

Functional analyses of the flowering time gene *OsMADS50*, the putative *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20)* ortholog in rice

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Summary

A late-flowering mutant was isolated from rice T-DNA-tagging lines. T-DNA had been integrated into the K-box region of *Oryza sativa* *MADS50* (*OsMADS50*), which shares 50.6% amino acid identity with the *Arabidopsis* MADS-box gene *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20)*. While overexpression of *OsMADS50* caused extremely early flowering at the callus stage, *OsMADS50* RNAi plants exhibited phenotypes of late flowering and an increase in the number of elongated internodes. This confirmed that the phenotypes observed in the knockout (KO) plants are because of the mutation in *OsMADS50*. RT-PCR analyses of the *OsMADS50* KO and ubiquitin (*ubi*):*OsMADS50* plants showed that *OsMADS50* is an upstream regulator of *OsMADS1*, *OsMADS14*, *OsMADS15*, *OsMADS18*, and *Hd (Heading date)3a*, but works either parallel with or downstream of *Hd1* and *O. sativa* *GIGANTEA (OsGI)*. These results suggest that *OsMADS50* is an important flowering activator that controls various floral regulators in rice.

Keywords: flowering, MADS, *OsMADS50*, rice, *SOC1/AGL20*, T-DNA.

Introduction

Heading date is a critical trait when determining cropping seasons and regional adaptability in rice. Its timing varies greatly depending on genotype and environmental factors such as photoperiod and light intensity (Dung *et al.*, 1998; Yamamoto *et al.*, 1998). While this may be of biological interest, researchers also wish to understand its molecular mechanism when developing breeding programs.

Genetic studies in rice (*Oryza sativa*) have led to the identity of several trait loci that control photoperiod sensitivity, including *Se (photoperiod-sensitivity)1*, *Se3–Se7*, and *E (photoperiod-sensitivity)1–E3* (Poonyarit *et al.*, 1989; Sano, 1992; Tsai, 1995; Yokoo and Okuno, 1993; Yokoo *et al.*, 1980). In addition, quantitative trait loci (QTL) analyses with molecular markers have detected tens of loci for heading date (Li *et al.*, 1995; Lin *et al.*, 1996, 1998; Maheswaran *et al.*, 2000; Xiao *et al.*, 1995; Yano *et al.*, 1997). Characterization of the photoperiod response, epistatic interaction analyses, and fine genetic linkage mapping for target QTLs have been performed using the near-isogenic lines (NILs)

derived from rice cv. Nipponbare (*japonica*) and rice cv. Kasalath (*indica*; Lin *et al.*, 1998, 2000, 2002; Monna *et al.*, 2002; Yamamoto *et al.*, 1998, 2000; Yano *et al.*, 1997). Three QTLs, *Hd1*, *Hd3*, and *Hd6*, are mapped precisely on the genetic linkage map as single Mendelian factors (Yamamoto *et al.*, 1998, 2000), and two tightly linked loci in the *Hd3* region, *Hd3a* and *Hd3b*, have been dissected (Monna *et al.*, 2002). These three QTLs were cloned via a map-based cloning strategy (Kojima *et al.*, 2002; Takahashi *et al.*, 2001; Yano *et al.*, 2000).

Hd1, which is allelic to *Se1*, encodes a protein containing the zinc finger domain and a nuclear localization signal (Yano *et al.*, 2000). Interestingly, under long-day (LD) conditions, *Hd1* inhibits flowering whereas *CONSTANS (CO)*, an *Arabidopsis* ortholog of *Hd1*, activates flowering. This is because *Hd1* inhibits the expression of *Hd3a*. In contrast, *CO* activates *FLOWERING LOCUS T (FT)*, which acts as a major flowering pathway integrator, being an ortholog of *Hd3a* in *Arabidopsis* (Hayama *et al.*, 2003; Izawa *et al.*,

2002). Under short-day (SD) conditions, however, *Hd1* activates *Hd3a* and promotes flowering (Izawa *et al.*, 2002; Kojima *et al.*, 2002). Another QTL, *Hd6*, encodes the α subunit of *CASEIN KINASE 2 (CK2)*; the functional Kasalath allele inhibits flowering under natural (April to August in Japan) and LD conditions but not under SD conditions (Takahashi *et al.*, 2001). In addition, the *Se5* gene, encoding a putative heme oxygenase, has been identified as a counterpart of *Arabidopsis LONG HYPOCOTYL 1 (HY1)*; Izawa *et al.*, 2000, 2002). That mutant is completely deficient in photoperiodic response and shows very early flowering. The role of *O. sativa GIGANTEA (OsGI)* also has been elucidated as an upstream activator of *Hd1* (Hayama *et al.*, 2003).

In rice, several major genes affecting heading date are related to either photoperiod sensitivity or basic vegetative growth (Yamamoto *et al.*, 1998, 2000). Thorough studies of the former influence have been based on the conserved nature between rice and *Arabidopsis* (reviewed by Mouradov *et al.*, 2002; Simpson, 2003; Yano *et al.*, 2001). The latter factor, however, has hardly been investigated at the molecular level. It is notable that MADS-box genes such as *FLC* (flowering locus C), *AGAMOUS-LIKE 27 (AGL27)* and *SHORT VEGETATIVE PHASE (SVP)* function as flowering repressors in *Arabidopsis* (Hartmann *et al.*, 2000; Michaels and Amasino, 1999; Scortecci *et al.*, 2001; Sheldon *et al.*, 1999), whereas *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20)* and *AGL24* act as activators (Lee *et al.*, 2000; Michaels *et al.*, 2003; Yu *et al.*, 2002). In rice, the SEP (sepallata)- and AGL6-group MADS-box genes accelerate heading date when overexpressed (Chung *et al.*, 1994; Jeon *et al.*, 2000c; Kang and An, 1997; Kang *et al.*, 1997). However, antisense transgenic plants do not show significantly delayed heading dates (Jeon *et al.*, unpublished results). Therefore, the effect of those genes on flowering time is considered to be indirect or redundant (Jang *et al.*, 2002).

Sequence analyses have revealed that the rice genome contains not only the SOC1/AGL20- and SVP-group MADS-box genes but also the *FCA* and *LUMINIDEPENDENS* homologs, which may be involved in an autonomous pathway (Goff *et al.*, 2002; Izawa *et al.*, 2003; Lee *et al.*, 2003). In this study, we have identified a knockout (KO) mutant of *O. sativa MADS50 (OsMADS50)* from T-DNA-tagging lines, and have elucidated its role as the SOC1/AGL20 ortholog in rice.

Results

Screening of flowering-time mutants in T-DNA-tagging lines

We previously reported the generation of 20 500 T-DNA insertional lines, using the Ti plasmid binary vector

pGA2144 (Jeon *et al.*, 2000b). For our forward genetic approach, the T₂ plants from 2933 lines were segregated in the field, and mutant lines that exhibited an alteration in flowering time were screened. We observed 25 lines (16 accelerated and 9 delayed) in which this deviation was more than 2 weeks from the average recorded for other transgenic plants.

Flanking sequence analyses of a late-flowering mutant line

Using inverse PCR, we isolated the DNA sequences flanking T-DNA from the flowering mutant lines (An *et al.*, 2003). The National Center for Biotechnology Information (NCBI) database was used to search for genes tagged by the T-DNA insertion. In line 0-153-43, which exhibited the late-flowering phenotype (Figure 1, right), the retrieved sequence was a MADS-box gene, *OsMADS50*, that showed 50.6% amino acid sequence identity with *SOC1/AGL20*, a flowering activator in *Arabidopsis* (Borner *et al.*, 2000; Lee *et al.*, 2000; Onouchi *et al.*, 2000). Among the 36 MIKC (MAPS, Intervening, Keratin-like, and C-terminal domains)-type MADS-box proteins present in rice (Lee *et al.*, 2003), *OsMADS50* is most homologous (60.8%) to *OsMADS56*. The *OsMADS50* protein also shares homology with maize *ZmMADS1*



Figure 1. Phenotype comparison between *OsMADS50* KO and WT plants. Photograph was taken when WT plant (W/W) flowered. The *OsMADS50* KO (T/T) plant had not bolted.

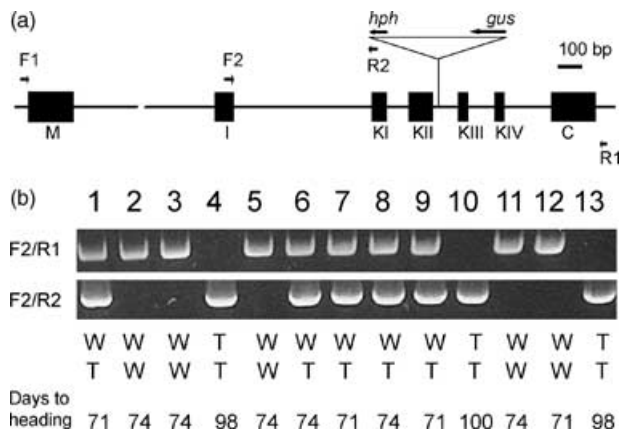


Figure 2. Schematic diagram of *OsMADS50*, position of T-DNA insertion, and genotyping of the *OsMADS50* KO progeny.

(a) Structure of *OsMADS50* and T-DNA insertion. Seven exons (filled boxes) and six introns (lines between the filled boxes) are shown. The M, I, K, and C regions are indicated below the exons. The K region consists of four exons, whereas the other regions comprise one exon each. T-DNA was inserted into the fourth intron. Arrows indicate primers: F1, forward primer at 5' UTR; F2, forward primer in the I region; R1, reverse primer in 3' UTR; R2, reverse primer in the hygromycin phosphotransferase (*hph*) gene in T-DNA.

(b) Genotyping of *OsMADS50* KO progeny. The F2 and R1 primers should have amplified 1.6-kb genomic DNA if no T-DNA insertion was made. If T-DNA was inserted, the length between two primers was too large to be amplified. The F2 and R2 primers should have amplified an approximately 2-kb band if T-DNA was inserted in the fourth intron. Samples 2, 3, 5, 11, and 12 amplified only the genomic DNA and therefore are the WT (W/W); samples 4, 10, and 13, which were harvested from late-flowering mutants, amplified only the 2-kb band and therefore are homozygous (T/T); the other samples showed amplification of both bands and are heterozygous (W/T). Days to heading after planting are indicated for each plant.

(75.2%), tobacco *TobMADS1* (51.1%), and mustard *SaMADSA* (50.0%) (Heuer *et al.*, 2001; Mandel *et al.*, 1994; Menzel *et al.*, 1996). Full-length protein sequences were aligned to calculate homologies.

This MADS-box gene is located on chromosome 3, and is composed of seven exons that encode a typical MIKC-type MADS-box protein (Figure 2a; Alvarez-Buylla *et al.*, 2000; Lee *et al.*, 2003). T-DNA was inserted into the fourth intron; the transcript direction of the β -glucuronidase (*gus*) gene in the T-DNA was opposite to that of *OsMADS50* (Figure 2a). We determined the genotypes of T₂ plants via PCR, using the primers located in *OsMADS50* and T-DNA. Among 13 plants, 3 were homozygous for the T-DNA insertion (Figure 2b, samples 4, 10, and 13). All of these plants flowered about 99 days after planting, whereas the other T₂ plants, being either heterozygous or wild-type (WT) segregants, flowered about 73 days after planting.

Suppression of *OsMADS50* causes late flowering

To confirm that the late-flowering phenotype is caused by the suppression of *OsMADS50* gene expression, we generated transgenic plants expressing RNAi constructs of the gene (Figure 3a). Among 82 T₁ plants, 76 manifested

the delayed flowering phenotype (Figure 3b). The majority of the plants flowered 1–2 months later than the WT and transgenic controls; eight did not flower until 140 days after planting.

RNA gel blot analysis showed that high levels of the *OsMADS50* RNAi transcript were present in the transgenic plants that displayed the late-flowering phenotype (Figure 4a). As expression was so high, it was difficult to visualize the endogenous *OsMADS50* transcript levels in the blots. Therefore, RT-PCR analyses were employed to verify suppression of *OsMADS50* expression (Figure 4b). In the late-flowering transgenic plants, transcript levels (confirmed by forward 1 (F1)/reverse 1 (R1) primers) were significantly reduced. These results indicate that reducing the expression of *OsMADS50* results in late flowering of rice plants. The degree of the late flowering was proportional to the RNAi levels as the RNAi plants 9 and 10 flowered later than the plants 6–8.

In addition to the late-flowering phenotype, transgenic plants carried more elongated internodes (Figure 3c), with the WT and transgenic control plants possessing five (38.1% and 47.4%, respectively) to six (52.4 and 47.4%, respectively). In contrast, most of the *OsMADS50* RNAi plants carried six (23.5%) to seven (62.7%) elongated internodes, but with some (13.7%) bearing as many as eight.

Ectopic expression of *OsMADS50* causes early flowering

To further study the functional roles of *OsMADS50* in flowering time, transgenic rice plants that overexpressed the sense constructs of the gene were generated. We used the maize ubiquitin (*ubi*) promoter to drive constitutive expression (Figure 5a). When transformed calli were transferred onto shoot induction media, about 20% developed into the structures that resembled floral organs, e.g. palea/lemma (Figure 5b), stigmas (Figure 5b), stamens (Figure 5c), ovaries (Figure 5c), and panicles (Figure 5d). We occasionally observed a spikelet containing all the floral organs. Although approximately 80% of the calli developed into shoots, two-thirds of those displayed the phenotype of extreme dwarfism and defective growth of the leaf blade (Figure 5e). These plants eventually died. The remaining one-third of the shoots differentiated into normal plants. Some then flowered earlier than the controls while others flowered at a time same as that for WT plants.

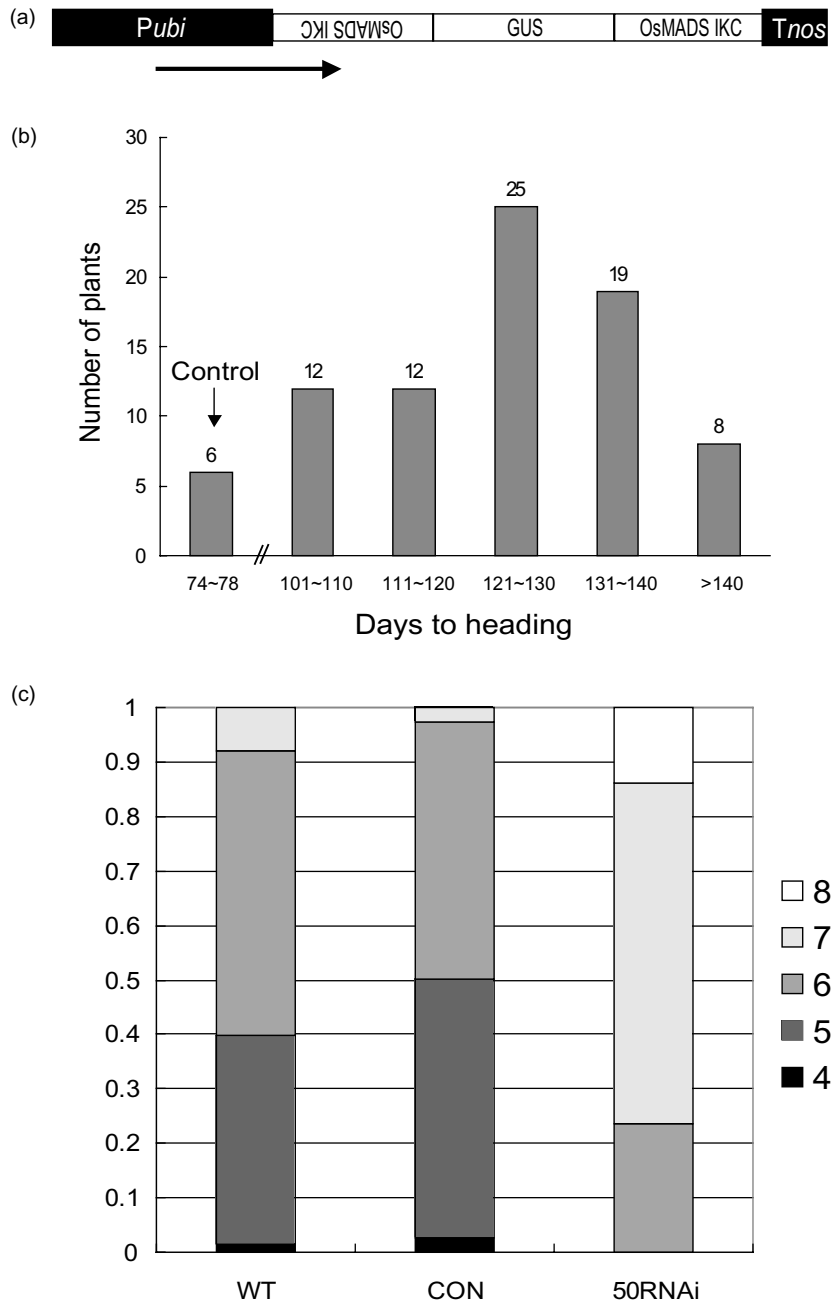
To examine whether the floral organ-like structures observed from the transgenic calli were indeed reproductive, we determined the transcript levels of the flower-specific MADS-box genes *OsMADS3* and *OsMADS4*. The former is the C function gene; the latter, the B function gene (Chung *et al.*, 1995; Kang *et al.*, 1995, 1998; Kyojuka *et al.*, 2000). These genes were expressed specifically in the floral organs that developed from the *ubi:OsMADS50* transgenic calli, indicating that they were authentic (Figure 5f).

Figure 3. Schematic diagram of *OsMADS50* RNAi construct and the phenotypes of the transgenic plants expressing the RNAi construct.

(a) *OsMADS50* RNAi construct, with GUS spacer inserted between two IKC regions of *OsMADS50*. The construct was inserted between the maize *ubi* promoter (*Pubi*) and the *nos* terminator (*Tnos*).

(b) Frequency distribution of days to heading in the *OsMADS50* RNAi T₁ transgenic plants, i.e. the number of days required for flowering from the time of transplanting. WT and other transgenic control plants flowered 74–78 days after planting. Nine lines did not flower until 140 days after planting. Broken bar indicates large gaps between two X-axis values.

(c) Proportion of elongated internode numbers in *OsMADS50* RNAi plants and WT controls. Values are averages from 63 WT, 152 transgenic control plants, and 102 *OsMADS50* RNAi stems. Y-axis indicates relative ratio of stems having elongated internodes, ranging from four to eight.



Expression patterns of *OsMADS50*

RNA gel blot analysis revealed that *OsMADS50* was variably expressed in most organs (Figure 6a). This gene was detected at a low level during the seedling stage, with transcripts increasing as the plant matured. In young panicles, expression was initially low, and continued to decline as these organs matured. RT-PCR analysis of leaves at four developmental stages confirmed the RNA gel blot analysis (Figure 6b). *OsMADS50* transcript was detected at all four stages, with the expression level slightly increasing in 49-day-old plants compared with those that were 20 days

old. The transcripts of *Hd3a*, *OsMADS14*, *OsMADS15*, and *OsMADS18* increased gradually, reaching a maximum at 80 days.

Relationship of *OsMADS50* with other flowering-time regulators

Genetic studies have shown that *OsMADS50* plays a major role in controlling flowering time in rice. Moreover, expression analyses have revealed that this gene acts early in plant development, being more abundantly expressed in the vegetative organs but decreasing to a very low level

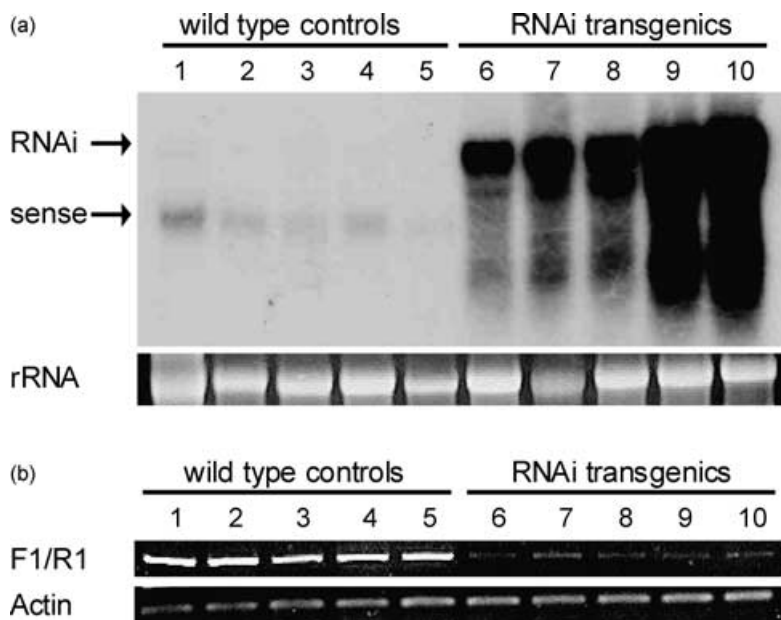


Figure 4. Endogenous level of *OsMADS50* in *OsMADS50* RNAi plants.

(a) RNA gel blot analysis for *OsMADS50* and *OsMADS50* RNAi transcripts in transgenic plants expressing *OsMADS50* RNAi constructs. Five WT plants (1–5) and five independent transgenic plants (6–10) were examined. The transgenic plants 6, 7, 8, 9, and 10 flowered 111, 115, 115, 130, and 135 days after planting, respectively. The rRNA level was observed as a control (bottom). RNAi, *OsMADS50* RNAi transcript; and sense, endogenous *OsMADS50* transcript. (b) RT-PCR analyses for *OsMADS50* and *OsMADS50* RNAi transcripts in WT plants (1–5) and transgenic plants (6–10). Identical RNA, isolated for RNA gel blot analysis, was reverse transcribed to synthesize cDNA. The primer pair used for detecting full-length *OsMADS50* was F1/R1, indicated in Figure 2(a). *Actin* was used as a control. PCR cycles for amplifying *OsMADS50* and *actin* were 26 and 23, respectively.

during the formation of floral organs. To further investigate the role of *OsMADS50*, we performed RT-PCR analyses for *Hd1*, *Hd3a*, and *OsGI*, all of which control flowering time in the photoperiod pathway (Hayama *et al.*, 2002, 2003; Kojima *et al.*, 2002; Yano *et al.*, 2000). We also examined four MADS-box genes (*OsMADS1*, *OsMADS14*, *OsMADS15*, and *OsMADS18*) that appear to be involved as well (Chung *et al.*, 1994; Jeon *et al.*, 2000a,c). In our experiments, expression of *Hd1* and *OsGI* was not changed in the *OsMADS50* KO plants (Figure 7). Interestingly, the *Hd3a* transcript was not detectable in the *OsMADS50* KO mutant, suggesting that *Hd3a* is downstream of *OsMADS50*. Expression levels of all the MADS-box genes were significantly decreased in the *OsMADS50* KO mutant plants, although the degree of reduction for *OsMADS18* transcript was not as significant as that for the other genes.

We also tested the expression levels of regulatory genes in the leaves of regenerating *ubi:OsMADS50* plants (Figure 7c). These plants flowered a few weeks after being transferred to the regeneration media. In contrast to the KO plants, the levels of *OsMADS14* and *OsMADS18* transcripts were increased in the *ubi:OsMADS50* plants, while those of *OsMADS1*, *OsGI*, and *Hd1* were not significantly changed. Expression levels of *OsMADS15* and *Hd3a* were too low to determine any changes in their transcripts.

Discussion

OsMADS50 is the putative ortholog of SOC1/AGL20

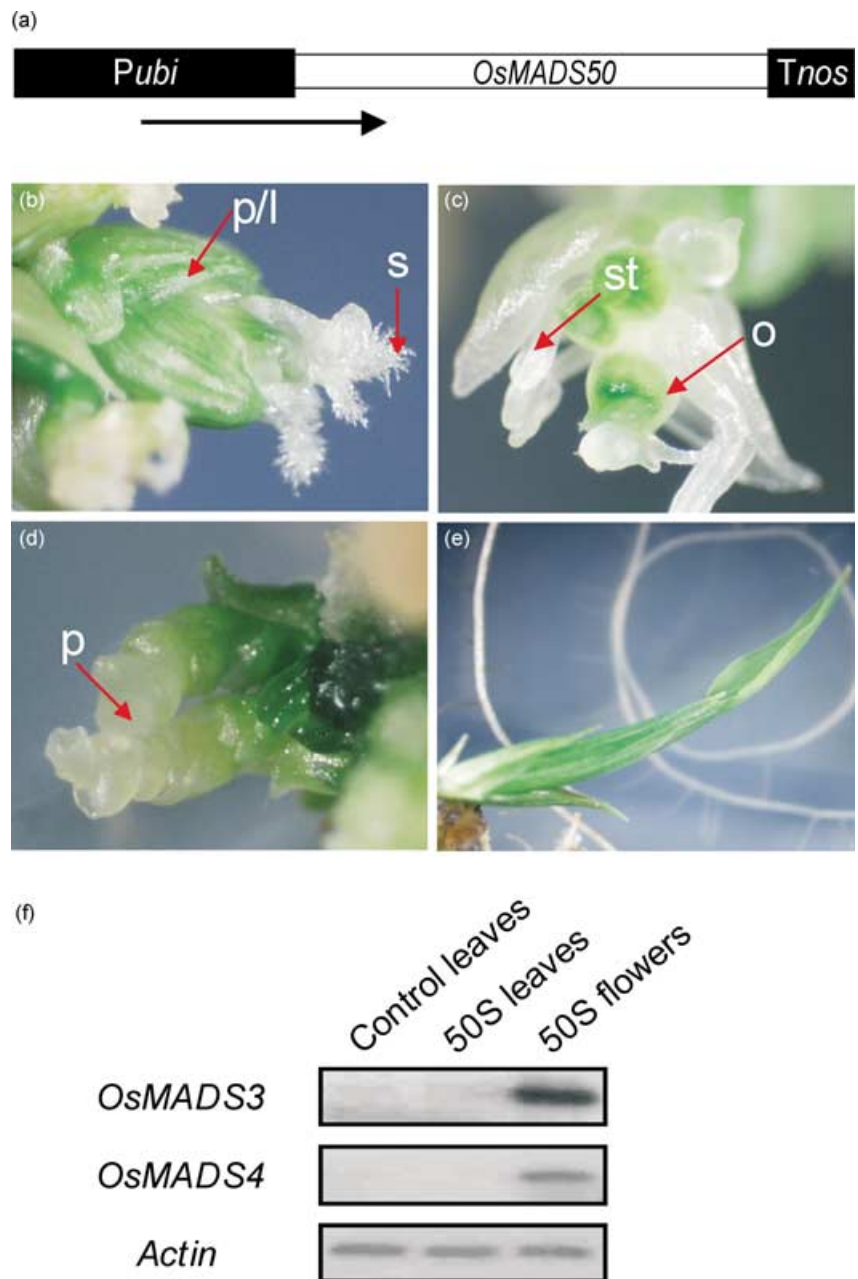
SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 is an important flowering activator, integrating autonomous and vernalization-, photoperiod-, and

GA-dependent flowering pathways in *Arabidopsis* (Borner *et al.*, 2000; Lee *et al.*, 2000; Moon *et al.*, 2003; Onouchi *et al.*, 2000). In rice, *OsMADS50* is the protein most homologous with SOC1/AGL20, showing 50.6% identity, whereas *OsMADS56* has 48.3% identity. Genome-wide phylogenetic analysis showed that *OsMADS50* is clustered with the SOC1-group MADS-box proteins (Lee *et al.*, 2003). Expression profiles are similar between *OsMADS50* and SOC1/AGL20. In *Arabidopsis*, SOC1/AGL20 is expressed in young leaves, with its level gradually increasing during vegetative growth (Lee *et al.*, 2000; Samach *et al.*, 2000). Likewise, we showed in this study that *OsMADS50* was most highly detected in leaves, and that expression was stronger in mature leaves than in young leaves or panicles. This result is also similar to those reported by Tadege *et al.* (2003). Both SOC1/AGL20 and *OsMADS50* are expressed in most organs, including leaves, roots, and inflorescences (Borner *et al.*, 2000; Lee *et al.*, 2000). Therefore, their sequence homology and expression patterns suggest that these two genes share similar biological functions. As expected, our functional analyses of *OsMADS50* elucidated that the gene is a flowering activator, causing early flowering when overexpressed and delayed flowering when suppressed. Our functional analysis of *OsMADS50* together with our previous phylogenetic analysis suggests that the mechanism of flowering activation by *OsMADS50/AGL20*-group genes was already established before the eudicot/monocot split and has been maintained in each of lineages.

OsMADS50 is a flowering activator involved in phase transition

Constitutive expression of *OsMADS50* directed early flowering at the callus stage. The transgenic calli developed

Figure 5. Analyses of *ubi:OsMADS50* plants. (a) Schematic diagram of *OsMADS50* sense construct. *Pubi*, maize *ubi* promoter; and *Tnos*, nos terminator. (b) Regenerated shoots with palea/lemma (p/l)- and stigma (s)-like structures. (c) Regenerated shoots with stamen (st)- and ovary (o)-like structures. (d) Regenerated shoots with panicle (p)-like structures. (e) Regenerated shoots displaying dwarfism and defective growth of leaf blade. (f) Expression portraits of *OsMADS3* and *OsMADS4* in the floral organ-like structures. Control leaves, leaves transformed with empty vector; 50S leaves, transgenic leaves overexpressing *OsMADS50*; and 50S flowers, floral organ-like structures overexpressing *OsMADS50*. *Actin* was used as a control.



floral organ-like structures that resembled palea/lemma, stigmas, and ovaries. We confirmed that they were genuine floral organs by measuring transcripts of the floral organ-specific MADS-box genes *OsMADS3* and *OsMADS4*. The former is an *AGAMOUS* (*AG*) ortholog that determines stamen and carpel development (Kang *et al.*, 1995, 1998; Kozuka *et al.*, 2000). The latter is the *PISTILLATA* (*PI*) ortholog involved in identifying lodicules and stamens (Kang *et al.*, 1998; Lee *et al.*, 2003; Moon *et al.*, 1999).

Our hypothesis for the function of *OsMADS50* as a flowering activator was supported genetically by the *OsMADS50* KO plants that showed the late-flowering phe-

notype. This trait was inherited in the next generation and was correlated with a reduction in the level of transcript. These observations were then confirmed with the *OsMADS50* RNAi plants. About 93% of the regenerated plants that overexpressed the *OsMADS50* RNAi construct showed significant late-flowering phenotypes, while also exhibiting a longer vegetative phase and developing more elongated internodes compared with our control plants. Internode elongation is a morphological marker of floral transition in rice; the increased number of elongated internodes was likely caused by a delayed phase transition in the transgenic plants. Some of the *OsMADS50* RNAi plants

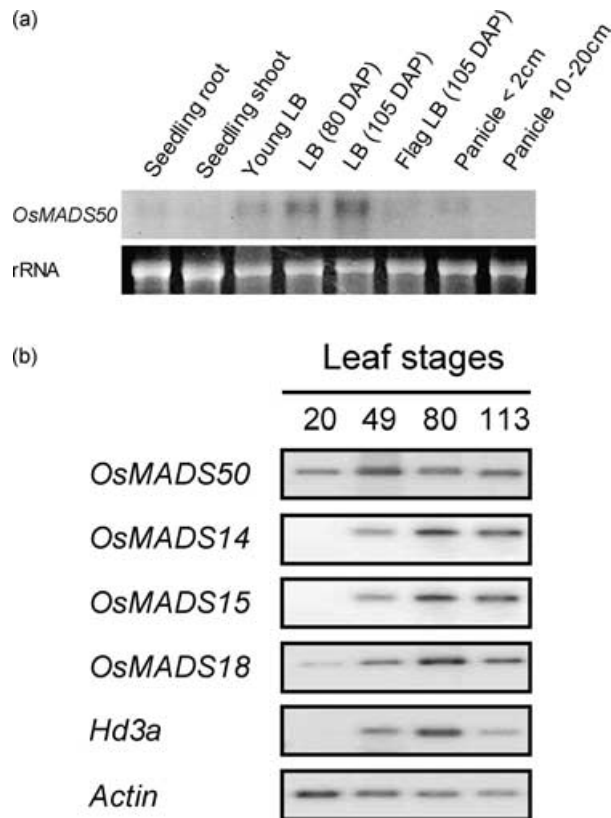


Figure 6. Expression profiles of *OsMADS50* and other flowering regulators. (a) RNA gel blot analysis, with 15 µg of total RNA used in each sample, and the KC region of *OsMADS50* serving as a probe. At bottom are the control ribosomal RNAs. From left, seedling roots and shoots at 7 days after germination, young leaf blades (LBs) at the 9–10 leaf stage (35 days after planting (DAP)), LBs 80 DAP, LBs 105 DAP, flag LBs 105 DAP, panicles < 2 cm long, and panicles between 10 and 20 cm. The plants flowered 90 DAP. (b) RT-PCR analyses of the putative flowering-time regulators at various developmental stages. WT plants were grown under SD conditions (10 h light/14 h dark, 30°C) in the growth chamber, and LBs were sampled 20, 49, 80, and 113 DAP. All samples were harvested 6 h after the light was turned on. Days to flowering were 87.

flowered later than *OsMADS50* KO plants. This may be caused by inhibition of other MADS-box gene expression. *OsMADS50* is closely related to *OsMADS56*. Therefore, we measured the transcript level of *OsMADS56* in the mature leaves of two *OsMADS50* RNAi plants, which flowered >135 days after planting. The experiment showed that expression of *OsMADS56* was not suppressed by *OsMADS50* RNAi (data not shown). However, it might be possible that expression of other MADS-box genes was suppressed. Alternatively, this may be because of different growing conditions. Further studies under controlled environmental conditions are needed.

Tadege *et al.* (2003) have also suggested that *OsMADS50* is a flowering activator because (i) constitutive expression of the gene in *Arabidopsis* causes early flowering and (ii)

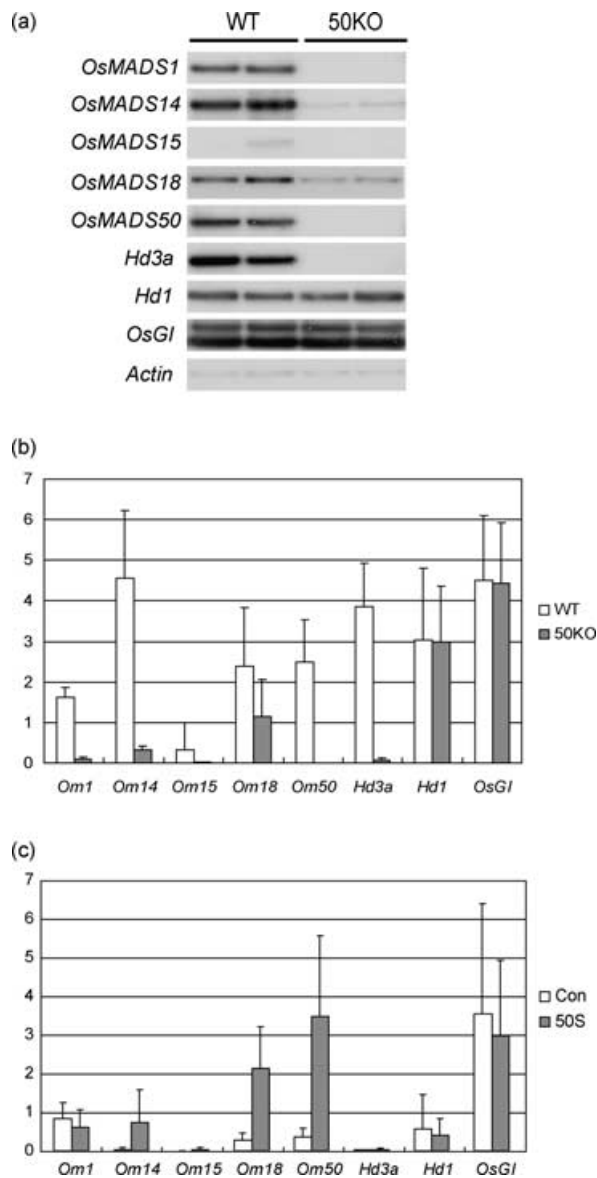


Figure 7. Expression profiles of MADS-box genes and photoperiod pathway genes in *OsMADS50* KO and *ubi:OsMADS50* leaves. (a) RT-PCR analyses in *OsMADS50* KO leaves. At 73 days after planting, leaf blades were harvested 2 h before sunset from KO plants. After RT-PCR, DNA gel blot analyses were performed with specific probes. Two independent WT segregants and two independent *OsMADS50* KO plants were examined. (b) Schematic representation of RT-PCR results in *OsMADS50* KO plants. DNA band intensity was measured and normalized against the *actin* transcript level. Results are an average of three independent experiments for each plant. Average values of two WT and two KO plants are represented. Bars, SDs. (c) Expression profiles in *ubi:OsMADS50* plants. Leaf blades were sampled from transgenic plants overexpressing *OsMADS50* and control plants, and were assayed for transcript levels of genes by quantitative RT-PCR analyses. Data are average of two to three independent samples. Con, T₁ control transformed with empty vector; and 50S, *ubi:OsMADS50* leaves. Bars, SDs. PCR primers and number of cycles for amplification of each gene are listed in Table 1.

the upregulation of *OsSOC1* at the onset of floral initiation is delayed in transgenic rice plants that overexpress *Arabidopsis FLC*. Their hypothesis is consistent with our results reported here.

OsMADS50 is upstream of *OsMADS1*, *OsMADS14*, *OsMADS15*, and *OsMADS18*

The *OsMADS1* gene is thought to be involved in the transition from the vegetative to the reproductive phase because its overexpression causes early flowering (Jeon *et al.*, 2000c). Overexpression of *OsMADS14* also promotes early flowering, and its effect is more severe (Jeon *et al.*, 2000c). Our transgenic calli produced floral organs, as observed from the *OsMADS50*-overexpressing transformants. Therefore, to study the relationship between *OsMADS50* and these MADS-box genes, we analyzed their transcripts in the *OsMADS50* KO and *ubi:OsMADS50* plants. Levels of *OsMADS14* were significantly decreased in the former but dramatically increased in the latter, indicating that *OsMADS14* is located downstream of *OsMADS50* in the floral induction pathway. Altering the expression of *OsMADS50* also affected the transcript levels of two other A-group MADS-box genes, *OsMADS15* and *OsMADS18*, suggesting that they are also downstream of *OsMADS50*. In *Arabidopsis*, the A-group gene *FRUITFUL* (*FUL*) acts to promote flowering (Ferrandiz *et al.*, 2000). Similarly, the rice A-group MADS-box genes may regulate flowering time, acting downstream from *OsMADS50*. Finally, we noted that the transcript level of *OsMADS1* was also affected in our *OsMADS50* KO plants.

Expression analyses of the MADS-box genes in WT plants also demonstrated that *OsMADS50* acts upstream of the other MADS-box genes. While a considerable amount of *OsMADS50* transcript was detectable in seedling shoots and young leaves, transcripts of the other MADS-box genes were not detectable or only weakly detected in these young vegetative organs (Figure 7; Chung *et al.*, 1994; Jang *et al.*, 2002; Tadege *et al.*, 2003). Among these genes, *OsMADS14* appears to be the most immediately downstream of *OsMADS50* because it is strongly expressed in vegetative organs and because its overexpression causes extremely early flowering, a phenotype similar to that found with *ubi:OsMADS50* plants (Jeon *et al.*, 2000c). It must still be investigated whether *OsMADS50* directly induces *OsMADS14* transcription by interacting with its promoter region and whether other factors also control *OsMADS14*.

OsMADS50 is epistatic to *Hd3a*

Three genes, *OsGI*, *Hd1*, and *Hd3a*, play major roles in the photoperiodic flowering pathway, corresponding to *Arabidopsis* *GI*, *CO*, and *FT*, respectively (Hayama *et al.*, 2003; Izawa *et al.*, 2002; Simpson, 2003). Although the overall

epistatic relationship is the same between the two model systems of *Arabidopsis* and rice, different regulatory mechanisms exist. Under LD conditions, flowering is promoted in *Arabidopsis* when *CO* activates *FT*, whereas delayed flowering in rice results from the repression of *Hd3a* by *Hd1* (Hayama *et al.*, 2003; Simpson, 2003). The photoperiodic pathway of *Arabidopsis* is integrated into other pathways through *SOC1/AGL20*, a direct target of *CO* (Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000). Therefore, this raises the possibility that *Hd1* is an upstream regulator of *OsMADS50*. This theory can be evaluated by examining the expression of *OsMADS50* and other MADS-box genes in *Hd1* mutants.

Our data showed that expression of *OsGI* and *Hd1* was not affected by *OsMADS50*, thereby indicating that they act upstream of or in parallel with *OsMADS50*. In contrast, *Hd3a* was downregulated in the *OsMADS50* KO plants, implying that *OsMADS50* is epistatic to *Hd3a*. However, the *Hd3a* transcript level was not altered in the *OsMADS50*-overexpressing plants. Therefore, we believe that an additional factor may be needed for the induction.

In *Arabidopsis*, *FLC* represses *SOC1/AGL20* antagonistically with *CO* via separate promoter motifs (Hepworth *et al.*, 2002). However, no *FLC* homolog is found in the rice genome (Goff *et al.*, 2002; Izawa *et al.*, 2003). Therefore, other repressors may exist in rice. Whether photoperiodic regulators, such as *OsGI* and *Hd1*, act as the negative component or other factors play that role should be investigated. For example, rice homologs of *FCA* and *LUMINIDEPENDENS* (Goff *et al.*, 2002; Izawa *et al.*, 2003), which activate the level of *SOC1/AGL20* on the autonomous pathway by repressing *FLC* transcription (Lee *et al.*, 2000; Michaels and Amasino, 1999; Quesada *et al.*, 2003; Samach *et al.*, 2000; Sheldon *et al.*, 1999), can be evaluated for their possible roles in controlling *OsMADS50* expression and flowering time.

Experimental procedures

Plant materials

The T-DNA-tagging lines used here were from *O. sativa* var. japonica cv. Dongjin (Jeon *et al.*, 2000b). Fifteen T₂ plants were grown for 1 month in the greenhouse, then transplanted to a paddy field until maturation.

Vector construction and transformation

To isolate the full-length cDNA clone of *OsMADS50* (GenBank Accession number AB003328; Shinozuka *et al.*, 1999) by nested PCR, we used the following four primers: F1, 5'-ATCAAGCTT-TACGGCCAAACCCTACAGC-3'; F2, 5'-ATCAAGCTTGTGGTTCATCGGCGATCG-3'; R1, 5'-TTGGGTACCGATGGGTAGTGGAGT-CTGC-3'; and R2, 5'-TTGGGTACCGAGATCCAGCTTATTCTGG-3'. These primers contained the restriction enzyme sites *HindIII*

Table 1 Primers and PCR cycles used in RT-PCR analyses

Gene	Forward primer	Reverse primer	PCR cycles 50 KO ^a	PCR cycles 50 S ^b
<i>OsMADS1</i>	tccatattgtcctggcaagat	aagagagcagcgcactt	28	32
<i>OsMADS14</i>	tctatgcagaaaaggtcctt	ggacgaagccaaaatacac	36	36
<i>OsMADS15</i>	gctcttatttcagctgaa	tcatatgtagcctgtagg	36	36
<i>OsMADS18</i>	ccaaactggatgcacttcag	atcaatatcgctggaagatg	23	32
<i>OsMADS50</i>	aaagctgacgctgatggttg	ttgggtaccgagatccagcttattcctgg	23	26
<i>OsGl</i>	tggagaaaggttggtgatgc	gatagacggcacttcagcagat	23	26
<i>Hd1</i>	ttctcctctccaaagattcc	catagcctttctgtttca	28	26
<i>Hd3a</i>	atggccggaagtggcagggac	atcgatcgggatcatcgtagg	36	36
<i>Actin</i>	gtatccatgatgactacatacaact	tactcagccttgccaatccaca	23	26

^aPCR cycles used for *OsMADS50* KO plants and WT segregants.

^bPCR cycles used for *ubi:OsMADS50* plants and WT controls.

and *Asp718* for subsequent cloning. The PCR product was first cloned into pBluescript SK- (Stratagene, La Jolla, CA, USA). Afterward, the cDNA was subcloned into the pGA1611 binary vector between the maize *ubi* promoter and the nopaline synthase (*nos*) terminator for sense construct (Kim *et al.*, 2003; Lee *et al.*, 1999). For the RNAi construct, the 1.2-kb GUS fragment was inserted between two IKC regions of *OsMADS50*, inversely oriented. This construct was cloned in pBluescript SK-, then subcloned into the pGA1611 binary vector under the *ubi* promoter. Rice transformation was performed according to the *Agrobacterium*-mediated methods described by Jeon *et al.* (1999) and Lee *et al.* (1999). All transgenic rice plants were grown in the greenhouse, then transferred to a confined paddy field.

RNA gel blot analysis and RT-PCR analysis

Total RNAs were isolated from fresh tissues with an RNA isolation kit (Tri Reagent; MRC Inc., Cincinnati, OH, USA). They were then fractionated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with a ³²P-labeled probe. All procedures for the blot analysis were performed as described by Kang *et al.* (1998). For the templates in RT-PCR analysis, first-strand cDNA was synthesized from 2 µg of total RNAs, using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The primers for the *OsGl*, *Hd1*, and *actin* genes were designed as reported previously (Hayama *et al.*, 2003; Takakura *et al.*, 2000; Yano *et al.*, 2000). In addition, gene-specific primers were designed for each rice MADS-box gene (Table 1). All PCR experiments were repeated more than three times, and PCR cycles were adjusted not to amplify target cDNA until saturation.

After PCR amplification, the products were separated on a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with a ³²P-labeled probe. The intensity of the signal was quantified with a PhosphorImager (FLA-2000; Fujifilm, Tokyo, Japan), and normalized against *actin*.

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References

- Alvarez-Buylla, E.R., Pelaz, S., Liljegren, S.J., Gold, S.E., Burgeff, C., Ditta, G.S., Ribas de Pouplana, L., Martinez-Castilla, L. and Yanofsky, M.F. (2000) An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. *Proc. Natl. Acad. Sci. USA*, **97**, 5328–5333.
- An, S., Park, S., Jeong, D.H. *et al.* (2003) Generation and analyses of end-sequence database for T-DNA tagging lines in rice. *Plant Physiol.* **133**, 2040–2047.
- Borner, R., Kampmann, G., Chandler, J., Gleissner, R., Wisman, E., Apel, K. and Melzer, S. (2000) A MADS domain gene involved in the transition to flowering in *Arabidopsis*. *Plant J.* **24**, 591–599.
- Chung, Y.Y., Kim, S.R., Finkel, D., Yanofsky, M.F. and An, G. (1994) Early flowering and reduced apical dominance result from ectopic expression of a rice MADS box gene. *Plant Mol. Biol.* **26**, 657–665.
- Chung, Y.Y., Kim, S.R., Kang, H.G., Noh, Y.S., Min, C.P., David, F. and An, G. (1995) Characterization of two rice MADS box genes homologous to *GLOBOSA*. *Plant Sci.* **109**, 45–56.
- Dung, L.V., Inukai, T. and Sano, Y. (1998) Dissection of a major QTL for photoperiod sensitivity in rice: its association with a gene expressed in an age-dependent manner. *Theor. Appl. Genet.* **97**, 714–720.
- Ferrandiz, C., Gu, Q., Martienssen, R. and Yanofsky, M.F. (2000) Redundant regulation of meristem identity and plant architecture by *FRUITFULL*, *APETALA1* and *CAULIFLOWER*. *Development*, **127**, 725–734.
- Goff, S.A., Ricke, D., Lan, T.H. *et al.* (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science*, **296**, 92–100.
- Hartmann, U., Hohmann, S., Nettesheim, K., Wisman, E., Saedler, H. and Huijser, P. (2000) Molecular cloning of *VSP*: a negative regulator of the floral transition in *Arabidopsis*. *Plant J.* **21**, 351–360.
- Hayama, R., Izawa, T. and Shimamoto, K. (2002) Isolation of rice genes possibly involved in the photoperiodic control of flowering by a fluorescent differential display method. *Plant Cell Physiol.* **43**, 494–504.
- Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature*, **422**, 719–722.

- Hepworth, S.R., Valverde, F., Ravenscroft, D., Mouradov, A. and Coupland, G. (2002) Antagonistic regulation of flowering-time gene *SOC1* by *CONSTANS* and *FLC* via separate promoter motifs. *EMBO J.* **21**, 4327–4337.
- Heuer, S., Hansen, S., Bantini, J., Brettschneider, R., Kranz, E., Lorz, H. and Dresselhaus, T. (2001) The maize MADS box gene *ZmMADS3* affects node number and spikelet development and is co-expressed with *ZmMADS1* during flower development, in egg cells, and early embryogenesis. *Plant Physiol.* **127**, 33–45.
- Izawa, T., Oikawa, T., Tokutomi, S., Okuno, K. and Shimamoto, K. (2000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). *Plant J.* **22**, 391–399.
- Izawa, T., Oikawa, T., Sugiyama, N., Tanisaka, T., Yano, M. and Shimamoto, K. (2002) Phytochrome mediates the external light signal to repress *FT* orthologs in photoperiodic flowering of rice. *Genes Dev.* **16**, 2006–2020.
- Izawa, T., Takahashi, Y. and Yano, M. (2003) Comparative biology comes into bloom: genomic and genetic comparison of flowering pathways in rice and *Arabidopsis*. *Curr. Opin. Plant Biol.* **6**, 113–120.
- Jang, S., An, K., Lee, S. and An, G. (2002) Characterization of tobacco MADS-box genes involved in floral initiation. *Plant Cell Physiol.* **43**, 230–238.
- Jeon, J.S., Chung, Y.Y., Lee, S., Yi, G.H., Oh, B.G. and An, G. (1999) Isolation and characterization of an anther-specific gene, *RA8*, from rice (*Oryza sativa* L.). *Plant Mol. Biol.* **39**, 35–44.
- Jeon, J.S., Jang, S., Lee, S. et al. (2000a) *Leafy hull sterile1* is a homeotic mutation in a rice MADS box gene affecting rice flower development. *Plant Cell*, **12**, 871–884.
- Jeon, J.S., Lee, S., Jung, K.H. et al. (2000b) T-DNA insertional mutagenesis for functional genomics in rice. *Plant J.* **22**, 561–570.
- Jeon, J.S., Lee, S., Jung, K.H., Yang, W.S., Yi, G.H., Oh, B.G. and An, G. (2000c) Production of transgenic rice plants showing reduced heading date and plant height by ectopic expression of rice MADS-box genes. *Mol. Breed.* **6**, 581–592.
- Kang, H.G. and An, G. (1997) Isolation and characterization of a rice MADS box gene belonging to the *AGL2* gene family. *Mol. Cells*, **7**, 45–51.
- Kang, H.G., Noh, Y.S., Chung, Y.Y., Costa, M.A., An, K. and An, G. (1995) Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. *Plant Mol. Biol.* **29**, 1–10.
- Kang, H.G., Jang, S., Chung, J.E., Cho, Y.G. and An, G. (1997) Characterization of two rice MADS box genes that control flowering time. *Mol. Cells*, **7**, 559–566.
- Kang, H.G., Jeon, J.S., Lee, S. and An, G. (1998) Identification of class B and class C floral organ identity genes from rice plants. *Plant Mol. Biol.* **38**, 1021–1029.
- Kim, S.-R., Lee, S., Kang, H.G., Jeon, J.-S., Kim, K.-M. and An, G. (2003) A complete sequence of the pGA1611 binary vector. *J. Plant Biol.* **46**, 211–214.
- Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T. and Yano, M. (2002) *Hd3a*, a rice ortholog of the *Arabidopsis FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant Cell Physiol.* **43**, 1096–1105.
- Kyozuka, J., Kobayashi, T., Morita, M. and Shimamoto, K. (2000) Spatially and temporally regulated expression of rice MADS box genes with similarity to *Arabidopsis* class A, B and C genes. *Plant Cell Physiol.* **41**, 710–718.
- Lee, S., Jeon, J.S., Jung, K.H. and An, G. (1999) Binary vector for efficient transformation of rice. *J. Plant Biol.* **42**, 310–316.
- Lee, H., Suh, S.S., Park, E., Cho, E., Ahn, J.H., Kim, S.G., Lee, J.S., Kwon, Y.M. and Lee, I. (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes Dev.* **14**, 2366–2376.
- Lee, S., Kim, J., Son, J.S. et al. (2003) Systematic reverse genetic screening of T-DNA tagged genes in rice for functional genomic analyses: MADS-box genes as a test case. *Plant Cell Physiol.* **44**, 1403–1411.
- Li, Z.K., Pinson, S.R.M., Stansel, J.W. and Park, W.D. (1995) Identification of quantitative trait loci (QTLs) for heading date and plant height in cultivated rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **91**, 374–381.
- Lin, H.X., Qian, H.R., Xiong, Z.M., Min, S.K. and Zhen, K.L. (1996) Mapping of major genes and minor genes for heading date in several rice varieties (*Oryza sativa* L.). *Chin. J. Genet.* **23**, 107–114.
- Lin, S.Y., Sasaki, T. and Yano, M. (1998) Mapping quantitative trait loci controlling seed dormancy and heading date in rice, *Oryza sativa* L. using backcross inbred lines. *Theor. Appl. Genet.* **96**, 997–1003.
- Lin, H.X., Yamamoto, T., Sasaki, T. and Yano, M. (2000) Characterization and detection of epistatic interactions of three QTLs, *Hd1*, *Hd2* and *Hd3*, controlling heading date in rice using nearly isogenic lines. *Theor. Appl. Genet.* **101**, 1021–1028.
- Lin, H.X., Ashikari, M., Yamanouchi, U., Sasaki, T. and Yano, M. (2002) Identification and characterization of a quantitative trait locus, *Hd9*, controlling heading date in rice. *Breed. Sci.* **52**, 35–41.
- Maheswaran, M., Ning Huang Sreerangasamy, S.R. and McCouch, S.R. (2000) Mapping quantitative trait loci associated with days to flowering and photoperiod sensitivity in rice (*Oryza sativa* L.). *Mol. Breed.* **6**, 145–155.
- Mandel, T., Lutziger, I. and Kuhlemeier, C. (1994) A ubiquitously expressed MADS-box gene from *Nicotiana tabacum*. *Plant Mol. Biol.* **25**, 319–321.
- Menzel, G., Apel, K. and Melzer, S. (1996) Identification of two MADS box genes that are expressed in the apical meristem of the long-day plant *Sinapis alba* in transition to flowering. *Plant J.* **9**, 399–408.
- Michaels, S.D. and Amasino, R.M. (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell*, **11**, 949–956.
- Michaels, S.D., Ditta, G., Gustafson-Brown, C., Pelaz, S., Yanofsky, M. and Amasino, R.M. (2003) *AGL24* acts as a promoter of flowering in *Arabidopsis* and is positively regulated by vernalization. *Plant J.* **33**, 867–874.
- Monna, L., Lin, H.X., Kojima, S., Sasaki, T. and Yano, M. (2002) Genetic dissection of a genomic region for a quantitative trait locus, *Hd3*, into two loci, *Hd3a* and *Hd3b*, controlling heading date in rice. *Theor. Appl. Genet.* **104**, 772–778.
- Moon, Y.H., Jung, J.Y., Kang, H.G. and An, G. (1999) Identification of a rice *APETALA3* homologue by yeast two-hybrid screening. *Plant Mol. Biol.* **40**, 167–177.
- Moon, J., Suh, S.S., Lee, H., Choi, K.R., Hong, C.B., Paek, N.C., Kim, S.G. and Lee, I. (2003) The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*. *Plant J.* **35**, 613–623.
- Mouradov, A., Cremer, F. and Coupland, G. (2002) Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell*, **14**, S111–S130.
- Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K. and Coupland, G. (2000) Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among *Arabidopsis* flowering-time genes. *Plant Cell*, **12**, 885–900.

- Poonyarit, M., Mackill, D.J. and Vergara, B.S. (1989) Genetics of photoperiod sensitivity and critical daylength in rice. *Crop Sci.* **29**, 647–652.
- Quesada, V., Macknight, R., Dean, C. and Simpson, G.G. (2003) Autoregulation of *FCA* pre-mRNA processing controls *Arabidopsis* flowering time. *EMBO J.* **22**, 3142–3152.
- Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. *Science*, **288**, 1613–1616.
- Sano, Y. (1992) Genetic comparisons of chromosome 6 between wild and cultivated rice. *Jpn. J. Breed.* **42**, 561–572.
- Scortecci, K.C., Michaels, S.D. and Amasino, R.M. (2001) Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. *Plant J.* **26**, 229–236.
- Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999) The *FLMADS* box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell*, **11**, 445–458.
- Shinozuka, Y., Kojima, S., Shomura, A., Ichimura, H., Yano, M., Yamamoto, K. and Sasaki, T. (1999) Isolation and characterization of rice MADS box gene homologues and their RFLP mapping. *DNA Res.* **6**, 123–129.
- Simpson, G.G. (2003) Evolution of flowering in response to day length: flipping the *CONSTANS* switch. *Bioessays*, **25**, 829–832.
- Tadege, M., Sheldon, C.C., Helliwell, C.A., Upadhyaya, N.M., Dennis, E.S. and Peacock, W.J. (2003) Reciprocal control of flowering time by *OsSOC1* in transgenic *Arabidopsis* and by *FLC* in transgenic rice. *Plant Biotechnol. J.* **1**, 361–369.
- Takahashi, Y., Shomura, A., Sasaki, T. and Yano, M. (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the alpha subunit of protein kinase CK2. *Proc. Natl. Acad. Sci. USA*, **98**, 7922–7927.
- Takakura, Y., Ito, T., Saito, H., Inoue, T., Komari, T. and Kuwata, S. (2000) Flower-predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L.). *Plant Mol. Biol.* **42**, 883–897.
- Tsai, K.H. (1995) Genetic analysis for heading time in wild rice strains. *Jpn. J. Genet.* **70**, 555–562.
- Xiao, J., Li, J., Yuan, L. and Tanksley, S.D. (1995) Dominance is the major genetic basis of heterosis in rice as revealed by QTL analysis using molecular markers. *Genetics*, **140**, 745–754.
- Yamamoto, T., Kuboki, Y., Lin, S.Y., Sasaki, T. and Yano, M. (1998) Fine mapping of quantitative trait loci *Hd-1*, *Hd-2*, and *Hd-3*, controlling heading date of rice, as single Mendelian factors. *Theor. Appl. Genet.* **97**, 37–44.
- Yamamoto, T., Lin, H.X., Sasaki, T. and Yano, M. (2000) Identification of heading date quantitative trait locus *Hd6* and characterization of its epistatic interactions with *Hd2* in rice using advanced backcross progeny. *Genetics*, **154**, 885–891.
- Yano, M., Harushima, Y., Nagamura, Y., Kurata, N., Minobe, Y. and Sasaki, T. (1997) Identification of quantitative trait loci controlling heading date in rice using a high-density linkage map. *Theor. Appl. Genet.* **95**, 1025–1032.
- Yano, M., Katayose, Y., Ashikari, M. et al. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell*, **12**, 2473–2484.
- Yano, M., Kojima, S., Takahashi, Y., Lin, H. and Sasaki, T. (2001) Genetic control of flowering time in rice, a short-day plant. *Plant Physiol.* **127**, 1425–1429.
- Yokoo, M. and Okuno, K. (1993) Genetic analysis of earliness mutations induced in the rice cultivar Norin 8. *Jpn. J. Breed.* **43**, 1–11.
- Yokoo, M., Kikuchi, F., Nakane, A. and Fujimaki, H. (1980) Genetic analyses of heading time in rice through its close linkage with blast resistance. *Jpn. J. Breed.* **27**, 123–130.
- Yu, H., Xu, Y., Tan, E.L. and Kumar, P.P. (2002) *AGAMOUS-LIKE 24*, a dosage-dependent mediator of the flowering signals. *Proc. Natl. Acad. Sci. USA*, **99**, 16336–16341.