SWI2/SNF2 Chromatin Remodeling ATPases SPLAYED and BRAHMA Control Embryo Development in Rice

Qian Xun

Nanjing Agricultural University

Min Mei

Nanjing Agricultural University

Ye Song

Nanjing Agricultural University

Chenyu Rong

Nanjing Agricultural University

Jiajun Liu

Nanjing Agricultural University

Tianhui Zhong

Nanjing Agricultural University

Yanfeng Ding

Nanjing Agricultural University

Chengqiang Ding (✉ dingcq@njau.edu.cn)

Nanjing Agricultural University <https://orcid.org/0000-0001-6061-1979>

Research Article

Keywords: chromatin remodeling, SPLAYED, BRAHMA, embryo, rice

Posted Date: February 2nd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1261353/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

In plants, *SPLAYED* (*SYD*) and *BRAHMA* (*BRM*) encode chromatin remodeling ATPases that use the energy derived from ATP hydrolysis to restructure nucleosomes and render certain genomic regions available to transcription factors. However, the function of *SYD* and *BRM* on rice growth and development is unknown. Here, we constructed *ossyd* and *osbrm* mutants using CRISPR/Cas9 technology and analyzed the effects of mutations on rice embryo development. We discovered that the *ossyd* and *osbrm* mutants exhibited severe defects during embryonic development, whereas endosperm development was normal. These results indicated that the development of the embryo and endosperm is independent of each other. Consequently, the *ossyd*- and *osbrm*-null mutants did not germinate due to the abnormal embryos. Furthermore, we observed the embryos of *ossyd*- and *osbrm*-null mutants, and they indeed had distinct differentiation defects in shoot establishment, acquired during embryogenesis. To verify the function of *OsSYD* and *OsBRM* in embryogenesis, we measured the transcript levels of marker genes at different stages. Compared with wild-type, the expression levels of multiple *OSH* genes were significantly reduced in the mutants, which was consistent with the defective shoot establishment phenotypes. The interaction between *SYD* and RICE FLORICAULA/LFY (*RFL*) was revealed using a yeast two-hybrid screening system, suggesting that the interaction between the *LFY* homolog and chromatin remodeling ATPases are ubiquitous in plants. Collectively, our findings provide the basis for elucidating the function of *OsSYD* and *OsBRM* during embryo development in rice.

Key Messages

Chromatin remodeling ATPases *OsSYD* and *OsBRM* are involved in shoot establishment, and both affect *OSH* gene transcription. *OsSYD* protein interacts with *RFL*, but *OsBRM* does not.

Introduction

In flowering plants, asymmetric division of the zygote is the first and most important process during embryo development, giving rise to apical and basal cells with different cell development fates. The apical cells subsequently develop into the shoot apical meristem and cotyledons, while the basal cells generate the root apical meristem (RAM) and hypocotyl (Palovaara, et al. 2016). Shoot apical meristem (SAM), cotyledon, RAM, and hypocotyl are formed along the apical-basal axis, while radial pattern consists of the epidermis (Layer 1), ground tissue (Layer 2), and central vascular cylinder (Layer 3), concentrically arranged from outside to inside (Mayer, et al. 1991; Yi, et al. 2016).

During the development of an embryo, some genes are expressed only in specific cell types and regions, revealing a radial pattern. For example, the GLABRA2 (GL2)-type homeobox gene Rice outermost cell-specific gene 1 (*Roc1*) is specifically expressed in the epidermis (L1) of the shoot and root crown from early embryogenesis (Ito, et al. 2003; Ito, et al. 2002). During embryonic development, tissue-specific marker gene *SCARECROW* (*SCR*) is expressed mainly in the ground tissue (L2) of late heart-stage embryos prior to the formation of cortex and endodermis (Di Laurenzio, et al. 1996). Consistent with the

expression pattern of *SCR*, *scr* mutant embryos are defective in the division of ground tissues. A rice homolog of *PINHEAD/ZWILLE* (*PNH/ZLL*) in *Arabidopsis* called *OsPNH1*, is specifically expressed in presumptive vascular areas of the central region and developing vascular tissues in the early embryonic stage (Nishimura, et al. 2002). Therefore, *OsPNH1* has been used as an L3 vascular tissue marker (Kamiya, et al. 2003). The function of the scutellum epithelium is nutrient transportation from the endosperm to the embryo during germination. Rice α -amylase 1 (*RAmy1A*) is a marker gene for the epithelium and is expressed on the dorsal side of the epithelium (Sugimoto 1997).

Land plant genomes contain two subfamilies of *KNOX* genes, namely class 1 (*KNOX1*) and class 2 (*KNOX2*) (Mukherjee, et al. 2009). *KNOX1* genes, which encode homeodomain (HD) transcription factors, play essential roles in SAM, while *KNOX2* genes are required for root formation (Truernit and Haseloff 2007). *KNOX1*-null mutants, such as *SHOOT MERISTEMLESS* in *Arabidopsis* result in the loss of SAM during embryogenesis (Long et al., 1996). The expression of other *KNOX1* genes is also localized around the region where SAM develops early during embryonic development (Sato, et al. 1996; Sato, et al. 1998; Smith, et al. 1995). These findings all reveal that *KNOX1* genes are indispensable for SAM formation and maintenance during embryogenesis. However, little is known about the regulation mechanism of *KNOX1* expression. In the rice genome, there are five functional *KNOX1* genes positively involved in shoot establishment called *ORYZA SATIVA HOMEBOX1* (*OSH1*), *OSH6*, *OSH15*, *OSH43* and *OSH71* (Sentoku, et al. 1999). *OSH1* and *OSH15* are both expressed in the same region where SAM develops later in early embryogenesis. In late embryogenesis, *OSH1* is expressed continuously in the SAM, while *OSH15* expression becomes visible around the boundaries of some embryonic organs (Sato, et al. 1998). Furthermore, *OSH1* can directly bind to five *KNOX* loci with conserved cis-elements and positively regulate the expression of all five *KNOX1* genes, which is essential for SAM formation and maintenance (Tsuda, et al. 2011). Because of the specific expression of *OSH1*, it has been studied as a marker gene for the apical region in rice (Kamiya, et al. 2003; Sato, et al. 1996; Yi, et al. 2016).

Switch/sucrose non-fermentable 2 (SWI2/SNF2) chromatin remodeling ATPases play essential roles in plant development and act in opposition to Polycomb repressors (Han, et al. 2015; Wu, et al. 2012). The four SWI2/SNF2 ATPases present in *Arabidopsis*, namely SYD, BRAHMA (BRM), MINUSCULE1 (MINU1), and MINU2 (Flaus, et al. 2006), use the energy derived from ATP hydrolysis to modify and restructure the nucleosomes (Clapier and Cairns 2009). Since the identification and maintenance of stem cells are closely related to chromatin status, aberrations during chromatin assembly, modification, or Polycomb repression will result in severe developmental defects of SAM or RAM (Kaya, et al. 2001; Sato, et al. 1998; Xu and Shen 2008; Xu, et al. 2013). In *Arabidopsis*, *SYD* is involved in both vegetative and floral development. In the SAM, stem cells maintain their active status and contribute to the continuous growth during the vegetative phase. For example, the homeobox gene *WUSCHEL* (*WUS*) is a master regulator that is mainly expressed in the organizing center of the SAM. During the vegetative phase, *SYD* regulates SAM maintenance by binding to the *WUS* promoter region (Kwon, et al. 2005). However, during reproductive organ development, *WUS* is repressed by *KNUCKLES* (*KNU*), a repressor activated by *AGAMOUS* (*AG*), through displacing *SYD* from the *WUS* promoter (Sun, et al. 2019). Genomic expression studies on *syd* or *brm* mutant seedlings revealed several genes that are uniquely dependent on either *SYD*

or *BRM* (Bezhani, et al. 2007). Functional loss of *BRM* in *Arabidopsis* results in dwarfism, male sterility, and developmental defects in the roots (Hurtado, et al. 2006; Kwon, et al. 2006). In *Arabidopsis*, *syd brm* double mutants showed embryonic lethality, in which embryonic development is arrested at the early heart stage (Bezhani, et al. 2007). In addition to *SYD* and *BRM*, *MINU1* and *MINU2* are two closely related proteins belonging to a single clade of SWI2/SNF2 ATPases present in land plants that are required for embryo and endosperm development. Notably, *minu1 minu2* double mutants were observed to develop defects in both the RAM and SAM. Collectively, these findings suggest that chromatin remodeling ATPases have critical roles in regulating embryonic development in plants.

In *Arabidopsis*, *LEAFY* (*LFY*), a key regulator of flowering, is highly expressed in the young floral primordium (Wagner and Meyerowitz 2002; Weigel, et al. 1992). Specifically, *LFY* controls floral development by recruiting *SYD* and *BRM* to overcome Polycomb repression (Wu, et al. 2012). Interestingly, *LFY* is also a regulator of embryogenesis. In moss (*Physcomitrella patens*), *PpLFY* is required for the first division after fertilization, and the development of *PpLFY* mutants are arrested at the single-cell stage (Tanahashi, et al. 2005). The function of *LFY* in fern (*Ceratopteris richardii*) is similar to that of *PpLFY*, revealing that *CrLFY* maintains normal cell division in the apical stem cells (Plackett, et al. 2018). However, the function of *LFY* in the embryogenesis of flowering plants is still unknown.

Recently, *LFY* was identified as a pioneer TF that directly binds to closed chromatin regions to activate the expression of target genes (Lai, et al. 2021). *LFY* displaces the linker histone (H1) and recruits SWI/SNF chromatin remodelers to loosen the DNA and modify the closed chromatin for transcription (Jin, et al. 2021). *RICE FLORICAULA LFY* (*RFL*)/*ABERRANT PANICLE ORGANIZATION 2* (*APO2*), the *LFY* homolog in rice, has a negative influence on branching-to-spikelet meristem transition, which directly impacts rice yield by facilitating the number of spikelets per panicle. Genetic analysis of different plant species, from mosses to angiosperms, revealed that *LFY* contains two highly conserved domains (Moyroud, et al. 2009). *RFL* shows distinct regulatory roles in plastochron, tiller formation, and panicle development (Ikeda-Kawakatsu, et al. 2012).

In this study, we aimed to investigate the function of *OsSYD* and *OsBRM* in rice embryo development using CRISPR/Cas9 mutagenesis. Embryo-lethal seeds in *ossyd*- and *osbrm*-null homozygotes and transcript levels of marker genes for embryogenesis reveal that *OsSYD* and *OsBRM* are required for shoot establishment during rice embryo development. In addition, we demonstrated that *OsSYD* can bind to the N-terminal domain of *RFL*, suggesting that *RFL* may be also involved in the embryogenesis, like the functions of other *LFY* homologous genes in moss or fern. Our results provide an intensive understanding of the function of chromatin remodeling in rice embryogenesis.

Materials And Methods

Plant materials and growth conditions

Transgenic plants were obtained using CRISPR/Cas9 technology (Mao *et al.*, 2013), including the *ossyd* and *osbrm* mutants with Nipponbare cultivar backgrounds. Each single-guide RNA (sgRNA) was fused to

the synthetic U3 promoter to construct *OsU3::sgRNA* and *UBI::Cas9* with hygromycin resistance. The *rfl* mutants were obtained from *apo2* mutants in the Taichung 65 cultivar backgrounds that were backcrossed to the Nipponbare cultivar five times (Ikeda-Kawakatsu, et al. 2012).

The mutant and WT plants were grown in paddy fields under natural field conditions and in a growth chamber under long-day conditions (14 h light at 28 °C and 10 h dark at 24 °C) with a relative humidity of 70%. To promote homozygous seed germination, the seeds were sown and grown in MS medium.

Genotyping and identification of *ossyd* or *osbrm* mutants

Seeds from rice heterozygote lines *syd-20/+* and *brm-1* (compound heterozygous mutation) were soaked in water overnight. After grains become softer, endosperm was extracted with a razor blade and embryo was kept for next seedling. DNA of endosperm was isolated by Sodium dodecyl sulfate (SDS) (Dellaporta 1994). Genotypes were identified by PCR, using gene-specific primers (Primers are listed in Table S3).

Genotypes of embryo is analyzed according to endosperm. The endosperm is a triploid formed by one sperm from the male parent and two polar nuclei from the female parent, and embryo is a diploid formed by one sperm and one egg cell. The two sperm are from the same pollen mother cell. Polar nuclei and the egg cell are also from the same embryo-sac mother cell. Therefore, we can analyze the genotype of embryo according to the endosperm genotype based on their formation principle.

Segregation ratio analysis in progeny of heterozygote lines

The source of the mutation allele was identified based on the peaks detected on the chromatogram. Since the endosperm is a triploid formed by one sperm from the male parent and two polar nuclei from the female parent, then the peak from the female parent should be two times higher than the peak from the male parent.

The segregation ratios of grains from heterozygous mutants or biallelic mutations were analyzed using the chi-squared test. The theoretical value (E) was calculated based on Mendel's law of segregation. The chi-squared statistics also was used to determine the distribution with several degrees of freedom (n value is determined by the number of groups) to judge extremeness (Pearson 1900). χ^2 tests were performed to evaluate the relationship between the actual data and the predicted ratio. Data with *P*-values <0.01 and <0.05 were considered to be statistically significant.

Phenotypes observation

Pictures of random seeds selected from heterozygote lines including *syd/+* and *brm-1*(compound heterozygous mutation) are taken with Canon camera. Embryo of Nip, *ossyd* and *brm-2* were observed under Stereo microscope called SZX16 microscope (Olympus, Tokyo, Japan).

Yeast two-hybrid

Matchmaker[®] Gold Yeast Two-Hybrid System (Takara, Shiga, Japan; <http://www.takarabio.com/>) was used for the Y2H experiments, following the manufacturer's instructions. All constructs were transformed into the Y2H Gold yeast strain, and the correct yeast derivatives were selected on medium lacking Leu, Trp, Ade, and His incubated at 30 °C for 2 days. RFL and RFL-N represent the full-length and the truncated form containing only the N-terminal domain (1–121 amino acids). A longer OsSYD protein (260–846 amino acids) was obtained from the Y2H screening library, while OsSYD-N (487–846 amino acids), and OsBRM-N (476–900 amino acids) were both truncated with deleted QLQ domains. The primers used for these experiments are listed in Table S3.

RNA isolation and quantitative reverse transcription PCR

Total RNA was extracted using E.Z.N.A.[®] Plant RNA Kit (Omega Bio-tek, Norcross, GA, USA; <http://www.omegabiotek.com/>) and subjected to reverse transcription using PrimeScript[™] RT Reagent Kit (Takara). The qRT-PCR assay was performed on ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA; <http://www.thermofisher.com/>) with SYBR[®] Premix Ex Taq[™] (Takara), following the manufacturer's protocols. The experiments were conducted using three biological replicates of each sample, with three technical replicates per biological sample. The mRNA levels were normalized using *OsUBQ* as the internal control. The primers used are shown in Table S3.

Pollen viability assay

We used two methods, namely I₂-KI staining and germination in liquid germination medium, to determine the pollen viability of mutant and WT seeds. The pollen grains of dehiscent anthers were placed on a glass slide with liquid germination medium containing 20% sucrose, 10% PEG4000, 40 mg/L H₃BO₃, 3 mmol/L Ca(NO₃)₂, and 10 mg/L VB1 and then incubated at 30 °C for 30 min (Khatun and Flowers 1995; Shenghua, et al. 2000). The experiments were performed in triplicate for each sample.

Section and microscopy

The samples were fixed in 5% agar and cut into 50 µm -thick sections using a new razor blade with vibrating microslicer (Leica VT1200). The microsections were stained with safranin for 45 min and then with toluidine blue for 5 s. The embryos were subsequently observed under SZX16 microscope (Olympus, Tokyo, Japan).

Sequence alignment

The amino acid sequences of RFL/APO2 and SYD were aligned using Illustrator for Biological Sequences (IBS) software (<http://ibs.biocuckoo.org/>) (Liu, et al. 2015). The DNA sequence and conserved domains of *SYD* were obtained from the NCBI (<https://www.ncbi.nlm.nih.gov>) database and analyzed using IBS software.

Results

OsSYD has an important role in embryonic development

To determine the specific function of *OsSYD*, we constructed a heterozygote mutant line (*syd-20/+*) from Nipponbare that had one base insertion (T) in the first conserved N-terminal domain using CRISPR/Cas9 genome editing technology (Fig. 1a). However, no *ossyd* homozygote mutant plants were obtained through segregation of posterity in seedling growth experiments. We obtained sufficient heterozygote seedlings, which meant that the homozygotes failed to germinate.

Since we did not obtain homozygote mutants through seedling growth, the homozygotes may have possessed defects that inhibited seeds from germinating. Typical rice seed is composed of an embryo and an endosperm that is formed via double fertilization. To achieve fertilization, pollen ruptures and discharges two sperm cells of the same genotype. During double fertilization, one sperm cell fertilizes the egg cell to form a diploid embryo, while the other sperm fuses with the two polar nuclei to create a triploid endosperm (Dresselhaus 2006; Yang, et al. 2013). To determine the genotypes of the rice embryos, we extracted the endosperm DNA from the half grains of *syd-20/+* plants and performed DNA sequencing. Genotype identification of the embryo was achieved according to the genotype of the endosperm. Then we obtained the homozygous mutant seeds used them the next experiment.

To determine the germination ability, we simultaneously cultured the wild-type (WT), *syd/+*, and *syd* seeds in MS medium. The WT and *syd/+* seeds germinated within 2 weeks after sowing (Fig. 2a). In contrast, homozygous seeds showed no apparent changes. These results suggested that the homozygous loss-of-function mutation of *OsSYD* inhibited seed germination. To validate these results, we focused on investigating the embryogenesis of the homozygotes. Generally, rice embryo consists of the scutellum, coleoptile, SAM, epiblast, and radicle. Notably, microscopic observations revealed that compared to that of the WT, the mature embryo of *syd-20* mutants exhibited severe developmental defects, specifically in SAM formation and organ differentiation (Fig. 2b), which caused embryo-lethal phenotypes.

OsSYD is not sufficient for the gametophyte formation

To determine whether *OsSYD* affects pistil and pollen development, we determined the plant genotypes and assessed whether the results adhered to Mendel's law of segregation. We found that the number of heterozygous mutants was two times higher than that of WT plants among germinated plants (Table S1). We then analyzed the segregation ratio in the progeny of *syd-20/+* mutant lines, including germinated and ungerminated seeds, and the results of chi-square tests proved that it conformed to Mendel's law of segregation (Table 1). Since the rice endosperm is triploid, we observed the source of mutant gametophytes in the heterozygotes based on the peaks in the chromatograms and discovered that the genotype ratio was 1:1 (Table S2). Together, these results suggested that the loss of *OsSYD* did not affect pistil and pollen development.

Moreover, the set percentage of *syd-20/+* did not decrease as expected, which indicated that the pistils in mutant plants were normal (Fig. 1b). In addition, the pollen viability assays showed no differences between the mutant *syd-20/+* and WT plants (Fig. 1c, d). Collectively, these results confirmed that *OsSYD* was not sufficient for both female and male gametophyte development.

Shoot establishment is arrested during embryonic development in *ossyd* mutants

We further measured the length and width of 50 randomly selected *syd-20* seeds and subsequently performed endosperm DNA extraction to identify the genotypes. As expected, there were no differences between the seed lengths of WT, heterozygous, and homozygous plants (Fig. 3a, b). This suggested that although the loss of *OsSYD* caused abnormal embryonic development, it did not influence seed length.

To determine the underlying cause for the abnormal *syd-20* embryos at the molecular level, we isolated *ossyd* mutant and WT mature embryos and measured transcript levels for existing marker genes during embryo development, such as *Roc1* (a marker for the L1 epidermis layer) (Ito, et al. 2003; Ito, et al. 2002), *OsSCR* (a marker for the L2 ground tissue layer) (Di Laurenzio, et al. 1996), *OsPNH1* (a marker for the L3 vascular tissue layer) (Nishimura, et al. 2002), *RAmy1A* (a marker for the epithelium) (Sugimoto 1997), and *OSH* (a marker for the shoot region) (Sato, et al. 1996). Phenotypically, the Nipponbare embryos were flatter and more oval-like compared to the *ossyd* mutant embryos, which were irregular and sunken (Fig. 4a). The quantitative real-time PCR (qRT-PCR) results suggested that the transcript levels of *Roc1*, *OsSCR*, *OsPNH1*, and *Ramy1A* were similar between WT and *ossyd* mutants (Fig. 4b-e). In contrast, *OsSYD* mutations triggered a sharp decrease in most *OSH* genes, except for *OSH1*, the expression of which was slightly decreased in *ossyd* mutants (Fig. 4f-i). *OSH1*, *OSH6*, *OSH15*, and *OSH71* are positively involved in shoot establishment, which is essential for SAM formation and maintenance (Sentoku, et al. 1999; Tsuda, et al. 2011). qRT-PCR results were consistent with the previously observed phenotypes of *syd-20* mutant embryos. Mutations of *OsSYD* caused defects in shoot establishment, while it did not affect cell differentiation of L1, L2, and L3.

Disrupting *OsBRM* function causes similar defects as *OsSYD* in embryogenesis

Previous studies have shown that N-terminal domains of chromatin remodeling ATPases SYD and BRM can both bind to LFY in *Arabidopsis*. However, our Y2H experiments for OsBRM-AD (240–630 amino acids) and RFL-BD revealed that there was no interaction between OsBRM and RFL in rice (Fig. 5a). To determine the function of *OsBRM*, we obtained a biallelic *brm-1* mutant (compound heterozygous mutation) using CRISPR/Cas9 technology. The mutation was different in both DNA strands, where one strand lacked TGG (deletion mutation in *brm-3*) and the other strand had an A insertion (insertion mutation in *brm-2*) (Fig. 5b). However, we were unable to obtain homozygous *brm-2* mutant plants. After we examined the endosperms of randomly selected grains, we found that the *brm-2* homozygous mutants were unable to germinate normally (Fig. 5c, d). As we mentioned before, *ossyd* homozygote mutants also inhibited seed germination through chromatin ATPases. Hence, we observed *brm-3* and *brm-2* embryos via vibration sectioning. Although they were both *OsBRM* mutants, *brm-3* was a weak mutation while *brm-2* was a null mutant because of different mutation modes. The results also showed

that the *brm-3* embryo was normal while *brm-2* embryo was similar to *ossyd*-null homozygotes, which exhibited severe defects in organ germination and SAM formation (Fig. 5e). This revealed that the function of *OsBRM* was similar to that of *OsSYD* in rice during embryonic development. In addition, there were no distinct differences in the seed sizes between the *brm-3* and *brm-2* homozygous mutants (Fig. 5f). To further verify that *OsBRM* has similar roles as *OsSYD* in embryogenesis, we also measured the transcript levels of marker genes in *brm-2* mutants and WT plants. The expression levels of marker genes in WT plants were the same as that in *ossyd* mutants (Figure 6a-h). *OsBRM* mutant embryo was arrested in shoot establishment, but it did not affect the cells in L1, L2, and L3.

However, we discovered that the segregation ratio of the gametophytes did not conform to Mendel's law of segregation (Fig. 5g). The source of the one-base (A) insertion mutation was also not consistent with Mendel's law, and the actual value (O) of the inserted allele from the male parent was distinctly less than that of the female parent (Fig. 5h). This suggested that the function of *OsBRM* might be similar to *OsSYD* during embryogenesis in rice, but it might have a different effect on the formation of male gametophytes.

OsSYD binds to the N-terminal domain of RFL

LFY is also a regulator of embryogenesis in other species like moss or fern (Plackett, et al. 2018; Tanahashi, et al. 2005). To investigate the possible association between the RFL and *OsSYD* in rice, we used the yeast two-hybrid (Y2H) screening system and successfully observed the interaction between them. In the AD-*OsSYD* and BD-RFL combination, *OsSYD* showed a strong interaction with RFL (Fig. 7a), which corroborated the findings previously observed in *Arabidopsis*. To identify the binding domain involved, we fused the N-terminal domain of RFL with the pGBKT7 vector. The results of the Y2H experiments proved that *OsSYD* could bind to the N-terminal domain of RFL, which contains the transcriptional activation regions. This suggested that *OsSYD* played an important role in the transcriptional activation of *RFL* in rice.

In previous studies, we have proved that *OsSYD* mutations cause severe defects in shoot establishment and transcript levels of *OSH6*, *OSH15*, and *OSH71* are all distinctly reduced in mutants. To investigate the relationship between *RFL* and *OsSYD*, we extracted RNA from *rfl* mutant embryos and measured the expression levels of *OSH6*, *OSH15*, and *OSH71*. They all decreased in *rfl* mutants and the results supported the previous findings that *OsSYD* affects the transcriptional activation of *RFL* in rice (Figure. 7b-d). Compared with those of *ossyd* mutant embryos, the seeds of *rfl* mutants germinated normally, while the leaf growth was significantly quicker than WT embryos (Ikeda-Kawakatsu, et al. 2012). This suggested that the deactivation of shoot meristem led to the activation of leaf primordium.

Discussion

Chromatin modification is an important regulatory mechanism that activates or represses gene expression. Several studies have already investigated the functions of different SWI2/SNF2 chromatin remodeling ATPases in this process. In the present study, we discovered that *OsSYD* did not affect rice gametophyte development, especially in female gametophyte formation. However, previous studies on

AtSYD single mutants revealed female sterility in *Arabidopsis*, in which the ovule does not contain an embryo sac. Furthermore, the fourth whorl carpels do not fuse at the tip and the stigma tissue does not properly form (Wagner and Meyerowitz 2002). These findings demonstrate that regardless of sequence homology, *AtSYD* and *OsSYD* most likely regulate gamete formation via different pathways. In addition, we found that the loss of *OsSYD* resulted in the development of severe defects during organ differentiation in mature embryos, specifically the failed shoot establishment. Although *SYD* was confirmed to function in SAM formation and maintenance by binding to the *WUS* promoter after plant germination (Kwon, et al. 2005), there was no direct evidence proving that *SYD* or its homologs can regulate SAM formation during embryogenesis. Hence, our data suggested that *SYD* was also involved in the regulation of shoot establishment during embryo development in flowering plants.

In *Arabidopsis*, the full-length *LFY* interacts with the N-terminal domains of *AtSYD* (465–726 amino acids) and *AtBRM* (631–885 amino acids), both of which contain two conserved regions: The HAS domain and an adjacent motif (Wu, et al. 2012). In contrast, the N-terminal QLQ domain is dispensable. Based on previous studies in *Arabidopsis*, we constructed the truncated N-terminal domains of *OsSYD-N* (487–846 amino acids) and *OsBRM-N* (476–900 amino acids), both with deleted QLQ domains. The results revealed that truncated *SYD-N* and *BRM-N* did not interact with *RFL* (Fig. S1a,b), while *OsSYD* (260–846 amino acids), containing the QLQ domain, bound to both the full-length protein and N-terminal domains of *RFL* (1–121 amino acids) (Fig. 7a). However, for *OsBRM* (240–630 amino acids) with the QLQ domain, there was still no interaction with *RFL* in rice (Fig. 5a). These findings suggested that the N-terminal QLQ domain in *SYD* was needed for normal interaction with *RFL*; however, its function in rice and *Arabidopsis* during growth and development may differ. Notably, the N-terminal domains of *RFL* contain the sterile alpha motif domain that is evolutionarily conserved in all plant species. In dicots, this domain confers *LFY* the ability to access condensed chromatin regions (Sayou, et al. 2016). This indicates that the sterile alpha motif domain is also required for *SYD* binding in both monocots and dicots. However, the full-length *OsSYD* and *OsBRM* are 4374 amino and 2197 amino acids long. The proteins are too large to perform bimolecular fluorescent complementary and pulldown experiments; therefore, these interactions were only based on yeast two-hybrid experiments. Therefore, we also need other feasible methods or assays to verify the interactions between *RFL* and *OsSYD* or *OsBRM*.

Pioneer TFs recognize and access the chromatin to regulate the expression of target genes. In plants, pioneer TFs are required to interact with accessible chromatin; however, the underlying mechanisms are still unclear. Recent studies have identified *LFY* as a pioneer TF that binds to the chromatin by recruiting *SWI2/SNF2* chromatin remodelers (Jin, et al. 2021). Similarly, the *LEAFY COTYLEDON1 (LEC1)* in embryos in *Arabidopsis* was reported to modulate the chromatin state for easier access before epigenetic modification (Tao, et al. 2017). These results demonstrate that the “unlocked” chromatin state is required for the normal function of pioneer TFs. Like *LFY*, several pioneer TFs require chromatin remodelers to support their transcriptional activity; this process has recently been reported in animals (King and Klose 2017). Collectively, these findings suggest that chromatin remodelers may be the “key” for regulating the chromatin state prior to the activity of pioneer TFs. Therefore, further research is warranted to confirm

this hypothesis and to determine the underlying mechanisms involved, especially for different plant species.

Chromatin remodeling ATPases are important for plant development and growth. Defects in ATPases cause different defect phenotypes in plants. Loss of *OsSYD* or *OsBRM* in rice resulted in abnormal seed germination. Owing to the severe defects observed in *OsSYD*- and *OsBRM*-null homozygotes, we did not obtain homozygous mutant plants and could not investigate *OsSYD* function in rice growth and development after germination. Thus, weak mutation must be studied to understand the regulatory mechanisms of chromatin remodeling ATPases in rice in the future.

Declarations

Conflict of interest The authors declare no conflict of interest.

Acknowledgements

The authors thank Pro. Jiankang Zhu and Pro. Caixia Gao for offering the CRISPR/Cas9 gene-editing vector. We also appreciate the Biogle and Biorun Genome Editing Center for producing transgenic rice. This study was supported by the National Natural Science Foundation of China (Grant number 31872855 and 31971842)

Author contribution statement

QX, CD and YD designed the research. QX, MM, YS, CR, JL, TZ and CD performed the experiments. QX and CD analysed the data and prepared the paper.

Conflict of interest The authors declare no conflict of interest.

Accession numbers

Gene information in this article can be found in Rice Annotation Project database, miRbase data libraries and the *Arabidopsis* Information Resource are under the corresponding accession numbers: *OsUBQ* (Os03g0234200), *OsSYD* (Os06g0255200), *RFL/APO2* (Os04g0598300), *OsBRM* (Os02g0114000), *Roc1* (Os08g0187500), *OsSCR* (Os12g0122000), *OsPNH1* (Os06g0597400), *OSH1* (Os03g0727000), *OSH6* (Os01g0302500), *OSH15* (Os07g0129700), *OSH71* (Os05g0129700), *RAmy1A* (Os02g0765600).

References

1. Bezhani S, Winter C, Hershman S, Wagner JD, Kennedy JF, Kwon CS, Pfluger J, Su Y, Wagner D (2007) Unique, shared, and redundant roles for the Arabidopsis SWI/SNF chromatin remodeling ATPases BRAHMA and SPLAYED. Plant Cell 19:403–416
2. Clapier CR, Cairns BR (2009) The biology of chromatin remodeling complexes. Annu Rev Biochem 78:273–304

3. Dellaporta S (1994) Plant DNA miniprep and microprep: versions 2.1–2.3. In: The maize handbook. Springer, pp 522–525
4. Di Laurenzio L, Wysocka-Diller J, Malamy JE, Pysh L, Helariutta Y, Freshour G, Hahn MG, Feldmann KA, Benfey PN (1996) The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. *Cell* 86:423–433
5. Dresselhaus T (2006) Cell-cell communication during double fertilization. *Curr Opin Plant Biol* 9:41–47
6. Flaus A, Martin DM, Barton GJ, Owen-Hughes T (2006) Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. *Nucleic Acids Res* 34:2887–2905
7. Han SK, Wu MF, Cui S, Wagner D (2015) Roles and activities of chromatin remodeling ATPases in plants. *Plant J* 83:62–77
8. Hurtado L, Farrona S, Reyes JC (2006) The putative SWI/SNF complex subunit BRAHMA activates flower homeotic genes in Arabidopsis thaliana. *Plant Mol Biol* 62:291–304
9. Ikeda-Kawakatsu K, Maekawa M, Izawa T, Itoh J, Nagato Y (2012) ABERRANT PANICLE ORGANIZATION 2/RFL, the rice ortholog of Arabidopsis LEAFY, suppresses the transition from inflorescence meristem to floral meristem through interaction with APO1. *Plant J* 69:168–180
10. Ito M, Sentoku N, Nishimura A, Hong S-K, Sato Y, Matsuoka M (2003) Roles of rice GL2-type homeobox genes in epidermis differentiation. *Breed Sci* 53:245–253
11. Ito M, Sentoku N, Nishimura A, Hong SK, Sato Y, Matsuoka M (2002) Position dependent expression of GL2-type homeobox gene, Roc1: significance for protoderm differentiation and radial pattern formation in early rice embryogenesis. *Plant J* 29:497–507
12. Jin R, Klasfeld S, Zhu Y, Garcia MF, Xiao J, Han S-K, Konkol A, Wagner D (2021) LEAFY is a pioneer transcription factor and licenses cell reprogramming to floral fate. *Nat Commun* 12:1–14
13. Kamiya N, Nishimura A, Sentoku N, Takabe E, Nagato Y, Kitano H, Matsuoka M (2003) Rice globular embryo 4 (gle4) mutant is defective in radial pattern formation during embryogenesis. *Plant Cell Physiol* 44:875–883
14. Kaya H, Shibahara KI, Taoka KI, Iwabuchi M, Stillman B, Araki T (2001) FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. *Cell* 104:131–142
15. Khatun S, Flowers T (1995) The estimation of pollen viability in rice. *J Exp Bot* 46:151–154
16. King HW, Klose RJ (2017) The pioneer factor OCT4 requires the chromatin remodeller BRG1 to support gene regulatory element function in mouse embryonic stem cells. *Elife* 6:e22631
17. Kwon CS, Chen C, Wagner D (2005) WUSCHEL is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in Arabidopsis. *Genes Dev* 19:992–1003
18. Kwon CS, Hibara K, Pfluger J, Bezghani S, Metha H, Aida M, Tasaka M, Wagner D (2006) A role for chromatin remodeling in regulation of CUC gene expression in the Arabidopsis cotyledon boundary. *Development* 133:3223–3230

19. Lai X, Blanc-Mathieu R, GrandVuillemin L, Huang Y, Stigliani A, Lucas J, Thevenon E, Loue-Manifel J, Turchi L, Daher H, Brun-Hernandez E, Vachon G, Latrasse D, Benhamed M, Dumas R, Zubieta C, Parcy F (2021) The LEAFY floral regulator displays pioneer transcription factor properties. *Mol Plant* 14:829–837
20. Liu W, Xie Y, Ma J, Luo X, Nie P, Zuo Z, Lahrmann U, Zhao Q, Zheng Y, Zhao Y, Xue Y, Ren J (2015) IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* 31:3359–3361
21. Mayer U, Ruiz RAT, Berleth T, Miséra S, Jürgens G (1991) Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* 353:402–407
22. Moyroud E, Tichtinsky G, Parcy F (2009) The LEAFY Floral Regulators in Angiosperms: Conserved Proteins with Diverse Roles. *Journal of Plant Biology* 52:177–185
23. Mukherjee K, Brocchieri L, Burglin TR (2009) A comprehensive classification and evolutionary analysis of plant homeobox genes. *Mol Biol Evol* 26:2775–2794
24. Nishimura A, Ito M, Kamiya N, Sato Y, Matsuoka M (2002) OsPNH1 regulates leaf development and maintenance of the shoot apical meristem in rice. *Plant J* 30:189–201
25. Palovaara J, de Zeeuw T, Weijers D (2016) Tissue and Organ Initiation in the Plant Embryo: A First Time for Everything. *Annu Rev Cell Dev Biol* 32:47–75
26. Pearson K (1900) X. On the criterion that a given system of deviations from the probable in the case of a correlated system of variables is such that it can be reasonably supposed to have arisen from random sampling. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science* 50:157-175
27. Plackett AR, Conway SJ, Hewett Hazelton KD, Rabbinowitsch EH, Langdale JA, Di Stilio VS (2018) LEAFY maintains apical stem cell activity during shoot development in the fern *Ceratopteris richardii*. *Elife* 7:e39625
28. Rao NN, Prasad K, Kumar PR, Vijayraghavan U (2008) Distinct regulatory role for RFL, the rice LFY homolog, in determining flowering time and plant architecture. *Proc Natl Acad Sci U S A* 105:3646–3651
29. Sato Y, Hong S-K, Tagiri A, Kitano H, Yamamoto N, Nagato Y, Matsuoka M (1996) A rice homeobox gene, OSH1, is expressed before organ differentiation in a specific region during early embryogenesis. *Proceedings of the National Academy of Sciences* 93:8117-8122
30. Sato Y, Sentoku N, Nagato Y, Matsuoka M (1998) Isolation and characterization of a rice homeobox gene, OSH15. *Plant Mol Biol* 38:983–997
31. Sayou C, Nanao MH, Jamin M, Pose D, Thevenon E, Gregoire L, Tichtinsky G, Denay G, Ott F, Peirats Llobet M, Schmid M, Dumas R, Parcy F (2016) A SAM oligomerization domain shapes the genomic binding landscape of the LEAFY transcription factor. *Nat Commun* 7:11222
32. 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* 11:1651–1663

33. Shenghua W, Fang C, Kaida Z (2000) In vitro pollen germination of rice (*Oryza sativa* L.). *Zuo wu xue bao* 26:609–612
34. Smith LG, Jackson D, Hake S (1995) Expression of knotted1 marks shoot meristem formation during maize embryogenesis. *Dev Genet* 16:344–348
35. Sugimoto N (1997) Temporal and spatial expression pattern of rice α -amylase gene *Ramy1A* during seed development. *Rice Genet Newsl* 14:148–150
36. Sun B, Zhou Y, Cai J, Shang E, Yamaguchi N, Xiao J, Looi L-S, Wee W-Y, Gao X, Wagner D, Ito T (2019) Integration of Transcriptional Repression and Polycomb-Mediated Silencing of *WUSCHEL* in Floral Meristems. *Plant Cell* 31:1488–1505
37. Tanahashi T, Sumikawa N, Kato M, Hasebe M (2005) Diversification of gene function: homologs of the floral regulator *FLO/LFY* control the first zygotic cell division in the moss *Physcomitrella patens*. *Development* 132:1727–1736
38. Tao Z, Shen L, Gu X, Wang Y, Yu H, He Y (2017) Embryonic epigenetic reprogramming by a pioneer transcription factor in plants. *Nature* 551:124–128
39. Truernit E, Haseloff J (2007) A Role for *KNAT* Class II Genes in Root Development. *Plant Signal Behav* 2:10–12
40. Tsuda K, Ito Y, Sato Y, Kurata N (2011) Positive autoregulation of a *KNOX* gene is essential for shoot apical meristem maintenance in rice. *Plant Cell* 23:4368–4381
41. Wagner D, Meyerowitz EM (2002) *SPLAYED*, a novel *SWI/SNF* ATPase homolog, controls reproductive development in *Arabidopsis*. *Curr Biol* 12:85–94
42. Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM (1992) *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* 69:843–859
43. Wu MF, Sang Y, Bezhani S, Yamaguchi N, Han SK, Li Z, Su Y, Slewinski TL, Wagner D (2012) *SWI2/SNF2* chromatin remodeling ATPases overcome polycomb repression and control floral organ identity with the *LEAFY* and *SEPALLATA3* transcription factors. *Proceedings of the National Academy of Sciences* 109:3576–3581
44. Xu L, Shen WH (2008) Polycomb silencing of *KNOX* genes confines shoot stem cell niches in *Arabidopsis*. *Curr Biol* 18:1966–1971
45. Xu P, Yuan D, Liu M, Li C, Liu Y, Zhang S, Yao N, Yang C (2013) *AtMMS21*, an *SMC5/6* complex subunit, is involved in stem cell niche maintenance and DNA damage responses in *Arabidopsis* roots. *Plant Physiol* 161:1755–1768
46. Yang W, Gao M, Yin X, Liu J, Xu Y, Zeng L, Li Q, Zhang S, Wang J, Zhang X, He Z (2013) Control of Rice Embryo Development, Shoot Apical Meristem Maintenance, and Grain Yield by a Novel Cytochrome P450. *Mol Plant* 6:1945–1960
47. Yi J, Lee Y-S, Lee D-Y, Cho M-H, Jeon J-S, An G (2016) *OsMPK6* plays a critical role in cell differentiation during early embryogenesis in *Oryza sativa*. *J Exp Bot* 67:2425–2437

Tables

Table 1. The segregation ratio in the progeny of *syd-20/+* mutant lines. The genotypes of grains from *syd-20/+* plants were identified using DNA sequencing. The theoretical values (E) were calculated according to Mendel's law of segregation. χ^2 tests were performed to evaluate the relationship between the actual data and the predicted ratio of 1:2:1 among WT, heterozygote and homozygote.

Genotype (n=2)	WT	heterozygote	homozygote
Actual Value (O)	33	64	23
Theoretical Value (E)	30	60	30
χ^2	2.2		
P	0.5		

Figures

Figure 1

Characterization of *OsSYD* one base insertion mutants. (a) Sequence alignment of *OsSYD* in wild-type (WT) and *syd* mutant plants. The DNA sequence and their corresponding amino acids in red font indicate the CRISPR/Cas9 targets in the mutant lines. (b) Comparison of the set percentage, pollen viability rate, and germination rate between WT and mutant *syd/+* plants. Values are presented as the mean $\pm$ standard deviation (SD). Determination of pollen viability via pollen staining with I_2 -KI (c), scale bar = 500 μ m, and germination in a liquid nutrient medium (d), scale bars = 0.1 mm.

Figure 2

Phenotype analysis of *ossyd* mutants. (a) Phenotypes of the wild-type (WT; top left), *sydl-20/+* (top right), and *ossyd* (bottom) seedlings after germination. The seeds were cultured in MS medium and incubated in the growth chamber under long-day conditions (14 h light at 28 °C and 10 h dark at 24 °C) for 2 weeks. Scale bars = 2.5 cm. (b) Representative images of mature rice embryo vibration sections obtained from WT (top) and mutant *sydl* (bottom) plants. The white arrow indicates the location of the shoot apical meristem (SAM). Scale bars = 100 μ m. SC, scutellum; CO, coleoptile; EP, epiblast; RA, radicle.

Figure 3

***OsSYD* mutants do not affect seed length.** (a) Phenotypes of seeds from 50 randomly selected *syd-20/+* plants. The genotypes of grain endosperm were determined via DNA sequencing. Scale bar = 1 cm. WT, wild-type; He, heterozygote; Ho, homozygote. (b) Average seed length of 50 randomly selected seeds in Figure 4a. Values are presented as the mean $\pm$ SD.

Figure 4

Comparison of transcription levels for marker genes in the embryos of *ossyd* mutants and WT control. (a) Phenotypes of the embryos between Nipponbare (Nip) and *ossyd* mutant plants under a stereo microscope. Scale bars = 1 mm. (b) - (i) qRT-PCR of *ROC1* (b), *OsSCR* (c), *OsPNH1* (d), *RAmy1A* (e), *OSH1* (f), *OSH6* (g), *OSH15* (h), *OSH71* (i). The gene expression is relative to *OsUBQ*. The expression level in the *ossyd* mutants was normalized to that in WT control. Experiments were performed in triplicate for each sample. Values are presented as the mean $\pm$ SD. ** $P < 0.01$; * $P < 0.05$ compared with WT using Student's t-test.

Figure 5

***OsBRM* functions during embryogenesis and male gametophyte formation.** (a) Yeast two-hybrid assay. AD, pGADT7; BD, pGBKT7; AbA, aureobasidin A. (b) Sequence alignment of *OsBRM* in wild-type (WT), *brm-3*, and *brm-2* plants. The DNA bases in red and blue fonts indicate the CRISPR/Cas9 targets for deletion and insertion mutations, respectively. (c) Comparison of seed phenotypes between Nipponbare (Nip) and biallelic mutant *brm-1* plants. Scale bar = 1 cm. (d) Comparison of germinated seedlings of *brm-1* (biallelic mutation/compound heterozygotes), *brm-3* (three base [TGG] deletion mutation), and *brm-2* (one-base [A] insertion mutation). The seeds were sown and grown in MS medium for 3 weeks. Scale bar = 5 cm. The amplified picture of embryo in *brm-2* was observed by stereo microscope. Scale bar = 100 μ m. (e) Representative images of mature rice embryo vibration sections obtained from *brm-3* (top) and mutant *brm-2* (bottom) plants. The white arrow indicates the location of the shoot apical meristem. Scale bars = 200 μ m. SC, scutellum; CO, coleoptile; EP, epiblast; RA, radicle. (f) Comparison of seed size (length, width, and thickness). Values are presented as the mean $\pm$ SD. ** $P < 0.01$; * $P < 0.05$. (g) Segregation ratio in biallelic mutant plants. The genotypes of the grain endosperm were determined via DNA sequencing. The theoretical values (E) were calculated according to Mendel's law of segregation. χ^2 tests were performed to evaluate the relationship between the actual data and the predicted ratio of 2:1:1 among *brm-1*, *brm-3*, and *brm-2*. (h) Statistical analysis of the one-base (A) insertion mutation allele from the male or female parents. The theoretical values (E) were calculated according to Mendel's law of segregation. χ^2 tests were performed to evaluate the relationship between the actual data and the predicted ratio of 1:1.

Figure 6

Comparison of transcription levels for specific marker genes in the embryos of *brm-2* mutants and WT control. qRT-PCR of *ROC1* (a), *OsSCR* (b), *OsPNH1* (c), *RAmy1A* (d), *OSH1* (e), *OSH6* (f), *OSH15* (g), and *OSH71* (h). The gene expression is relative to *OsUBQ*. The expression level in the *brm-2* mutants was normalized to that in WT control. Experiments were performed in triplicate for each sample. Values are presented as the mean $\pm$ SD. ** $P < 0.01$; * $P < 0.05$ compared with WT using Student's t-test.

Figure 7

***OsSYD* interacts with RFL, the *LFY* homolog in rice.** (a) Yeast two-hybrid (Y2H) assay results (bottom). RFL and RFL-N represent the full-length and the truncated form containing only the N-terminal domain

(1–121 amino acids [aa]), respectively (top). The long OsSYD (260–846 aa) was obtained from the Y2H library. AD, pGADT7; BD, pGBKT7; AbA, aureobasidin A. **(b) - (d)** qRT-PCR of *OSH6* (b), *OSH15* (c), and *OSH71* (d). The gene expression is relative to *OsUBQ*. The expression level in the *ossyd* mutants was normalized to that in WT control. Experiments were performed in triplicate for each sample. Values are presented as the mean $\pm$ SD. $**P < 0.01$; $*P < 0.05$ compared with WT using Student's t-test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplementfigureandtable.pdf](#)