Homeobox Is Pivotal for OsWUS Controlling Tiller Development and Female Fertility in Rice

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ABSTRACT OsWUS has recently been shown to be a transcription factor gene critical for tiller development and fertility in rice. The OsWUS protein consists of three conserved structural domains, but their biological functions are still unclear. We discovered a new rice mutant resulting from tissue culture, which hardly produced tillers and exhibited complete female sterility. The male and female floral organs of the mutant were morphologically indistinguishable from those of the wild type. We named the mutant srt1 for completely sterile and reduced tillering 1. Map-based cloning revealed that the mutant phenotypes were caused by a mutation in OsWUS. Compared with the two previously reported null allelic mutants of OsWUS (tab1-1 and moc3-1), which could produce partial N-terminal peptides of OsWUS, the srt1 protein contained a deletion of only seven amino acids within the conserved homeobox domain of OsWUS. However, the mutant phenotypes (monoculm and female sterility) displayed in srt1 were as typical and severe as those in tab1-1 and moc3-1. This indicates that the homeobox domain of SRT1 is essential for the regulation of tillering and sterility in rice. In addition, srt1 showed an opposite effect on panicle development to that of the two null allelic mutants, implying that the srt1 protein might still have partial or even new functions on panicle development. The results of this study suggest that the homeobox domain is pivotal for OsWUS function.

WOX (WUSCHEL related homeobox) proteins, a subgroup of homeodomain transcription factors specific for plants, are involved in several developmental processes of plants, such as embryonic patterning, stem cell maintenance, and organ formation (van der Graaff et al. 2009).

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WUSCHEL (WUS) is the founding gene of the WOX gene family, originally identified in Arabidopsis (AtWUS). It serves as a central regulator in the maintenance of stem cell identity in the central zone (CZ) of the shoot and floral meristems (Laux et al. 1996; Mayer et al. 1998). AtWUS interacts with other genes to regulate the maintenance of meristems at both the vegetative and reproductive stages of Arabidopsis, through a feedback loop mechanism involving at least the CLAVATA 3 (CLV3) and AGAMOUS (AG) genes. It has been found that the development of shoot apical meristem (SAM) and floral meristem (FM) at the early stages is regulated by the WUS-CLV3 signaling pathway. In Arabidopsis, WUS is produced in the organization center (OC) of SAM and migrates to the stem cells of CZ (Yadav et al. 2011), where it binds to the promoter of CLV3 to activate CLV3 transcription (Brand et al. 2000; Schoof et al. 2000). The expression of CLV3 negatively regulates AtWUS expression in OC via the CLAVATA ligand-receptor system to maintain stem cells in a constant population (Brand et al. 2000). During floral development, the FM identity is controlled by the WUS-AG feedback loop. At the reproductive phase, AtWUS activates
the expression of AG in the early stages of floral initiation. After stage 6 of floral development, the stem cells in FM are terminated by AG that represses WUS expression, which allows gynoecium differentiation (Lohmann et al. 2001; Lenhard et al. 2001). A mutation in wus-1 causes shoot meristem failure after seed germination and the mutant plants repetitively initiate defective shoot meristems, leading to many disorganized bunches of leaves at the base and the tip of stems. The flower of wus-1 lacks most of the central organs and prematurely terminates in a single central stamen. AtWUS has also been found to regulate the anther and ovule development (Deyhle et al. 2007; Groß-Hardt et al. 2002; Sieber et al. 2004).

OsWUS in rice has not been subjected to intense studies until recently. An early report using in situ RNA hybridization has suggested that OsWUS is expressed in young leaf primordia with a prominent expression pattern in the lateral leaf margins (Nardmann and Werr 2006). Different from the expression pattern of AtWUS in Arabidopsis, the presence of OsWUS transcripts in the SAM of rice appears to be transient. During the later stage of development, OsWUS is expressed at the abaxial face of the emerging axillary meristems (AMs). Two recent reports on rice mutants defective in tillering have demonstrated that OsWUS is required for AM initiation and is not associated with the maintenance of the SAM (Tanaka et al. 2015; Lu et al. 2015). Both OsWUS mutants, tab1-1 (Tanaka et al. 2015) and moc3-1 (Lu et al. 2015), exhibit severe defects in tillering and flower development. Mutant plants produce no tiller and form a small panicle in the main culm. The floral structures are defective in some flowers of tab1-1, but look normal in all the flowers of moc3-1. The fertility defect in moc3-1 is believed to be female sterile, but the cause of sterility in tab1-1 has not been investigated.

Figure 1 Growth and reproduction phenotypes of srt1. (A) Phenotype of srt1 at the tillering stage. The parental cultivar MH86 served as the wild-type (WT) control. (B) Phenotypes of srt1 at the ripening stage. The srt1 mutant plant produced only one panicle that was completely sterile. (C) Defective tillers of srt1. Three tillers separated from a wild-type (WT) plant all produced a panicle. Three main culms from three individual srt1 mutant plants all produced no valid tiller. Panicles were formed only in the main culms of srt1. (D) Highlights of the basal portions of stem of a WT tiller and a srt1 main culm. Note adventitious root generated from the basal stem nodes of srt1. (E) Frequency of plants with various numbers of tillers. Note that most of the srt1 plants had no tiller and some had one to four tillers per plant. All the wild-type plants produced more than five tillers per plant.

Figure 2 Reproductive phenotypes of srt1. (A–B) Comparison of young (A) and mature panicles (B) between MH86 (WT) and srt1 mutant. Note that panicles of srt1 were larger than those of WT, but produced no filled seed at all. (C) Some spikelets of srt1 had deformed lemma and palea and could not close tightly. Bar = 5 mm. (D) Floral organs of spikelets with the lemma and palea detached were indistinguishable between srt1 and WT. Bar = 2 mm. (E) Pollen grains of srt1 were as viable as those of WT, when stained with iodine-potassium iodide solution. Bar = 200 μm.
The homeobox domain of WUS proteins has been implicated in DNA binding, an essential function of a transcriptional factor. It is a highly conserved motif with about 60–66 amino acid residues folded into a helix-loop-helix-turn-helix structure, which serves as a classic and specific DNA binding domain (Pabo and Sauer 1984; Gehring et al. 1994). Target genes of the WUS transcriptional factor in Arabidopsis have not been identified. The C-terminal structures of AtWUS, including an acidic domain, a WUS box, and an EAR-like motif, have been shown to be necessary for its biological function (Kieffer et al. 2006).

The acidic domain serves as a transcriptional activator, while the WUS box is a repressor essential for all the activities of AtWUS (Ikeda et al. 2009). The EAR-like motif is conserved in plants and appears to be involved in transcriptional repression (Ohta et al. 2009). These multi-functional domains allow AtWUS to act as a bifunctional transcriptional factor, serving mainly as a repressor in stem cell regulation and becoming an activator for AG gene expression in floral patterning (Ikeda et al. 2009).

Three (homebox, WUS box, and EAR-like motif) of the four domains of AtWUS are conserved in OsWUS (Nardmann and Werr 2006). The exact functions of these domains in OsWUS have not been investigated. In this study, we discovered a new independent mutant that forms large panicles with deformed and sterile spikelets and produces a drastically reduced number of tillers per plant. We refer to the mutant as completely sterile and reduced tillering 1 (srt1). We applied a pooled whole-genome sequencing approach to map and clone the candidate gene and found that a 21-bp deletion of OsWUS was responsible for the mutant phenotype. Compared with the two published null mutants tab1-1 and moc3-1, srt1 retains a complete WUS box and EAR-like motif as in the wide-type WUS protein. Phenotypically, srt1 produced few tillers and was completely female sterile, suggesting that the homeobox is essential for tillering development and female fertility in rice.

### MATERIALS AND METHODS

#### Plant materials and field experiments

The main plant materials used in this study included two rice (Oryza sativa L. ssp. indica) cultivars Minghui86 (MH86) and 93-11, and the srt1 mutant and its corresponding heterozygote (abbreviated as srt1-het) isolated from a population of plants regenerated from tissue culture of MH86. The mutant allele was inherited through segregation at the srt1 locus were developed from crosses of srt1-het (♀ × 93-11 (♂) and MH86 (♀) × srt1 (♂). F1 individual plants were allowed for selfing to produce F2 seeds. Rice plants were grown in paddy fields under normal growth conditions in Fuzhou city, China. Several traits including the number of tillers per plant, length of panicle, number of primary branches, and number of secondary branches were investigated. Student t-test was performed in statistical analysis.

#### Fast mapping of SRT1 using bulked segregant analysis by sequencing (BSA-seq)

From the F2 population of srt1-het × 93-11, leaf samples of 50 mutant plants (M-pool) and 50 phenotypically normal plants (N-pool) were collected and bulked respectively for genomic DNA extraction using the CTAB method (Murray and Thompson 1980). Paired-end DNA-seq libraries with an average insert size of 400 bp were constructed for each pool using the Illumina TruSeq DNA LT kit (ID: FC-121-2001) according to the manufacturer’s instructions (Illumina, San Diego, CA). The libraries were sequenced on Illumina HiSeq version 2500, each generating 4.6 Gb DNA sequencing data, representing an average of

#### Table 1 Seed setting rates of reciprocal crosses between srt1 and its parental cultivar MH86

| Cross (♀/♂) | No. of Panicles | No. of Spikelets | No. of Hybrid Seeds | Seed Setting Rate (%) |
|-------------|-----------------|-----------------|--------------------|----------------------|
| MH86/srt1   | 8               | 859             | 77                 | 9.9 ± 6.8             |
| srt1/MH86   | 6               | 466             | 0                  | 0                    |

*aTotal number of spikelets from the panicles in the cross.

*bTotal number of F1 hybrid seeds from the panicles in the cross.

*cMean ± SD of seed setting rates of panicles in the cross.
In addition, Illumina sequencing data (unpublished data) of the genomes of MH86 and 93-11 with a coverage of \(~\times 50\) and \(~\times 30\), respectively, were available from our previous work conducted at the Institute of Biotechnology, Fujian Academy of Agricultural Sciences.

Adaptor-trimmed reads from the four sequencing datasets (M-pool, N-pool, MH86, and 93-11) were aligned to the reference genome of rice \((O. sativa L. ssp. japonica)\) cultivar Nipponbare (version IRGSP-1.0) using BWA \((Li et al. 2009a)\) with default parameters. The uniquely mapped and properly paired alignments from the four datasets were picked by our custom Perl scripts and sorted by SAM tools \((Li et al. 2009b)\). The alignments were then imported into FreeBayes \((Garrison and Marth 2012)\) for identification of variants, including single nucleotide polymorphisms (SNPs) and short insertion/deletion (InDel)s, using default parameters. A subset of these variants, exhibiting explicit polymorphisms between MH86 and 93-11 and having a mean value of allele frequencies in the two pools ranging from 0.3 to 0.7, were selected as markers for genome typing. Based on these markers, a cubic average allele frequency difference (CAAFD) profile was plotted by scanning the whole genome with a 2000-kb sliding window at a step length of \(~10\) kb. The CAAFD of a window was represented by its center point and was calculated with the following formula:

\[
\text{CAAFD} = \left( \frac{1}{n} \sum_{i=1}^{n} \left( \frac{m_{1i} n_{2i} - m_{2i} n_{1i}}{m_{1i} + m_{2i} - n_{1i} - n_{2i}} \right) \right)^3
\]

where \(n\) is the total number of markers in the window; \(m_{1i}\) and \(m_{2i}\) are the counts of the alleles from the genomes of MH86 and 93-11, respectively, at the \(i\)th marker in the M-pool; \(n_{1i}\) and \(n_{2i}\) are the counts of the alleles from the genomes of MH86 and 93-11, respectively, at the \(i\)th marker in the N-pool. The highest peak of CAAFD was considered to be the target region harboring SRT1.

**Fine mapping of SRT1**

A set of InDel and SNP markers were developed in the target region according to the genomic sequence data of the two parents (MH86 and 93-11). These markers were then used to genotype 199 mutant plants selected from the \(F_2\) population of \(srt1\)-het \(\times\) 93-11. Primers of the markers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and were synthesized by Shanghai Sangon Biological Engineering...
Pollen viability assay
To evaluate pollen viability of the wild-type and mutant plants, developing panicles were collected and stained with I2-KI staining buffer. To evaluate pollen viability of the wild-type and mutant plants, developing panicles were collected and stained with I2-KI staining buffer. To evaluate pollen viability of the wild-type and mutant plants, developing panicles were collected and stained with I2-KI staining buffer. To evaluate pollen viability of the wild-type and mutant plants, developing panicles were collected and stained with I2-KI staining buffer.

Multiple sequence alignment
Amino acid sequences of OsWUS, OsWUS, and 12 other WUS-related proteins (WOX) in rice were downloaded from UniProt (http://www.uniprot.org/uniref/). Names of rice WOX proteins were adopted as proposed by Zhang et al. (2010). Multiple sequence alignment was performed using Clustal W (http://www.ebi.ac.uk/clustalw).

Protein 3D modeling
Three-dimensional images of the mutant and the wild-type OsWUS proteins were obtained by simulation of the amino acid sequences using bioinformatics software (Chen et al. 2006).

Data availability
The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.
P = 0.137), further confirming that the SRT1 mutation was a typical recessive Mendelian trait. The fact that the F2 population exhibited a clear segregation ratio of 3:1 for fertile plants (+/+ and 2/+) and sterile plants (2/2) from self-pollinated F1 (+/+), implied that srt1 was a recessive sporophytic mutation.

Cosegregation of sterility and tillering defects in srt1
In the F2 population described above, all the fertile plants exhibited normal tillering phenotype as the control MH86, and all the sterile plants had tillering defects, none having more than four tillers per plant. Among the 71 sterile mutants, 47 plants remained as monoculm with no tiller at all and the remaining 24 plants had only 1–4 tillers per plant, which were significantly fewer than those of the normal fertile plants under the same growth condition. This observation suggests that both defective traits, sterility and reduced tillering, were linked completely and controlled by the same srt1 mutation.

Mapping of SRT1
A total of 656,084 variants (SNPs and short InDels) were selected as markers for mapping SRT1. Analysis showed that there was a major peak of CAAFD at the end of the long arm of chromosome 4, covering a region of ~5 Mb (from ~29 Mb to 34 Mb in the physical map), with the climax at ~32.8 Mb (Figure 4). This region is evidently very likely to contain the causal gene of the observed mutation.

To fine map the target gene SRT1, we developed a set of InDel markers in the target 5 Mb region using the information from the sequencing data. By genotyping the 199 mutant plants from the F2 of srt1-het · 93-11 with these InDel markers, we narrowed down the SRT1 interval from 32.9 cM (between ST_8 and ST_23) to 12.6 cM (between ST_18 and ST_23) (Figure 5). Further linkage analysis using markers developed in this region delimited SRT1 within an interval of 231 kb between ST_18 and STSNP_3, tightly linked to ST_5 (Figure 5).

Identification of srt1 as LOC_Os04g56780
Analysis of the reference rice genome revealed that the 231-kb genomic region between ST_18 and STSNP_3 contains 37 coding sequences (CDSs). The directions and gene ID of these 37 CDSs are depicted above the chromosome 4 nucleotide sequence. Analysis of the genome sequencing data in this region detected only a 21-bp deletion in LOC_Os04g56780 in the M-pool. InDel marker ST_5 was found ~5 kb upstream of the start codon (ATG) of LOC_Os04g56780. No nucleotide change was found in all other 36 CDSs. Black/blue arrows indicate the directions of CDSs opposite to/the same as that of the chromosome 4 nucleotide sequence. The red arrow highlights LOC_Os04g56780 as the candidate gene for SRT1. The exon–intron structures of LOC_Os04g56780 and its two neighboring CDSs are depicted below the chromosome 4 nucleotide sequence. (B) A pair of primers (Loc-780-4) flanking the 21-bp deletion of LOC_Os04g56780 was used for genotyping 280 F2 plants of MH86×srt1 by PCR amplification. Lanes 1 and 2: positive controls of genomic DNA from srt1 mutant and WT cultivar 93-11; lanes 3, 5, 8, 9, 16, and 17: genomic DNA samples from the mutant plants of the F2 population; lanes 4, 6, 7, 10–15, 18–20: genomic DNA samples from the phenotypically normal plants of the F2 population. Note that the 21-bp deletion at LOC_Os04g56780 cosegregated with the mutant phenotype.

Figure 6 Identification of SRT1 as a deletion mutant of OsWUS. (A) The 231-kb genomic region between ST_18 and STSNP_3 contains 37 coding sequences (CDSs). The directions and gene ID of these 37 CDSs are depicted above the chromosome 4 nucleotide sequence. (B) A pair of primers (Loc-780-4) flanking the 21-bp deletion of LOC_Os04g56780 was used for genotyping 280 F2 plants of MH86×srt1 by PCR amplification. Lanes 1 and 2: positive controls of genomic DNA from srt1 mutant and WT cultivar 93-11; lanes 3, 5, 8, 9, 16, and 17: genomic DNA samples from the mutant plants of the F2 population; lanes 4, 6, 7, 10–15, 18–20: genomic DNA samples from the phenotypically normal plants of the F2 population. Note that the 21-bp deletion at LOC_Os04g56780 cosegregated with the mutant phenotype.
ST_5 that was tightly linked to SRT1 was found to be present upstream of the start codon (ATG) of LOC_Os04g56780 (Figure 6A). Furthermore, for a random sample of 280 plants from the F2 population of MH86·srt1, a pair of primers (Loc-780-4F: CAACGTAC CAGCTGCTGTAG and Loc-780-4-R: ATCAGGTCGCCCAACTCG) flanking the 21-bp deletion of LOC_Os04g56780 was able to detect the deletion as homozygous mutant (2/2) in all sterile F2 plants, and heterozygous state (2/+), or homozygous wild-type state (+/+) in all fertile F2 plants (Figure 6B), suggesting cosegregation of the 21-bp deletion in LOC_Os04g56780 with the mutant phenotype. Taken together, these three lines of evidence allow us to conclude that LOC_Os04g56780 is the candidate gene of SRT1 and the 21-bp deletion is the mutation that causes the mutant phenotypes.

**DISCUSSION**

In this study, we have identified a new mutant of OsWUS named srt1, which would produce a nearly full-length WUS peptide except for the deletion of seven amino acids in the HD. The mutation resulted in a dramatic reduction of tillers and complete female sterility. In addition, the mutation also caused morphological alterations in panicles and

**Table 2 Phenotype comparison among srt1, tab1-1, and moc3-1**

| Mutant      | srt1 | tab1-1 | moc3-1 |
|-------------|------|--------|--------|
| Tiller number | 0.5 (0~4) | 0  | 0 |
| Panicle architecture | Denser | Shorter branch | Smaller |
| Flower structure | Defect in lemma and palea | Defective organs in partial flowers | Normal |
| Seed setting rate | 0 | Unknown | 0 |
| Fertility | Female sterility | Unknown | Female sterility |
spikelets. Our findings suggest that OsWUS is an important gene with pleiotropic effects on plant development in rice.

As a new mutant allele of OsWUS, srt1 is quite different from moc3-1 and tab1-1, two other mutants of OsWUS reported recently (Lu et al. 2015; Tanaka et al. 2015). Both moc3-1 and tab1-1 are truncated at the whole C-terminal half of the peptides, and are expected to completely lose their biochemical functions as transcription factors. As compared to them, srt1 has only a short deletion (~2.4% of the whole peptide) and probably retains partial functions. Hence, although two other mutants of OsWUS have already been reported, the results of this study are still highly valuable and useful for our understanding of the biochemical functions of different domains of WUS proteins. The data presented in this report not only confirm and clarify the important functions of OsWUS in the regulation of tillering and flower development, but also unveil potential function of the HD of OsWUS in rice.

The functions of OsWUS

Our observations have clearly shown that the OsWUS mutation affects four major traits of rice: tillering, fertility, panicle development, and spikelet development. As compared with two other recent reports, the main common defect found in all three mutants of OsWUS (Table 2) is tillering inhibition. Hence, controlling tiller development is undoubtedly the most significant function of OsWUS in rice. The incomplete inhibition of tillering in srt1 implies that the srt1 protein might still have a partial activity.

Although the sterility phenotype of tab1-1 has not been fully observed and described, female sterility appears to be a common defect in OsWUS mutant lines (Table 2). Lu et al. (2015) speculate that moc3-1 is likely to be female sterile. In our study, using reciprocal cross tests and pollen staining, we confirmed that srt1 is female sterile. Hence, we conclude that OsWUS controls the female fertility in rice. Like srt1, both moc3-1 and tab1-1 exhibit morphological changes in panicles as compared with the wild type (Table 2). However, while moc3-1 and tab1-1 panicles are smaller with fewer branches and spikelets than the wild-type control, panicles of srt1 were larger and produced more branches and spikelets than the wild-type control. The opposite effects on panicle development between srt1 and the other two mutants further imply that the srt1 mutant protein might still maintain a partial activity, or even have acquired a new function.

The effect of OsWUS mutation on spikelet development does not appear to be consistent among the three mutants (Table 2). In our study, we found that srt1 spikelets developed deformed lemma and palea and could not close tightly, but their inner floral organs seemed indistinguishable from those of wild type (Figure 2C). By contrast, only some tab1-1 spikelets show abnormal phenotypes (lack of one or more floral organs), and all moc3-1 spikelets appear morphologically normal. The discrepancy among these results has prompted a question whether or not OsWUS is really involved in the regulation of spikelet development in rice. Perhaps, the function of OsWUS on spikelet development is dependent on the genetic background of the mutants.

The function of homeobox in OsWUS

OsWUS possesses three conserved domains, the homeobox, WUS box, and EAR-like box (Figure 7C), of which the biological functions have not been studied. In both tab1-1 and moc3-1, the homeobox remains intact, but the whole C-terminal half of the protein containing the WUS box and EAR-like box is not synthesized (Figure 8). Therefore, the functions of tab1-1 and moc3-1 are likely to be completely lost. In contrast, srt1 retains almost the whole WUS protein except for the short deletion within the homeobox (Figure 8). Protein 3D modeling analysis of the N-terminal region revealed that the homeobox of srt1 lacks a short B-strand, suggesting that a small alteration of the homeobox may destroy the function of the protein. As compared to the other two mutants, the defects in srt1 were almost as severe as those in tab1-1 and moc3-1, especially for the phenotypes of tillering inhibition and female sterility. The fact that a mutation of the homeobox can result in almost complete loss-of-function indicates that the homeobox is pivotal to the biological function of OsWUS.

In Arabidopsis, a loss-of-function mutation in AtWUS results in the development of defective flowers lacking most of the central floral...
organisms and having only a single central stamen (Mayer et al. 1998). Interestingly, the Arabidopsis mutant was-3, which contains a proline to leucine missense mutation in the HD, also produces abnormal flowers, with about three stamens per flower. This suggests that the homebox is also an important domain for the full function of AtWUS in Arabidopsis.

In plants, homeodomain (HD)-containing proteins have been implicated in various developmental processes, such as flower and root development, cell fate specification in meristems, and establishment of leaf polarity (Benfey and Weigel 2001). Analysis of 350 WOX proteins from 50 species of the plant kingdom reveals that all WOX members contain three highly conserved residues in the homeodomain: L (145), I (152), and V (157) (Lian et al. 2014). In the 3D structures of AtWUS and OsWUS, these three amino acid residues form an angle of 110.19°, indicating that the three residues may have crucial roles in maintaining the primary function of WUS proteins (Lian et al. 2014). The deletion of the seven amino acid residues (KNVFYYFW) in srt1 (Figure 8) includes residue V (157). It is speculated that absence of this key residue may result in structural alteration and functional loss of OsWUS. Further characterization of the DNA-binding properties of srt1 and comparison with those of animal homebox proteins may help decipher the molecular mechanisms underlying the function of WUS proteins in plants.

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