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ORIGINAL ARTICLE

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# Morphogenesis and molecular basis on *naked seed rice*, a novel homeotic mutation of *OsMADS1* regulating transcript level of *AP3* homologue in rice

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**Abstract** The floral organs are formed from floral meristem with a regular initiation pattern in angiosperm species. Flowers of *naked seed rice* (*nsr*) were characterized by the overdeveloped lemma and palea, the transformation of lodicules to palea-/lemma-like organs, the decreased number of stamens and occasionally extra pistils. Some *nsr* spikelets contained additional floral organs of four whorls and/or abnormal internal florets. The floral primordium of *nsr* spikelet is differentiated under an irregular pattern and an incomplete determination. And molecular analysis indicated that *nsr* was a novel homeotic mutation in *OsMADS1*, suggesting that *OsMADS1* played a distinct role in regulating the differentiation pattern of floral primordium and in conferring the determination of flower meristem. The gain-of-function of *OsMADS1* transgenic lines presented the transformation of outer glumes to lemma-/palea-like organs and no changes in length of lemma and palea, but loss-of-function of *OsMADS1* transgenic lines displayed the overdeveloped lemma and palea. Both findings revealed that *OsMADS1* played a role in specifying lemma and palea and acted as a repressor of overdevelopment of lemma and palea. Moreover, it was indicated that *OsMADS1* upregulated the transcript level of *AP3* homologue *OsMADS16*, using real-time PCR analysis on gain- and loss-of-function of *OsMADS1* transgenic lines.

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**Abbreviations** *AG*: *AGAMOUS* · *AP*: *APETALA* · *DEF*: *DEFICIENS* · *GLO*: *GLOBOSA* · *nsr*: *Naked seed rice* · *PI*: *PISTILLATA* · SEM: Scanning electron microscopy · *SEP*: *SEPALLATA* · *SQUA*: *SQUAMOSA*

## Introduction

Based on the genetic and molecular analyses on specific floral mutants of *Arabidopsis* and *Antirrhinum*, the classic ABC model was proposed (Coen and Meyerowitz 1991; Weigel and Meyerowitz 1994). According to the model, A genes function for sepals, and C genes activity specify the identity of carpel. A genes determine the formation of petal with B genes, while C genes specify stamens identity with B genes. The example of the homeotic genes includes the A gene *APETALA1* (*AP1*), the B gene *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and the C gene *AGAMOUS* (*AG*) in *Arabidopsis*. In *Antirrhinum*, *SQUAMOSA* (*SQUA*) provides the function of A homeotic gene, *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) are members of B homeotic genes, and *PLENA* (*PLE*) is identified as C homeotic gene (Thomas 2001). Except *AP2*, ABC homeotic genes are members of MADS-box genes family, which encode MADS-box proteins with MADS, I, K and C domains in plants. The classic ABC model appears generally applicable to distantly related dicotyledon, while it has been extended by the identifications of D genes *FBP7* and *FBP11* and E genes *SEPALLATA1/2/3* (Angenent and Colombo 1996; Pelaz et al. 2000). Ectopic expression of *PI-AP3-SEP3* or *PI-AP3-AP1* is sufficient to transform leaves into petaloid organs and that of *PI-AP3-SEP3-AG* converts cauline leaves into staminoid organs (Honma and Goto 2001). Furthermore, the quartet model of

floral organs identity has been proposed that complexes of MADS-box proteins bind to the specific genes, respectively, and determine the identity of floral organs of four whorls (Theissen and Saedler 2001).

In monocot rice, MADS-box genes involved in floral development have been isolated and studied. *OsMADS15* shares similar sequence and expression pattern with *AP1* and is a putative ortholog and functional equivalent of *AP1* (Kyozuka et al. 2000). *OsMADS4* is *PI*/*GLO* paralog and flowers of its transgenic lines exhibit conversion of lodicules into palea-/lemma-like structures (Kang et al. 1998). *OsMADS16* is a member of the *AP3* family and OsMADS16 does not form a homodimer, but the protein interacts with OsMADS4, OsMADS6 and OsMADS8 (Lee et al. 2003; Xiao et al. 2003). *OsMADS3* is highly homologous to *AG* and *PLE* in sequence and expression patterns, and loss-of-function of *OsMADS3* shows the changes in the stamens and pistil (Kang et al. 1998). *OsMADS1* belongs to the *AP1*/*AGL9* sub-group and OsMADS1 protein interacts with the OsMADS14 and OsMADS15 (Lim et al. 2000). The functions of floral organ identity genes appear to be broadly conserved between dicot and monocot plants.

In angiosperm species, the particular number of floral organs is formed with the precise pattern. In dicotyledon *Arabidopsis*, the abnormal number of floral organs in flowers is resulted from some causes, such as the mutations in ABC homeotic genes (Coen and Meyerowitz 1991), the changes of floral meristem size in *clavata* and *wiggum* (Clark et al. 1993; Running et al. 1998), the alteration of organ spacing in *perianthia* (Running and Meyerowitz 1996), or the change in floral meristem determination in *wuschel* (Laux et al. 1996). In monocot rice, several mutations associated with floral organ number have been identified. *superwoman1* is a homeotic mutant of *OsMADS16* and displays the conversion of stamens and lodicules into carpels and palea-/lemma-like organs, respectively (Nagasawa et al. 2002). *drooping leaf* (*dl*) exhibits the complete homeotic transformation of gynoecium to stamens, and *DL* is a member of the *YABBY* gene family and regulates carpel specification in rice (Yamaguchi et al. 2004). *floral organ number1* (*fon1*) exhibits the enlargement of the floral meristem and contains the increased number of all floral organs, and *FON1* is orthologous to *Arabidopsis CLAVATA1*, which encodes a leucine-rich repeat receptor kinase (Suzaki et al. 2004). Two missense mutations at MADS domain of *OsMADS1* generate *lhs1* mutant, in which all four floral whorls are affected (Jeon et al. 2000).

*nsr* mutant was derived from the hybrid progeny between *indica* rice and *Triticum aestivum* wheat, and the morphological and physiological characters and cytological mechanism of low fertility were studied briefly in *nsr* (Tang et al. 1981; Wu et al. 2004). *nsr* shows normal karyotype and 12 bivalents during meiosis, and is allelic to *lhs1* (Khush and Librojo 1985). According to data published, the morphology of *nsr* is different from *lhs1* in abnormal panicle, internal florets, variation of stamens and abnormality of pistils. In this experiment, the elucidation on the morphogenesis and molecular basis of *nsr* mutant was conducted. The results showed that the variation of *nsr* spikelets was caused by the irregular differentiation pattern and incomplete determination of floral primordium. And *nsr* was a novel homeotic mutation in *OsMADS1*, which played a role in specifying lemma and palea, acted as a repressor of overdevelopment of lemma and palea and regulated transcript level of *AP3* homologue *OsMADS16*.

## Materials and methods

### Plant materials

*Naked seed rice* was a natural mutant derived from the hybrid progeny between *indica* rice (Tieguai 1) and *Triticum aestivum* wheat (Kangxiu 1). The $F_1$ cross was made between *nsr* mutant and Zhenongda 104 (*japonica*). The mapping population of 1160 $F_2$ mutant plants was used in this experiment. Tiefuai 1 (*indica*) was selected as a control plant for morphogenesis and sequence analyses. Nipponbare (*japonica*) was applied in transgenic experiments.

### Microscopic observation

After heading, spikelets were selected randomly and floral organs were investigated under a light microscopy. For scanning electron microscopy (SEM), young panicles at differentiation stages were fixed in fixative solution of 2.5% glutaric dialdehyde and washed with a sodium phosphate buffer (0.1 M, pH 7.2). Then the samples were fixed in 1% osmic acid, dehydrated with an ethanol series, incubated in an ethanol–isoamyl acetate (1:1[v/v]) and isoamyl acetate, in turn. The samples were dried, mounted and coated with gold. The mounted specimens were observed with a scanning electronic microscope (model KYKY-1000B) at an accelerating voltage of 15 kV.

### Genetic analysis on nsr mutant

At heading stage, genomic DNA was extracted from $F_2$ mutant plants and used for genetic mapping by microsatellite markers. The total RNA from young panicles was isolated and the cDNA molecules were synthesized with oligo-$dT_{15}$ primers and a first-strand cDNA synthesis kit (SuperScriptsII; Invitrogen). The coding sequences of *OsMADS1* cDNA were amplified by RT-PCR with a set of primers, 5′-TGCAAAGGGGATAGAGTAGTAGAGA-3′ and 5′-GGGGAGAAGGTCGTAAGAGA-3′. The amplified fragments were sequenced by MegaBACE 1000 DNA Analysis System.

### Construction of binary vector

Binary vector pCAMBIA1301 was modified for ectopic expression. The cauliflower mosaic virus 35S promoter (35S) was cloned in as a *Hin*dIII-*Bam*HI fragment in the region of multi-cloning site. Subsequently, the nopaline synthase terminator was cloned as a *Sac*I–*Eco*RI fragment downstream of the 35S promoter.

For construction of overexpressing *OsMADS1*, the coding sequence of *OsMADS1* cDNA was isolated with specific primer sets. PCR products were cloned into pUCm-T. The sense orientation of the cDNA was verified by *Eco*RV and *Sal*I digestion and was inserted into the *Sma*I and *Sal*I sites of the modified pCAMBIA1301. The recombinant was called 35S-*OsMADS1*.

To make the construct for expressing dsRNA in plant cells, we first applied a bridge vector pBS-in, in which a 605-bp intron fragment was placed between two multi-cloning sites. The 351 bp-length fragment of coding sequence of *OsMADS1* was amplified by RT-PCR from wild-type rice with primer sets, 5′-GAGCAGCTTGAGAACCAGATAGA-3′ and 5′-TCATTGCTCAGATGGTCCATGTAG-3′. PCR products were cloned into pUCm-T. The subcloned products were placed upstream and downstream of the intron fragment in opposite directions, in turn. The constructed bridge vector was inserted into the *Kpn*I and *Sac*I sites of the modified pCAMBIA1301. The resulting RNAi construct was denoted as 35S-dsRNAi*OsMADS1*.

### Rice transformation

The *Agrobacterium* strains EHA105 harboring 35S-*OsMADS1* or 35S-dsRNAi*OsMADS1* plasmid were used to transform rice calli induced from the mature embryos of Nipponbare, respectively, according to the methods by Hiei et al. (1994). Co-cultivation was for 2–3 days in the dark at 25°C and the co-cultivated calli were transferred onto an NCH medium containing 50 mg $l^{-1}$ hygromycin and 50 mg $l^{-1}$ cefotaxime in the light at 28°C until actively proliferating calli developed. The actively growing calli were transferred onto a regeneration MS medium supplemented with 0.05 mg $l^{-1}$ NAA, 3 mg $l^{-1}$ kinetin, 1% sorbitol, 0.8% phytagar for 2–4 weeks. A light/dark cycle of 16/8 h was provided during the regeneration. The regenerated plantlets were grown in a greenhouse.

### Real-time PCR

For quantitative real-time PCR experiments, iCycler iQ™ Real-Time PCR Detection System (DIO-RAD, USA) was used. For PCR reactions, a mastermix of reaction components was prepared, according to Instruction of iQ SYBR Green Supermix (DIO-RAD, USA). The following primers were used for real-time PCR experiments: *OsMADS1* forward primer, 5′-CATGGACCATCTGAGCAATGA-3′, and reverse primer, 5′-AAGAGAGCACGCACGTACTTAG-3′; *RAP1B* forward primer, 5′-GCGAAAGGATAGAGGATGTACCAG-3′, and reverse primer, 5′-GCAACCGCAAGATGACAATAG-3′; *OsMADS4* forward primer, 5′-AGCACAAGATGTTGGCTTTTAGGG-3′, and reverse primer, 5′-CATCTAGCAGCGCATGAGG-3′; *OsMADS16* forward primer, 5′-TCAAGGACATCAACCGCAACCTG-3′, and reverse primer, 5′-ATGATACTTCCTGTGGCGAACCTC-3′; *OsMADS3* forward primer, 5′-AACGCAAACAGTAGGACCATAGTG-3′, and reverse primer, 5′-CCCCTCTCATTCTCAACAACC-3′; *OsMADS13* forward primer, 5′-GCGATAATGTGAGCAACCTGT-3′, and reverse primer, 5′-TTCTGAGGTCCATGTTGTCGTTCT-3′; *OsMADS15* forward primer, 5′-TCTTCCACCACAAAATATCTGCTAC-3′, and reverse primer, 5′-GGTACGTGCTGATGATTACACAA-3′; *ACT1* forward primer, 5′-CTTCTAATTCTTCGGACCCA-3′, and reverse primer, 5′-TTGAAAACTTTGTCCACGCTAATC-3′.

## Results

### Morphology of spikelets in *nsr* plants

Wild-type rice spikelet comprised a pair of rudimentary outer glumes at its base and four whorls of floral organs, namely lemma/palea, a pair of lodicules, six stamens and a pistil from the periphery to the center, and lodicules were located inner to lemma (Fig. 1a). Wild-type spikelet was closed (Fig. 1b), and its palea/lemma was characterized by abundant and large epidermal cells and long trichomes (Fig. 1j). No obvious alteration was observed during vegetative stage, while abnormal spikelets were investigated after heading in *nsr* plants. Compared to wild-type plant, *nsr* mutant showed leafy and overdeveloped lemma and palea in spikelet, which was open (Fig. 1c), and the palea/lemma displayed fewer and smaller epidermal cells and shorter trichomes with higher density (Fig. 1k). *nsr* spikelet generated internal florets with palea-/lemma-like organ (Fig. 1d). Two pairs of lodicules became leafy and arranged inner to both lemma and palea, and three stamens and one pistil were formed in *nsr* spikelet (Fig. 1e), although eight stamens, three pistils and three pairs of lodicules were rarely found in *nsr* spikelet (Fig. 1f). Occasionally, an ovary tipped with five stigmas (Fig. 1g) or the conversion of anther into stigma (Fig. 1h) was observed in the *nsr* spikelet. Mature florets were formed on one rachilla axis of *nsr* mature spikelet (Fig. 1i), indicating that *nsr* spikelets had an incomplete determination of floral meristem.

To study further on the floral development in the spikelets of *nsr* mutant, an investigation on 3,600 spikelets was conducted. 24.4% of *nsr* spikelets generated abnormal florets with palea-/lemma-like organs, whereas 91.3% of *nsr* spikelets contained two pairs of lodicules, which were transformed to palea-/lemma-like organs.

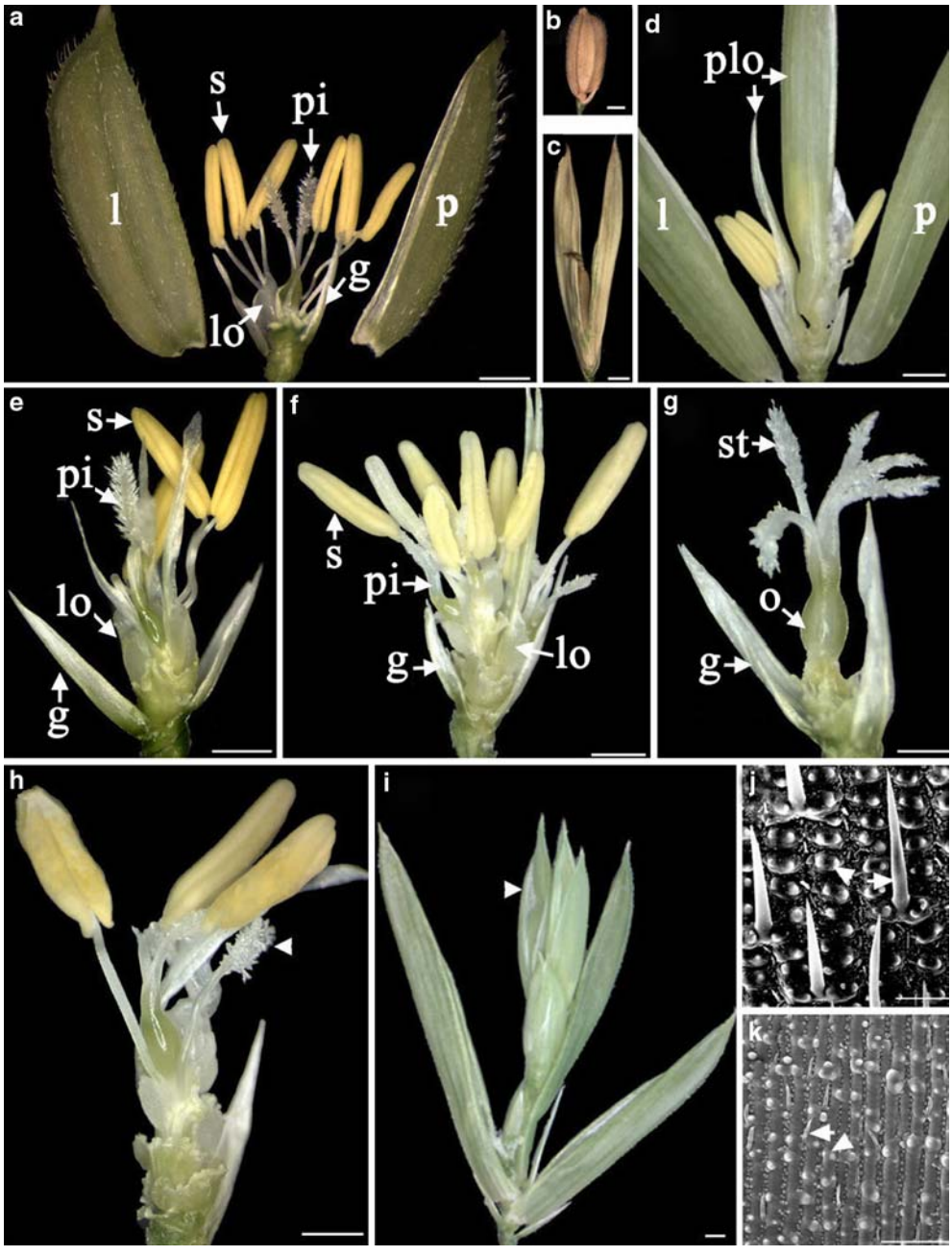


**Fig. 1** Phenotypes of wild-type and *naked seed rice* (*nsr*) mutant rice. **a**–**b** the spikelet of wild-type rice. **c**–**i** phenotypic alteration of floral organs in *nsr* spikelets. Lemma and palea were removed from *nsr* spikelets (**e**–**h**), and the outer three whorls of floral organs were removed from the *nsr* spikelet (**g**). *Arrowhead* indicated the conversion of anther into stigma and internal floret in *nsr* spikelets (**h**) and (**i**), respectively. *Arrow* indicated trichome and *arrowhead* showed the epidermis of wild-type (**j**) and *nsr* (**k**) spikelet. *g* glume; *l* lemma; *p* palea; *lo* lodicule; *plo* palea-/lemma-like organ; *s* stamen; *o* ovary; *pi* pistil; *st* stigma. *Bars* from **a**–**i** = 1 mm, *Bars* in **j, k** = 100 μm

97.9% of spikelets contained less than six stamens and the number of stamens was reduced to three, on an average. One pistil was mostly observed in the *nsr* spikelets, of which 20.9% formed over one ovary. *nsr* spikelets were characterized by overdeveloped lemma and palea, an increased number of lodicules, a decreased number of stamens and extra pistils. *nsr* spikelets aslo formed internal florets, which contained floral organs of four whorls.

## Microscopic investigation of *nsr* spikelets

Scanning electron microscopy was conducted to compare the morphogenesis of wild-type flowers with that of *nsr* at different differentiation stages (Fig. 2). In wild-type flower, floral primordium generated outer glumes, lemma and palea first (Fig. 2a). Then lodicule and six stamens primordia were generated and the former was located inner to the lemma (Fig. 2b). Floral primordium gave a rise to generate a gibbous carpel primordium, while other floral organs primordia were continuing to grow (Fig. 2c).

The morphogenesis of *nsr* spikelet was different from that of wild-type spikelet. In *nsr* spikelet, outer glumes, palea and lemma were formed (Fig. 2d). Then lodicule primordium was initiated inner to the lemma (Fig. 2e). Alternatively, two lodicule primordia were formed and located inner to both the lemma and palea, respectively (Fig. 2f). Lodicule began to overdevelop at the early stage

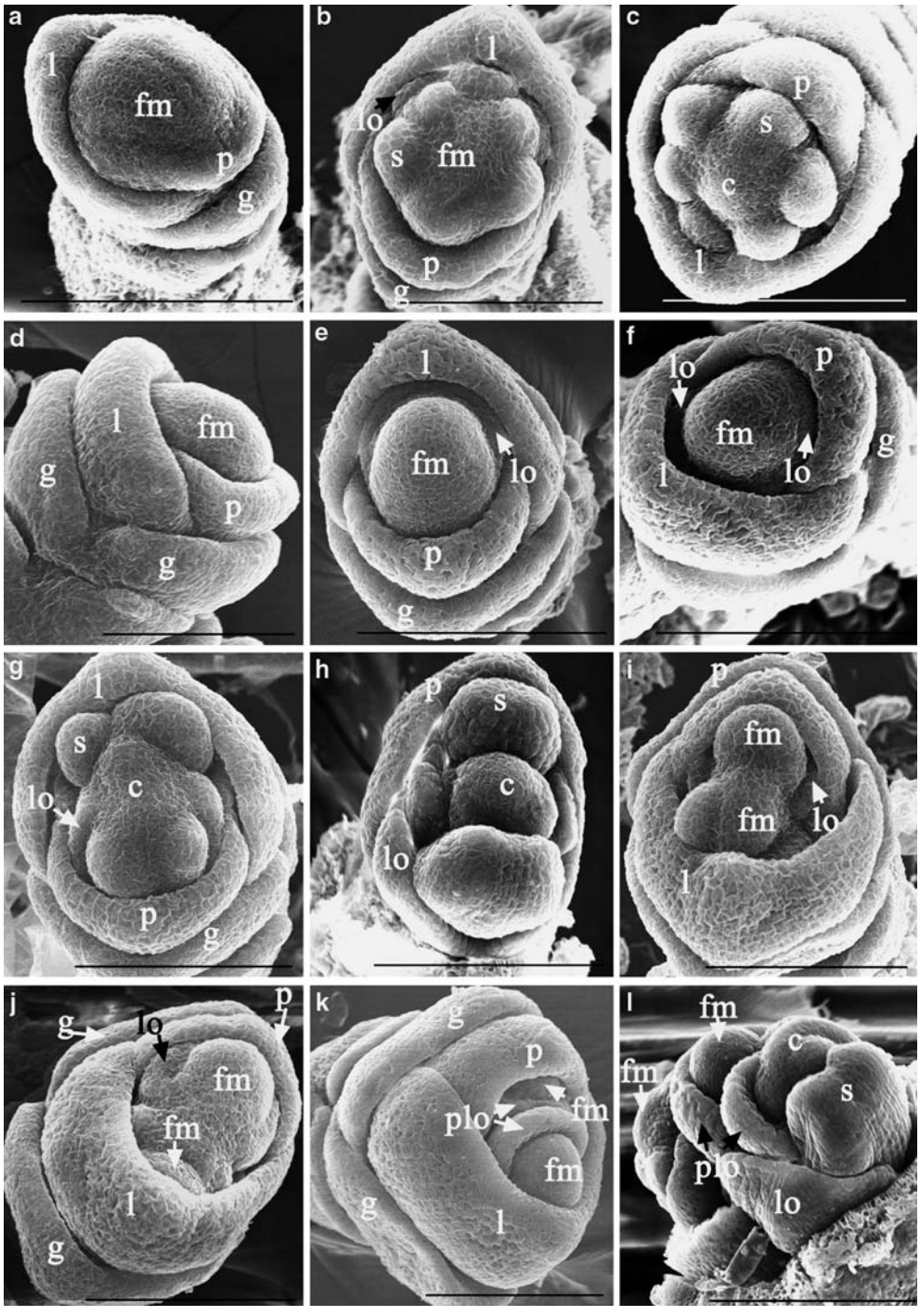


**Fig. 2** Scanning electron microscopy of wild-type and *nsr* spikelets. **a**–**c** and **d**–**l**, the differentiation of floral primordium in wild-type spikelets and *nsr* spikelets, respectively. Lemma was ripped off the *nsr* spikelet (**h**), and lemma and palea were removed from *nsr* spikelet (**i**). *g* glume; *l* lemma; *p* palea; *lo* lodicule; *plo* palea-/lemma-like organ; *s* stamen; *c* carpel; *fm* floral primordium. *Bars* = 100 μm

of flower development (Fig. 2h). Stamens and carpel primordia were generated irregularly, with the number of stamens varying from two to four (Fig. 2g, h). Interestingly, floral primordium was elongated in the direction of lemma and palea and divided (Fig. 2i). Two separate floral primordia were formed in *nsr* spikelet (Fig. 2j), and two newly formed floral primordia generated palea-/lemma-like organs, respectively (Fig. 2k). Three floral primordia developed in one *nsr* spikelet (Fig. 2l). The floral primordium of *nsr* spikelet differentiated under the irregular pattern and incomplete determination.

## Genetic analysis and gene mapping

The $F_1$ cross was made between *nsr* and *japonica* rice Zhenongda 104, which showed normal spikelet phenotype. The $F_2$ population of 460 plants segregated wild-type and mutant plants in a ratio of 3:1 ($\chi^2 = 0.23$, $P > 0.95$), indicating *nsr* was a monogenic recessive trait. *nsr* locus was primarily mapped to chromosome 3 between microsatellite markers, RM3548 (2.26 cM) and RM2326 (1.7 cM). Subsequent fine mapping showed that *nsr* locus was located between RM3417 (0.26 cM)

and RM7576 (0.21 cM) (Fig. 3a). With the sequence of RM3417 and RM7576, a blast homology search was carried out and identified two BAC clones, AC105928 and AC135138, between which there were three overlapping BAC clones (AC104179, AC146619 and AC134241). Alignment of sequence of the five overlapping BAC clones showed that the physical distance between RM3417 and RM7576 was about 400 kb. Annotation of this region identified an open reading frame (ORF) encoding a MADS-box protein, OsMADS1 (http://www.gramene.org).

Jeon et al. (2000) reported that *lhs1* was a homeotic mutation in *OsMADS1*, which was mapped between RG100 and RZ313. The region included *nsr* locus. Thereby, the coding regions of the *OsMADS1* gene were amplified from *nsr* and the wild-type rice with primers located at *OsMADS1* cDNA sequence. Sequence analysis of the amplified fragments showed that 5 nucleotides of A, G, A, A and T at positions 58, 80, 287, 527 and 666 in coding region were changed to G, A, G, G and C in the *nsr* mutant, respectively. Consequently, the deduced amino acids of $Thr^{20}$, $Gly^{27}$, $Lys^{96}$ and $Asn^{176}$ were replaced with Ala, Asp, Arg and Ser, respectively, and $His^{222}$ was not changed in OsMADS1 protein of *nsr* (Fig. 3b). *nsr* contained both missense and nonsense mutations in the coding region of *OsMADS1*. In *lhs1* mutant, the nucleotides C and G at position 70 and 80 in the coding region of *OsMADS1* were changed to T and A, respectively (Jeon et al. 2000). Thereby, *nsr* was a novel mutation of homeotic gene *OsMADS1*.

## Floral organs alteration in transgenic rice plants overexpressing *OsMADS1*

To address the role of *OsMADS1* in floral organ development, the cDNA clone containing the full-length *OsMADS1* ORF was placed under the cauliflower mosaic virus 35S promoter. The construct was introduced into rice cells by *Agrobacterium*-mediated transformation. Two independent transgenic lines were generated and each included a number of transgenic plants.

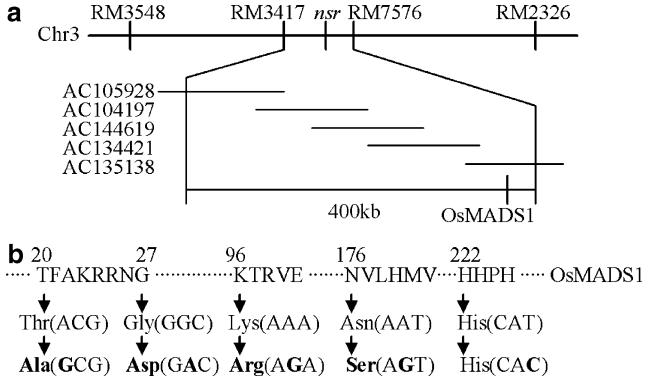


**Fig. 3** Molecular identification of *nsr* mutant. **a** *nsr* was mapped between RM3417 and RM7576. **b** deduced amino acid and its corresponding nucleotide. *Bold letters* indicated the changes in *nsr* mutant

The wild-type rice spikelet had a pair of rudimentary outer glumes at its base and comprised lemma, palea, two lodicules, six stamens and one pistil with two stigmas (Fig. 4a, b). In 35S-*OsMADS1* transgenic lines, no obvious abnormality was observed during vegetative stage, but the alternations of flower phenotypes were investigated after heading. A significant proportion (17.9%) of flowers in 35S-*OsMADS1* transgenic lines generated the enlarged outer glumes, which approached the size of lemma or palea (Fig. 4c). The stamen was characterized by the decreased number, which was mostly five (Fig. 4e). Occasionally, the flowers did not bear any internal floral organs (Fig. 4d). RT-PCR analysis revealed that the transgenic lines expressed the *OsMADS1* transcript in their leaves, while no transcript was detected in the wild-type leaves (Fig. 4j), suggesting that ectopic expression of *OsMADS1* was responsible for the alteration of floral organs in transgenic rice plants.

The real-time quantitative reverse transcriptase technique offers both high sensitivity and specificity (Bustin 2000), and it was applied in an analysis of the expression level of genes belonging to a very conserved gene family (Yokoyama and Nishitani 2001). Therefore, real-time PCR was applied to investigate the transcript levels of several MADS-box genes, which presumably specify floral organ identity in rice. Figure 5 illustrated the relative transcript levels of several MADS-box genes in the young panicles (5 cm) of 35S-*OsMADS1* transgenic rice and the control plants. The transcript level of *OsMADS1* was about two times higher than that of wild-type rice. The expression of *RAP1A*, *OsMADS4*, *OsMADS16*, *OsMADS13* and *OsMADS15* were increased significantly, but *OsMADS3* transcript was affected less.

## Floral organs alteration in 35S-dsRNAi*OsMADS1* transgenic rice plants

In plants, the transgenic plants expressing dsRNAs (RNA hairpins) can significantly knock down the transcription level of their targeted endogenous genes in *Arabidopsis*, and mimic their loss-of-function mutants (Chuang and Meyerowitz 2000). In rice, RNAi was applied to address the function of *AP3* homologue *OsMADS16* (Xiao et al. 2003). In this study, the *OsMADS1* cDNA fragment was introduced into an RNAi construct under 35S promoter. Three transgenic rice lines (independent transformants) were successfully recovered.

All 35S-dsRNAi*OsMADS1* transgenic lines grew normally as wild-type rice during vegetative stage. Obvious changes in spikelets were observed after heading. Compared with that in wild-type rice (Fig. 4a, b), the overdevelopment of lemma and palea was observed in 35S-dsRNAi*OsMADS1* spikelet, which was open (Fig. 4f). 35S-dsRNAi*OsMADS1* transgenic spikelet

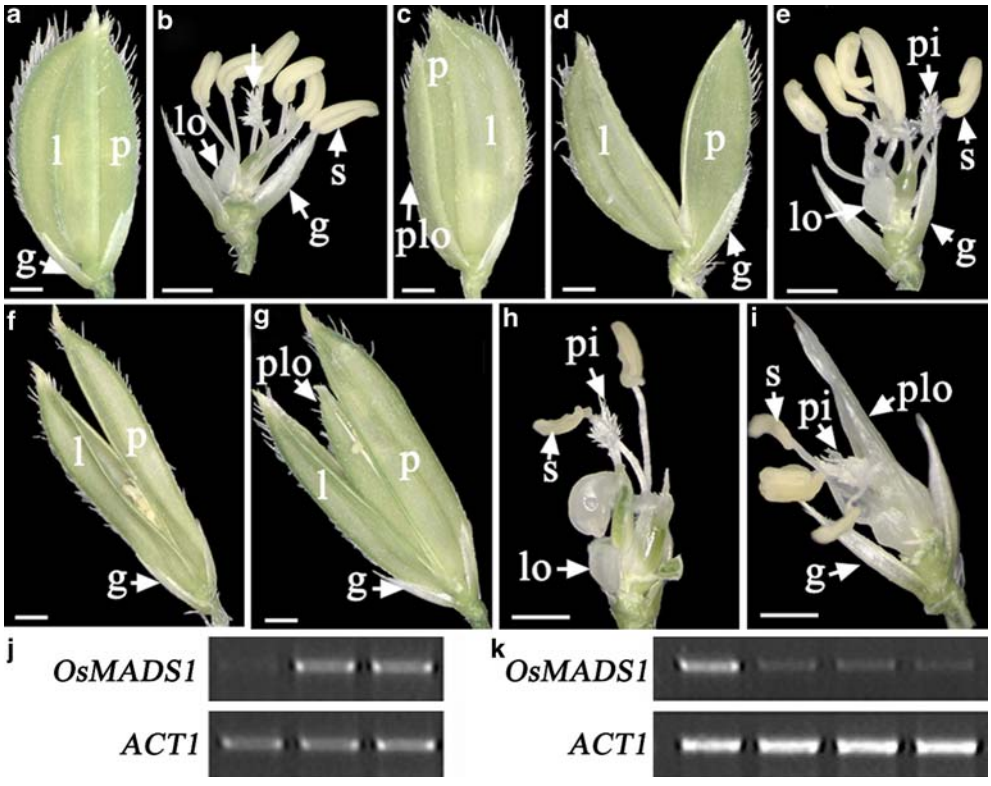


**Fig. 4** Floral alterations of transgenic rice carrying 35S-*OsMADS1* and 35S-dsRNAi*OsMADS1* construct. **a**–**b** wild-type flower; **c**–**e** the flowers of 35S-*OsMADS1* transgenic plants; **f**–**i** the flowers of 35S-dsRNAi*OsMADS1* transgenic plants. Lemma and palea were removed from spikelet (**b**), (**e**), (**h**) and (**i**). **j**–**k** RT-PCR analysis of *OsMADS1* transcripts in the 35S-*OsMADS1* and 35S-dsRNAi*OsMADS1* transgenic plants; *Lane 1*, RT-PCR product from wild-type rice; the other lanes, RT-PCR products of transgenic lines. *g* glume; *l* lemma; *p* palea; *lo* lodicule; *plo* palea-/lemma-like organ; *s* stamen; *pi* pistil. *Bars* = 1 mm

generated one extra palea-/lemma-like organ inside (Fig. 4g) or the transformation of two lodicules into four palea-/lemma-like organs (Fig. 4h). The stamens were characterized by the diverse number, which varied from two (Fig. 4i) to six (data not shown). Occasionally, two separate pistils appeared in one spikelet (Fig. 4h).

An investigation on floral organs was made to study further on the flowers development of the 35S-dsRNAi*OsMADS1* transgenic plants. The averaged length of lemmas and paleas amounted to 0.90 and 0.73 cm, respectively, and were significantly longer than those of wild-type spikelets. 36.7% of the investigated flowers had five stamens, 11.4% of flowers contained two, three or four stamens. Whereas 5.1% of lodicules in investigated flowers was transformed into palea-/lemma-like organs. The different efficiencies of the floral organ variation indicated that lodicules and stamens might have different responses to the introduced dsRNA of *OsMADS1* cDNA fragment.

RT-PCR analysis was conducted to compare the transcripts of *OsMADS1* in the 35S-dsRNAi*OsMADS1* transgenic plants with the control plants. As shown in Fig. 4k, a weaker band *OsMADS1* transcripts were detected in flowers of transgenic plants after 35 cycles of amplification, compared to that in wild-type panicles. When 28 cycles was applied, the *OsMADS1* transcript was not detected in the flowers of transgenic plants (data not shown). This result showed that the reduced level of *OsMADS1* transcription led to the changes in floral organs of transgenic plants.

The real-time PCR was applied to investigate the transcription levels of several MADS-box genes in young panicles (5 cm) of 35S-dsRNAi*OsMADS1* transgenic plants and the control plants (Fig. 6). In 35S-dsRNAi*OsMADS1* lines, *OsMADS1* transcript was reduced to approximately 20% of that in wild-type rice. The remaining *OsMADS1* transcript indicated that the introduced dsRNA did not completely suppress the endogenous transcription in this transgenic plant. The transcript levels of *OsMADS16*, *OsMADS3* and *OsMADS13* were significantly reduced when compared with those of wild-type rice. In contrast, *RAP1B* expression was increased greatly. *OsMADS4* and *OsMADS15* transcript levels were affected slightly.

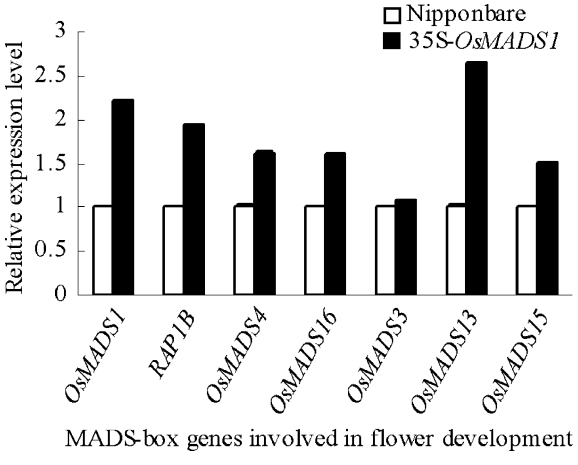


**Fig. 5** Real-time PCR analysis on MADS-box genes in 35S-*OsMADS1* lines

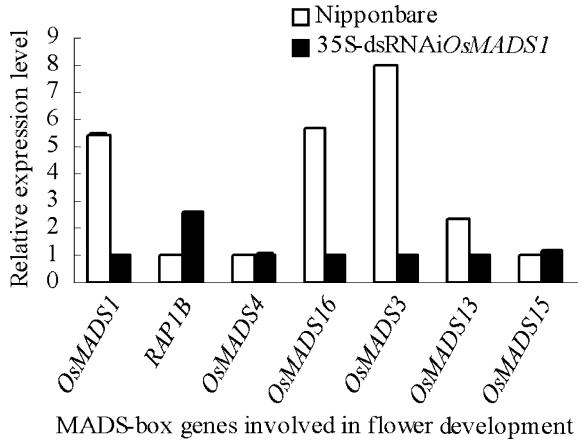


**Fig. 6** Real-time PCR analysis on MADS-box genes in 35S-dsRNAi*OsMADS1* lines

## Discussion

*Naked seed rice* was characterized by the overdeveloped palea and lemma, leafy lodicules, decreased stamens, occasionally extra pistils and internal florets. The observation on the morphogenesis of floral organs indicated that floral primordium of *nsr* spikelets differentiated into floral organs under the irregular pattern, compared with that of wild-type rice. First, floral primordium initiated one lodicule inner to lemma, or one more lodicule inner to palea alternatively after the proper formation of palea and lemma primordia. As a result, two pairs of lodicules were observed in most *nsr* spikelets. Second, floral primordium in *nsr* spikelets formed stamens and carple primordia with an irregular shape and a decreased number of stamens. Third, the division floral primordium was also observed in the *nsr* spikelets. Consequently, the syncarpy or abnormal florets might be formed, which resulted in an increased number of floral organs subsequently. The results suggested that the mutant spikelets of *nsr* plants resulted from the irregular differentiation pattern of floral primordium and incomplete determination of floral meristem.

In rice, several floral mutants have been studied on a molecular basis, such as *fon1*, *spw1*, *dl* (Nagasawa et al. 2002; Suzaki et al. 2004). The *lhs1* mutant is derived from *japanica* rice and caused by missense mutations of code $Arg^{24}$ and $Gly^{27}$ in MADS domain of *OsMADS1* (Kinoshita et al. 1977; Jeon et al. 2000). *nsr* has been reported to be allelic to *lhs1* (Khush and Librojo 1985). This result was supported by the findings in this study, but missense mutations of $Thr^{20}$, $Gly^{27}$, $Lys^{96}$ and $Asn^{176}$ and nonsense mutation of $His^{222}$ of *OsMADS1* in *nsr* were different from the missense mutations in *lhs1*. Therefore, *nsr* was a novel mutant of homeotic gene *OsMADS1*. Pellegrini et al. (1995) reported that the $Gly^{27}$ is located at the DNA binding position. Similarly, the missense mutation of code $Gly^{27}$ to Asp results in *ap1–2* and *cal-3* mutants (Mandel et al. 1992; Kempin et al. 1995). Transgenic lines from the introduced gene expressing double-stranded RNA with the *OsMADS1* cDNA fragment generated mutant spikelets, which comprised overdeveloped palea and lemma, increased lodicules, decreased stamens and occasionally extra pistil. The spikelets of 35S-dsRNAi*OsMADS1* lines were similar to those of *nsr* plants, providing further evidence that *nsr* was a homeotic mutant in *OsMADS1*.

The overdeveloped lemma and palea were present in the 35S-dsRNAi*OsMADS1* transgenic plants, in which the transcript level of endogenous *OsMADS1* was reduced significantly. No significant changes were observed in the length of lemma and palea, and the transcripts of *OsMADS1* were expressed abundantly in 35S-*OsMADS1* transgenic plants. In consideration of a later expression of *OsMADS1*, which is confined to lemma and palea (Chung et al. 1994; Prasad et al. 2001), it was suggested that wild-type *OsMADS1* might function as a repressor of the overdevelopment of lemma and palea. Although the transcript level of mutant *OsMADS1* in *nsr* spikelets appeared higher than wild-type *OsMADS1* in the control plants (data not shown), the missense mutations contributed to the loss of proper function of *OsMADS1* in *nsr* plants, as a result, the overdeveloped lemma and palea were observed.

Genetic studies on antisense suppression mutants of *OsMADS4* and the maize *silky1* mutant suggest that lodicules are equivalent to petals, although lodicules are morphologically different from petals (Schmidt and Ambrose 1998). Transgenic plants expressing double-stranded RNA with *OsMADS16* cDNA fragment displays the conversion of two lodicules into palea-/lemma-like organs (Xiao et al. 2003). In this study, most of the *nsr* spikelets contained two pairs of lodicules, which were converted to palea-/lemma-like organs. The similar phenotypic conversion was observed in the 35S-dsRNAi*OsMADS1* transgenic plants, in which the transcript level of *OsMADS16* was reduced greatly. However, the conversion and increased number of lodicules were not observed in 35S-*OsMADS1* transgenic plants, which had a higher transcript level of *OsMADS16* than the wild-type rice. These results indicated that *OsMADS1* played its role in upregulation of transcript activity of *OsMADS16*, an *AP3* homologous gene. The transcripts of *OsMADS16* are present in the lodicules and stamens (Moon et al. 1999). *OsMADS1* is expressed uniformly in young flower primordia and its expression is confined to the lemma and palea later, with weak expression in the carpel (Chung et al. 1994; Prasad et al. 2001). It was speculated that the role of *OsMADS1* in affecting the *OsMADS16* transcripts might require a co-factor, which was expressed in both floral meristem and primordia of lodicule and stamens.

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