCt). Each measurement was performed with at least two biological replicates or independently repeated at least twice, and each biological sample was analyzed with three technical replicates. Nucleotide sequences of the gene-specific primers are listed in Table S1.

**Quantification of endogenous IAA and cytokinin**

Calli of Osarid3 and WT cultured on CIM were transferred onto hormone-free MS medium containing 2% sucrose for 20 days, then the calli (0.2 g) were harvested and used for IAA and cytokinin quantification. The extraction and measurement of auxin was performed as described by Liu et al. (2012). Cytokinins were extracted as described previously (Kojima et al., 2009), and measured using a liquid chromatography-tandem mass spectrometry system (AQUITY UPLC® System/Quattro Ultima LT; Waters; http://www.waters.com/) with an ODS column (AQUITY UPLC BEH C18, 1.7 μm, 2.1 × 100 mm; Waters).

**Chromatin immunoprecipitation assays**

For ChIP assays, calli of Osarid3 and WT were cultured on SIM for 6 days, and then used for chromatin extraction and immunoprecipitation as described previously (Zong et al., 2013) using a rabbit polyclonal antibody made by Yi Ji SHENG WU (http://www.immunogen.com.cn/) against the full-length OsARID3. Briefly, 1 g of samples were harvested and cross-linked in 1% (v/v) formaldehyde under vacuum. Chromatin was then extracted and sonicated to achieve an average DNA size between 0.2 and 1 kb. Immunoprecipitation was performed as described using the specific OsARID3 antibody (Figure 2c). The precipitated DNA was then analyzed by quantitative PCR using gene-specific primers listed in Table S1.

**EMSA**

To express ARID3 in Escherichia coli, the full-length CDS of OsARID3 was amplified with the ARID3ORF-F and ARID3ORF-R1 primers (Table S1) was inserted into the BamHI and Xhol sites of pGEX-6P-1 (GE Healthcare; http://www3.gehealthcare.com/) and introduced into Escherichia coli BL21 cells (Novagen; http://www.novagen.com). The GST-tagged ARID3 protein was expressed in the transformed cells cultured at 28°C induced with 1 mmol/L isopropyl-β-D-thiogalactoside (IPTG), and then purified with Glutathione Sepharose 4B resin (GE Healthcare). To prepare double-strand DNA probes, two complementary oligonucleotides labeled by biotin at the 5’-end were mixed, heat-denatured at 95°C for 10 min, and annealed by gradually cooling to room temperature. The EMSA reaction and detection was performed using the Light Shift EMSA Kit (Thermo Fisher Scientific; http://www.thermoshifer.com/) according to the manufacturer’s instructions.

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