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PANICLE PHYTOMER2 (*PAP2*), encoding a SEPALLATA subfamily MADS-box protein, positively controls spikelet meristem identity in rice

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In rice panicle development, new meristems are generated sequentially in an organized manner and acquire their identity in a time- and position-dependent manner. In the panicle of the *panicle phytomer2-1* (*pap2-1*) mutant, the pattern of meristem initiation is disorganized and newly formed meristems show reduced competency to become spikelet meristems, resulting in the transformation of early arising spikelets into rachis branches. In addition, rudimentary glumes and sterile lemmas, the outermost organs of the spikelet, elongate into a leafy morphology. We propose that *PAP2* is a positive regulator of spikelet meristem identity. Map-based cloning revealed that *PAP2* encodes OsMADS34, a member of the SEPALLATA (SEP) subfamily of MADS-box proteins. *PAP2*/*OsMADS34* belongs to the *LOFSEP* subgroup of MADS-box genes that show grass-specific diversification caused by gene duplication events. All five *SEP* subfamily genes in rice are expressed exclusively during panicle development, while their spatial and temporal expression patterns vary. *PAP2* expression starts the earliest among the five *SEP* genes, and a low but significant level of *PAP2* mRNA was detected in the inflorescence meristem, in branch meristems immediately after the transition, and in glume primordia, consistent with its role in the early development of spikelet formation. Our study provides new evidence supporting the hypothesis that the genes of the *LOFSEP* subgroup control developmental processes that are unique to grass species.

Keywords: *LOFSEP* • *OsMADS34* • *PANCILE PHYTOMER2* (*PAP2*) • Rice inflorescence • *SEPALLATA* (*SEP*) • Spikelet.

Abbreviations: BAC, bacterial artificial chromosome; IM, inflorescence meristem; RNAi, RNA interference; RT–PCR, reverse transcription–PCR.

Introduction

The basic architecture of the inflorescence is determined by the identity and activity of meristems. Studies in the model eudicot species, mostly Arabidopsis, have allowed the basic model of the genetic control of flower initiation to be built (for a review, see Blázquez et al. 2006). *LEAFY* (*LFY*) and *APETALA1* (*AP1*) are key regulators of floral meristem identity in Arabidopsis (Mandel et al. 1992, Weigel et al. 1992, Wagner et al. 1999). Other genes, such as *CAULIFLOWER* (*CAL*) and *SEPALLATA* (*SEP*), together with *LFY* and *AP1*, control floral meristem identity (Ferrándiz et al. 2000, Ditta et al. 2004). Once the floral meristem fate is established, the meristem starts to produce floral organs according to the mechanism known as the ABCE model (Goto et al. 2001).

Grass species show unique features of inflorescence development (Clark and Pohl 1969). A conspicuous characteristic of grass inflorescence is the spikelet, a small branch containing a variable number of flowers called florets. Because the spikelet is a terminal structure, the arrangement of spikelets rather than flowers is considered to define inflorescence architecture in grass species. A schematic of the rice inflorescence, called the panicle, is shown in **Fig. 1A**. During rice panicle formation, new meristems are sequentially generated. Newly formed meristems acquire the identity of a rachis branch meristem or terminate as a spikelet meristem, depending upon their position and the timing of their occurrence. The meristem that becomes a rachis branch meristem continues to generate next order lateral meristems that will eventually acquire the spikelet identity and terminate (Itoh et al. 2005). The key factor that determines the grass inflorescence form is the spatial and temporal regulation of spikelet meristem fate.

Because the spikelet is unique to grasses, unveiling the molecular and genetic control of spikelet initiation and development

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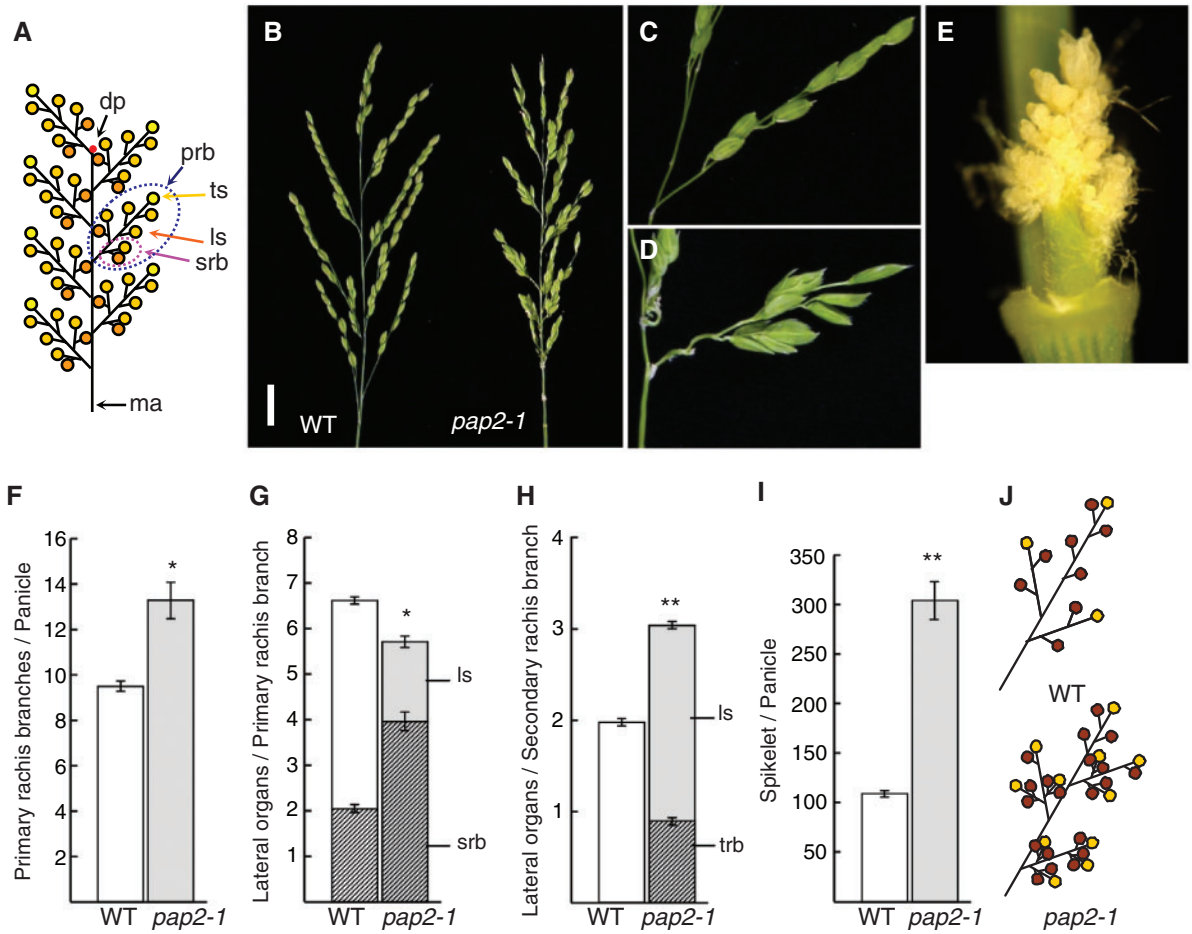

Fig. 1 Phenotype of the *pap2-1* mutant in panicle development. (A) Schematic of a rice panicle. ma, main axis; prb, primary rachis branch; srb, secondary rachis branch; ls, lateral spikelet; ts, terminal spikelet; dp, degeneration point of the IM. (B) Morphology of panicles in the wild-type (left) and *pap2-1* (right). Scale bar = 2 cm. (C and D) Spikelets on the primary rachis branch in the wild-type (C) and *pap2-1* (D). (E) Magnified view of aggregated branches on the *pap2-1* primary rachis branch shown in D. (F) Number of primary branches per panicle. Sample size, $n = 10$. (G) Number of lateral organs generated on a primary branch. srb, secondary rachis branch; ls, lateral spikelet. Sample size, $n = 10$. (H) Number of lateral organs generated on a secondary branch. trb, tertiary rachis branch; ls, lateral spikelet. Sample size, $n = 10$. (I) Number of spikelets per panicle. All spikelets generated were counted under the stereomicroscope irrespective of whether they were normal or abortive. Sample size, $n = 10$. (J) Schematics of a spikelet of the wild-type (top) and *pap2-1* mutant (bottom). The asterisks show that the difference is significant at the *1% and **0.1% level, respectively.

has been of great interest from both evolutionary and developmental points of view. So far, several genes that control panicle and spikelet development have been identified in grass species such as maize and rice (Bommert et al. 2005, Bortiri and Hake 2007). They can be classified based on the developmental steps they control. Genes of the first class, such as *ABERRANT PANICLE ORGANIZATION1* (*APO1*), *RICE CENTROLADIALLIS* (*RCN*), *GRAIN NUMBER1* (*GN1*) and *DENSE AND ERECT PANICLE1* (*DEP1*), control the initiation of meristem identity (Ashikari et al. 2005, Ikeda et al. 2005, Ikeda et al. 2007, Huang et al. 2009, Ikeda et al. 2009). *APO1*, which encodes an F-box protein related to Arabidopsis *UNUSUAL FLORAL ORGANS* (*UFO*), negatively regulates the spikelet meristem identity (Ikeda et al. 2005, Ikeda et al. 2007, Ikeda et al. 2009). In *apo1* mutants, specification of the spikelet identity is accelerated, resulting in the formation of a small panicle. In contrast, an increased expression of *APO1* caused the delay of spikelet identity determination, leading to an increased number of branches in the panicle. Constitutive expression of *RCN*, a *TERMINAL FLOWER1* (*TFL1*) ortholog, delays spikelet meristem identity and causes an extreme increase in the number and in the level of ramification of rachis branches, indicating that *RCN* can suppress the spikelet meristem identity (Nakagawa et al. 2002). *GN1* and *DEP1* were identified as quantitative trait loci that modulate grain yield through the control of the meristem phase transition to a spikelet meristem (Ashikari et al. 2005, Huang et al. 2009). *GN1* encodes a cytokinin oxidase, an enzyme that catalyzes the degradation of cytokinins, implying an involvement of cytokinins in the control of spikelet meristem identity. Arrangement of spikelets in the panicle is disturbed in

panicle phytomer1 (*pap1*) mutants, suggesting that the *PAP1* gene may play a role in the initiation of the spikelet meristems although its molecular nature remains to be determined (Takahashi et al. 1998).

Genes of the second class are required to maintain the spikelet meristem identity and/or to control the transition from spikelet to floret meristem identity. Mutations of these genes do not affect the arrangement of the spikelets but spikelet development is impaired. *SUERNUMEROUS BRACT* (*SNB*), *FRIZZY PANICLE* (*FZP*), *LEAFY HULL STERILE1* (*LHS1*) and *EXTRA GLUME1* (*EG1*) belong to this class (Jeon et al. 2000, Komatsu et al. 2003, Agrawal et al. 2005, Chen et al. 2006, Lee et al. 2006, Li et al. 2009). Finally, a third group of genes including *SUPERWOMAN1*, *OsMADS3*, *OsMADS58* and *DROOPING LEAF* is required for floral organ development (Nagasawa et al., 2003, Yamaguchi et al. 2004, Yamaguchi et al. 2006).

Despite successful isolation and characterization of all the genes mentioned above, our understanding of the molecular basis of rice panicle development remains fragmented, and more molecular studies are needed for a full understanding of the process. In this study, we isolated a new mutant named *panicle phytomer2* (*pap2*) that shows abnormal panicle development including a disordered arrangement of spikelets, an increase of rachis branches and the elongation of rudimentary glumes and sterile lemmas. We show that *PAP2* encodes OsMADS34, which belongs to the SEP subfamily of the MADS-box proteins. We suggest that *PAP2* is one of the key regulators of spikelet meristem identity in rice.

Results

The panicle of the *pap2-1* mutant produces more rachis branches

A wild-type panicle contains several primary rachis branches that are produced by the inflorescence meristem (IM). After producing several rachis branch meristems the IM loses its activity and is left as a vestige called a degenerate point at the base of the uppermost primary branch (**Fig. 1A**). The primary rachis branch contains a few secondary rachis branches at the bottom, several lateral spikelets and a terminal spikelet. Secondary rachis branches produce a few spikelets.

A recessive mutant with abnormal panicle morphology was identified through a screening of a rice population derived from tissue culture regenerated plants (Hirochika 2001). The morphological resemblance between the panicle of the mutant identified and that of a previously reported *pap1* mutant (Takahashi et al. 1998) prompted us to test the genetic relationship between the two mutations. Because the genetic complementation test indicated that the mutation was not located in the *PAP1* locus (data not shown), the mutant was named *pap2-1*. In the *pap2-1* mutant, the arrangement of lateral organs, such as rachis branches and spikelets, was disordered and the panicle became irregularly denser, in sharp contrast to the well-defined phyllotaxy of the wild-type panicle (**Fig. 1B–D**). Furthermore, agglomerates of a large number of branches and spikelets with arrested development were often observed (**Fig. 1B, D, E**).

The *pap2-1* panicle appears smaller than that of the wild-type due to the suppressed elongation of nodes in the panicle (**Fig. 1B**), but it contains more branches. We examined the branching pattern of *pap2-1* in more detail. In this analysis, all spikelets were counted regardless of their development. The number of primary rachis branches in a rice panicle depends on the timing of IM deactivation. A significant increase in the number of primary rachis branches per panicle was observed in *pap2-1*, indicating that the IM activity was maintained longer in *pap2-1* than in the wild-type (**Fig. 1F**). The total number of lateral organs generated on primary rachis branches is slightly reduced in *pap2-1* while it is significantly increased on the secondary branches (**Fig. 1G, H**). In rice panicles, a few basal lateral organs on the rachis branches grow as next order branches and distal ones grow as spikelets. The shift from rachis branch formation to spikelet formation is delayed in *pap2-1*, resulting in the transformation of spikelets that arise early into rachis branches (**Fig. 1G, H**). In the wild-type panicle, 31.0% of lateral organs produced in the primary rachis branches grow as secondary rachis branches, whereas 70.8% become secondary branches in *pap2-1* (**Fig. 1G**). This characteristic, namely production of more rachis branches, was again observed on the secondary rachis branches in *pap2-1*, leading to the formation of tertiary branches, which are not usually seen in the wild-type panicle (**Fig. 1H**). The increase in the number of branches resulted in an increase in the total number of spikelets initiated per panicle (**Fig. 1I**). Defects observed in *pap2-1* panicles are schematically summarized in **Fig. 1J**. The panicle phenotypes observed in *pap2-1* suggest that *PAP2* positively controls spikelet meristem identity.

In contrast to the defects in the panicle, no alteration in the overall growth phenotype, including plant height, growth of tillers, the plastochron and the total number of leaves produced, was observed in *pap2-1* during vegetative development (**Supplementary Fig. S1**). Although flag leaves of the wild-type and *pap2-1* emerged at the same time, the *pap2-1* panicle appeared approximately 5 d later than that of the wild-type (82.6 ± 0.7 days in the wild-type, vs. 87.5 ± 0.7 d in *pap2-1*), most probably due to a delay in the elongation of the panicle node.

Abnormal morphology of the *pap2-1* spikelet

The spikelet meristem produces lateral organs in a distichous phyllotaxy. The first set is two bracts called glumes. Then, one or more bracts known as lemmas are generated and a floral meristem initiates in the axil of the lemma. Because glumes in the rice spikelet are reduced in size, they are called rudimentary glumes. Inside of the rudimentary glumes are empty glumes and a floret containing a lemma, a palea, two lodicules, six stamens and a pistil (Yamaguchi and Hirano 2006). Only one floret is formed in a rice spikelet, but it is interpreted that a rice spikelet originally generated three florets and empty glumes are lemmas of two florets that have been lost during the course of

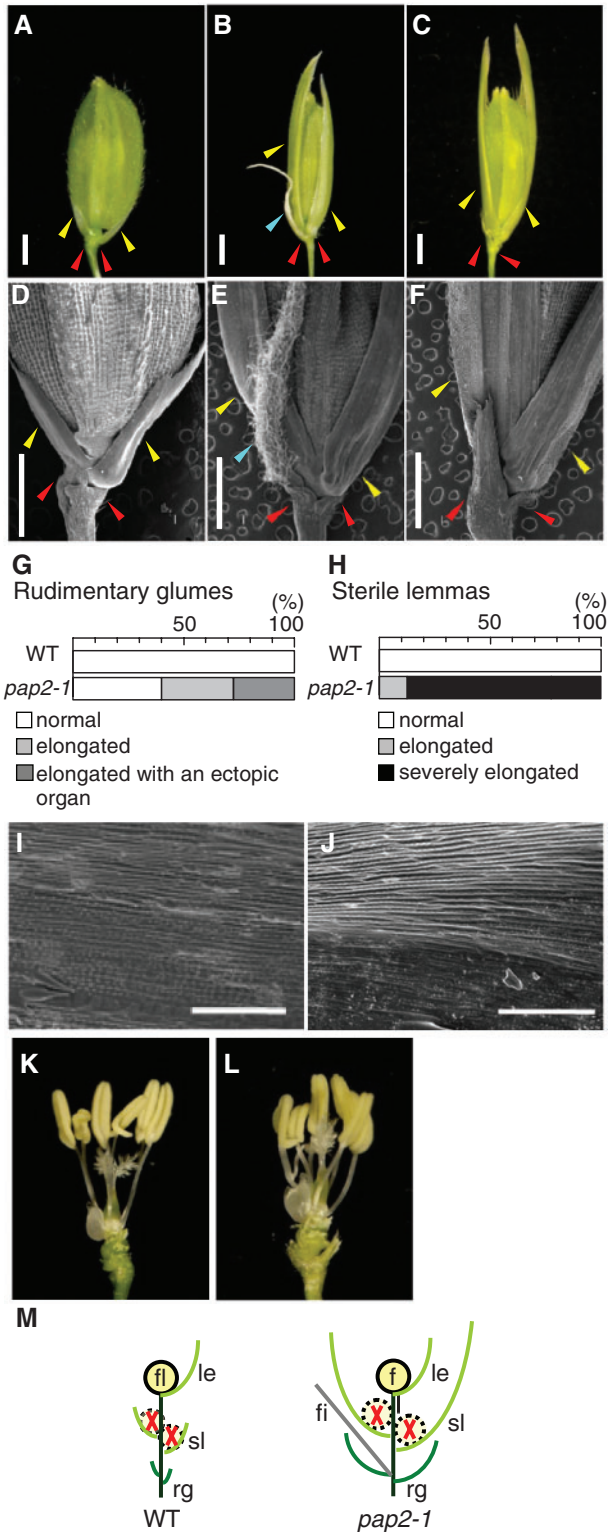

Fig. 2 Phenotype of *pap2-1* in spikelet development. (A–C) Spikelets in the wild-type (A) and *pap2-1* (B, C). Rudimentary glumes (red arrowheads) and sterile lemmas (yellow arrowheads) elongate in *pap2-1*. An ectopic organ (blue arrowhead) often developed in the axil of the rudimentary glume in *pap2-1* (B, C). Scale bars = 1 mm. (D–F) SEM analysis of the spikelet of wild-type (D) and *pap2-1* (E, F). Scale bars = 500 µm. (G and H) Number of rudimentary glumes (G) and sterile lemmas (H) in the wild-type and *pap2-1*. Sample size, $n = 40$. (I and J) Surface of sterile lemmas in the wild-type (I) and *pap2-1* (J). Scale bars = 100 µm. (K and L) Floral organs in the wild-type (K) and *pap2-1* (L) spikelets. (M) Schematics of spikelets from the wild-type (left) and *pap2-1* (right). rg, rudimentary glume; sl, sterile lemma; fi, filamentous organ; le, lemma; fl, floret.

evolution (Komatsu et al. 2003, Bommert et al. 2005). According to this interpretation, empty glumes would correspond to sterile lemmas in other grass species. Herein we use the term sterile lemmas instead of empty glumes to better compare the spikelet structure with that of other grass species.

The most conspicuous abnormality in *pap2-1* spikelets is the elongation of the sterile lemmas (**Fig. 2A–F**). Rudimentary glumes are also elongated, and an ectopic filamentous organ often develops in the axils of the rudimentary glumes, which are usually barren (**Fig. 2B, E**, blue arrowhead). Approximately 60% of the spikelets in a *pap2-1* panicle exhibited elongated rudimentary glumes, and 40% of them developed an ectopic filamentous organ (**Fig. 2G**). Elongation of sterile lemmas was observed in all spikelets examined (**Fig. 2H**). Approximately 13% of the sterile lemmas elongated to about half the size of the lemma, 65% grew to a similar size and 23% grew longer. In spite of the elongation, the appearance of the surface of the elongated sterile lemmas remained unchanged, indicating that their identity as sterile lemmas was maintained (**Fig. 2I, J**). No detectable abnormality was observed in the floret (**Fig. 2K, L**), including the morphology of the lemma and palea (data not shown). The abnormalities in *pap2-1* spikelets are illustrated in **Fig. 2M**.

PAP2 encodes a MADS-box transcription factor similar to SEPALLATA (SEP)

We isolated the *PAP2* gene by map-based cloning. Fine mapping using 2,832 F_2 plants allowed the *PAP2* locus to be mapped to a 10,000 bp interval on chromosome 3, where only one gene, *OsMADS34* (Os03g0753100), was predicted (**Fig. 3A**). Sequence analysis revealed an insertion of *Tos17*, an endogenous retrotransposon, in the fourth exon of *OsMADS34* in *pap2-1* (**Fig. 3B**). This insertion introduces a termination codon in the K-box of the protein (**Supplementary Fig. S2**). The *OsMADS34* mRNA accumulation was decreased to an undetectable level in *pap2-1* (**Fig. 3C**). These data suggest that *PAP2* is *OsMADS34* and that the defects in *pap2-1* are most probably caused by the insertion of *Tos17*. We confirmed this hypothesis by introducing a wild-type genomic DNA fragment containing the *OsMADS34* region into *pap2-1* and achieving full complementation of the *pap2-1* phenotype (**Fig. 3D**). Finally, a reduction of *OsMADS34* expression by RNA interference (RNAi) mimicked the *pap2-1* phenotype (**Fig. 3E, F**). Based on these results, we concluded that *PAP2* is Os03g0753100, encoding a MADS-box protein, OsMADS34.

OsMADS34 is a member of the MADS-box gene family that regulates many aspects of plant growth and development (Malcomber and Kellogg 2005, Kater et al. 2006). *PAP2/OsMADS34*

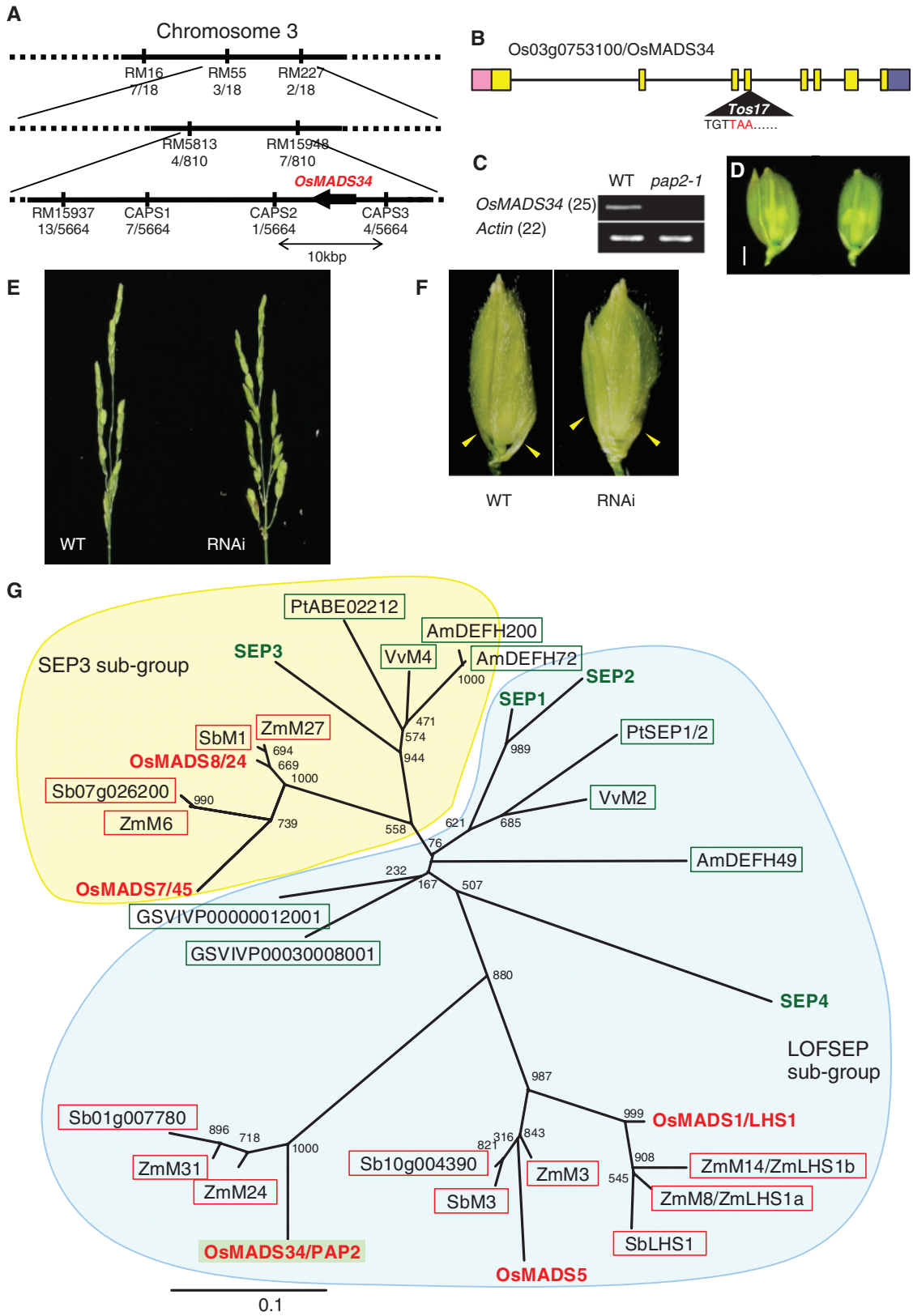

Fig. 3 Isolation of *PAP2* by positional cloning. (A) Location of the *PAP2* locus on rice chromosome 3. The numbers of recombinants are indicated under each marker. (B) Structure of the *PAP2/OsMADS34* gene. The boxes indicate exons, the line indicates introns and the triangle shows the insertion of *Tos17*. (C) Steady-state level of *PAP2/OsMADS34* mRNA in the shoot apex of the wild-type and *pap2-1*. (D) Complementation test of *pap2-1* by *PAP2/OsMADS34*. The elongated sterile lemma phenotype of *pap2-1* (left) was rescued in the complemented plant (right).
Continued

is a type II MADS-box gene (there are >44 known type II MADS-box genes), a member of the *SEPALLATA* (*SEP*) subfamily (**Fig. 3G**). *SEP* genes are further divided into two major subgroups, namely the *SEP3* and *LOFSEP* subgroups (Malcomber and Kellogg 2005, Zahn et al. 2005, Arora et al. 2007). Among the five genes of the *SEP* subfamily in rice, *LHS1/OsMADS1*, *OsMADS5* and *PAP2/OsMADS34* belong to the *LOFSEP* subgroup, and *OsMADS7* and *OsMADS8* are members of the *SEP3* subgroup.

Expression pattern of the five rice *SEP* genes during early stages of panicle development

For a better understanding of the functions of the rice *SEP* genes in panicle development, the expression patterns of the five *SEP* genes during early stages of panicle development were examined (**Fig. 4**). Although a comprehensive study of MADS-box gene expression of rice was reported previously, the earliest stage examined in the study was at the 0–3 cm panicle stage (Arora et al. 2007). Because all critical steps of spikelet and floral organ differentiation end before the panicle reaches 2 mm in size, and maturation and elongation of organs occur in the later stages, we decided to examine expression of the five *SEP* genes during much earlier stages of panicle development. We collected, under the microscope, panicles that were approximately 0.1–1 mm in length and further categorized them into five developmental stages as reported by Furutani et al. (2006). The expression of the five *SEP* genes started at various time during panicle development. A low level of *PAP2/OsMADS34* mRNA accumulation could be observed from stage 1 when the primary rachis branches differentiate. The expression of *PAP2/OsMADS34* continued through stages 2 and 3 and increased at stage 4 when spikelet organs differentiate. In contrast to the early onset of *PAP2/OsMADS34*, *LHS1/OsMADS1* expression started from the spikelet meristem initiation stage (stage 4). High levels of *LHS1/OsMADS1* expression were observed at the floral organ differentiation stage (stage 5). *OsMADS5* showed a pattern of mRNA accumulation similar to that of *PAP2/OsMADS34* but at relatively lower levels. Both *OsMADS7* and *OsMADS8* mRNAs are expressed predominantly at stage 5 as previously reported (Pelucchi et al. 2002).

Finally, we examined the spatial distribution of the expression of the three *LOFSEP* subgroup genes by in situ hybridization (**Fig. 5**). *PAP2/OsMADS34* mRNA accumulation was first observed in the IM and initiating primary rachis branches (**Fig. 5A, B**), and continued in the lateral spikelet meristem and the floret meristem (**Fig. 5C, D**). *PAP2/OsMADS34* mRNA was also detected at the tips of the rudimentary glumes and sterile lemmas, as previously described by Pelucchi et al. (2002) (**Fig. 5E**). *LHS1/OsMADS1* expression was first observed in the entire region of the floret meristem (**Fig. 5H**), and it started to be excluded from the meristem region as the lemma and palea primordial are formed, as previously reported (**Fig. 5I, J**) (Prasad et al. 2001). *OsMADS5* expression started slightly earlier than that of *LHS1/OsMADS1* and was localized in the floret meristem (**Fig. 5L, M**) and the palea primordia (**Fig. 5N, O**).

Discussion

PAP2 is a positive regulator of spikelet meristem identity in rice

Here we describe *pap2*, a new rice mutant that exhibits abnormal development of the panicle. In *pap2-1*, the early arising spikelet meristems are converted to rachis branch meristems, resulting in an increase in the number and the ramification level of rachis branches. Based on this observation, we propose that *PAP2* is a spikelet meristem identity gene of rice. So far, only a few rice genes that control spikelet meristem identity have been reported. *APO1* and *GN1* work in the control of spikelet meristem identity, however, they suppress the spikelet's identity, an action opposite to that of *PAP2* (Ashikari et al. 2005, Ikeda et al. 2005). On the other hand, *DEP1* controls spikelet identity in a positive manner, as does *PAP2* (Huang et al. 2009). *RFL*, a rice *LFY* ortholog, is another gene that negatively regulates spikelet identity (Rao et al. 2008). Elucidation of the genetic interactions among these genes is required in order to understand the mechanisms controlling rice panicle development. In addition to the conversion of spikelets to rachis branches, rudimentary glumes and sterile lemmas showed leafy morphology in *pap2-1*. This may be an indication of reduced floral fate, but we cannot rule out the possibility that *PAP2* controls the identity of rudimentary glumes and sterile lemmas.

Among the spikelet meristem identity genes of rice, *PAP2* is unique. *pap2-1* is the only mutant in which disorganized positioning of branches and spikelets was observed in addition to the increase in the number of branches. In the other mutants, although the patterns of meristem identity are altered, the arrangement of lateral organs is well organized as in the wild-type panicle. Considering that all meristems in the rice panicle initiate as axillary meristems in the axils of bracts, the irregular

Fig. 3 (caption continued)
(E) Reduction of *OsMADS34* mRNA accumulation phenocopied the *pap2-1* phenotype. Panicles in an untransformed wild-type plant (left) and the *OsMADS34* knock-down plant (right). (F) Spikelets in an untransformed wild-type plant (left) and the *OsMADS34* knock-down plant (right). Yellow arrows show sterile lemmas. Sterile lemmas are elongated in spikelets of *OsMADS34* knock-down plants, resembling those of the *pap2-1* mutant. (G) Phylogenetic tree of *SEP* genes in plants. Amino acid sequences in the M-box and K-box were used for the analysis. The phylogenetic analysis was conducted using the Clustal W program, and the phylogenetic tree was constructed by the Neighbor–Joining method. Bootstrap values from 1,000 replicates are indicated at each node. Letters in red indicate rice genes and letters in green indicate Arabidopsis genes. Genes shown in the red boxes are from grass species, whereas genes in the green boxes are from eudicots. Os, rice; Zm, maize; Vv or GSVIVP, grape; Pt, poplar; Sb, sorghum (*Sorghum bicolor*); At, Arabidopsis; Am, snapdragon.

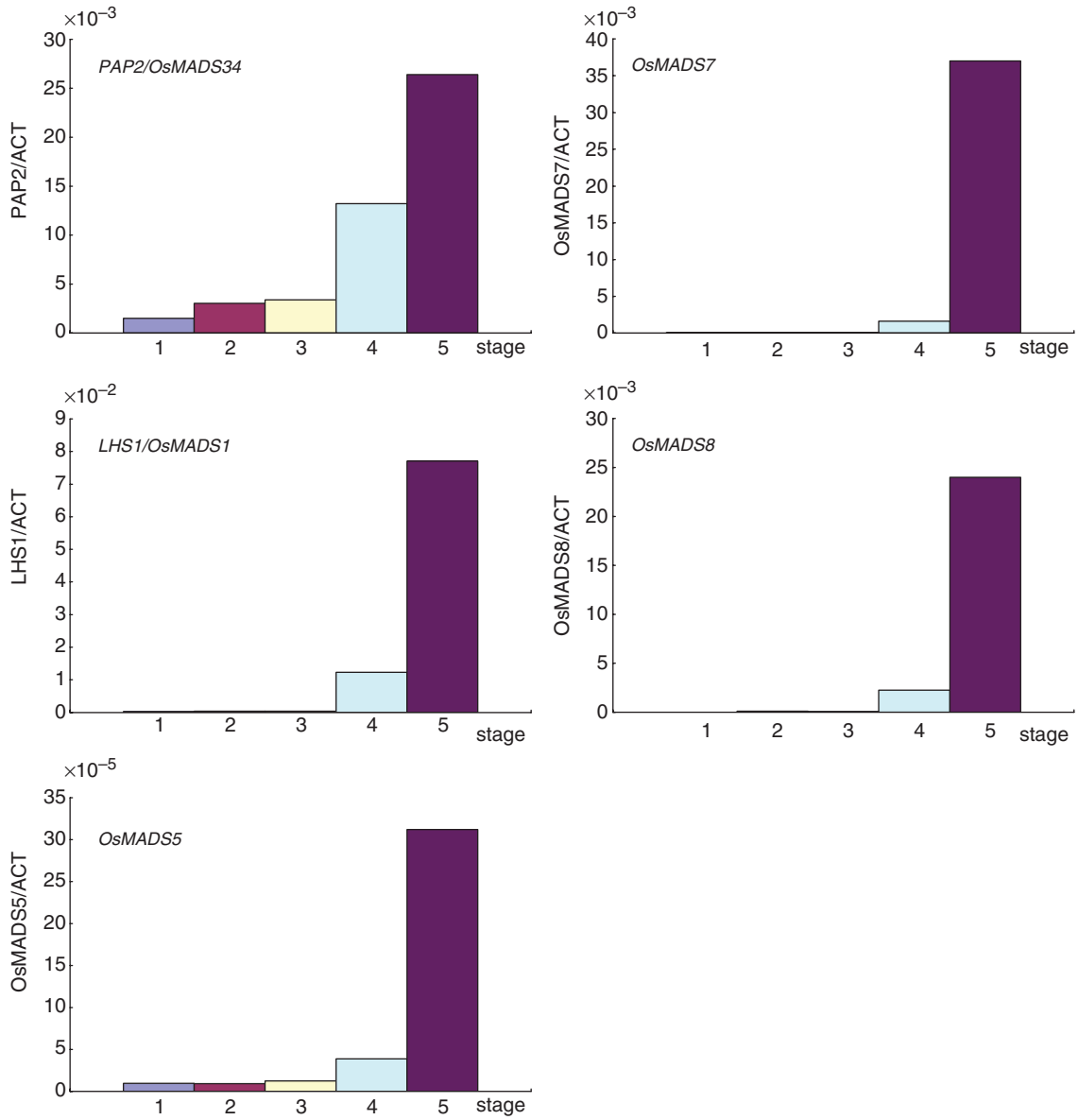

Fig. 4 Expression pattern of five rice *SEP* genes during panicle development. Levels of mRNA accumulation during panicle development were examined by quantitative RT–PCR. Panicle development was divided into five stages according to Furutani et al. (2006). Stage 1, IM after the transition to the reproductive phase to the panicle at primary branch differentiation; stage 2, late primary branch differentiation to early secondary branch initiation; stage 3, young panicles at secondary branch differentiation; stage 4, spikelet differentiation; stage 5, floral organ differentiation. Values are averages of three biological repeats.

pattern of branch formation in *pap2-1* suggests that *PAP2* may play a role in the control of phyllotaxy. This hypothesis should be examined in future studies.

Function of *SEP* subfamily MADS-box genes in rice panicle development

A large number of studies have shown that molecular mechanisms controlling the development of inner floral organs are relatively well conserved between grasses and eudicots (Kater et al. 2006, Yamaguchi and Hirano 2006). In contrast, little is known about the regulatory mechanisms that control the formation of outside organs that are unique to grass species and yet show a great variation among grass species. In this study, we show that *PAP2/OsMADS34*, belonging to the *LOFSEP* subgroup of the *SEP* family MADS-box genes, regulates the identity of the spikelet meristem and development of rudimentary glumes and sterile lemmas, the outermost organs in the spikelet. *PAP2/OsMADS34* branches out with a group of monocot genes on a phylogenetic tree (**Fig. 3E**). Phylogenetic analyses have shown that duplications of *LOFSEP* genes occurred

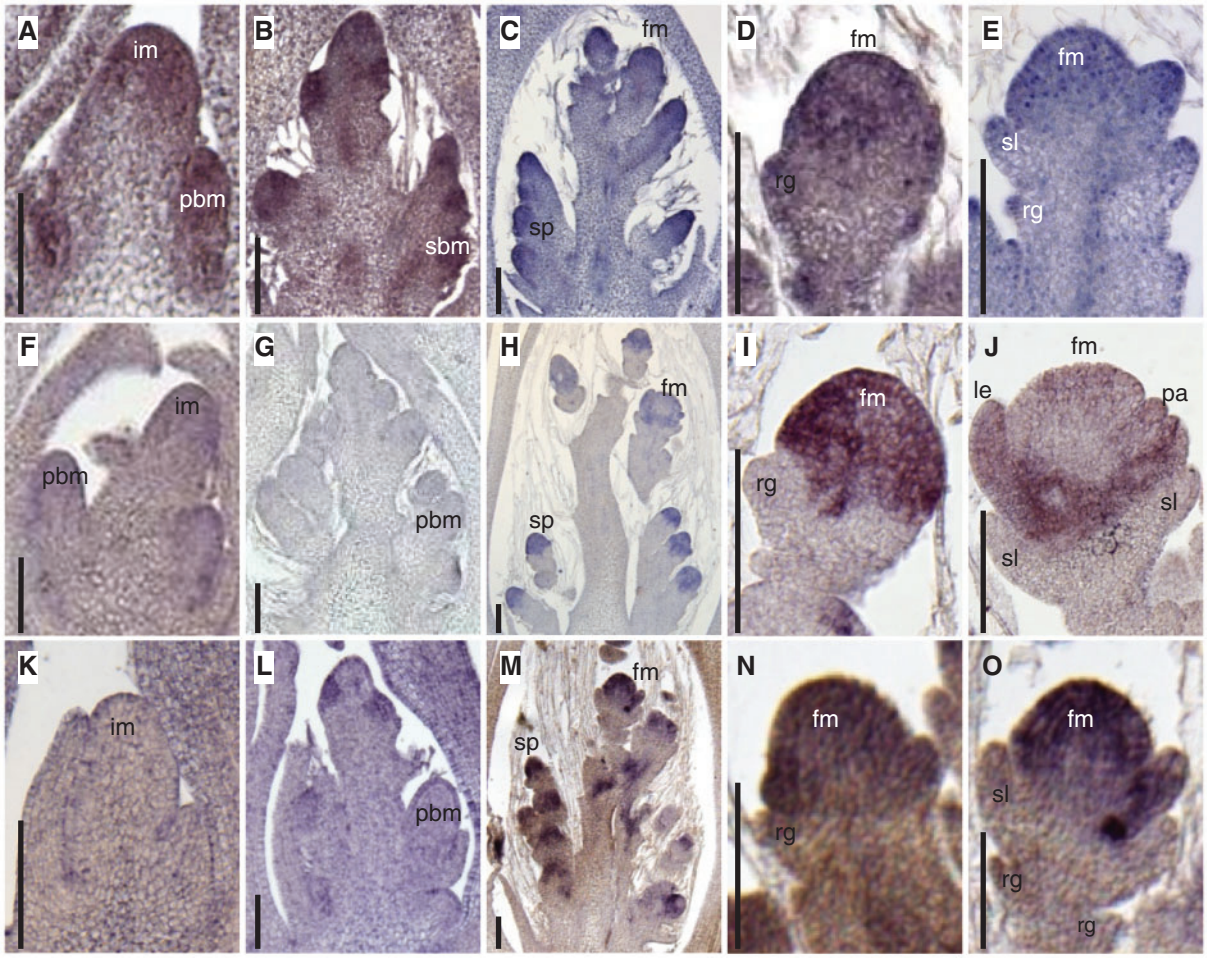

Fig. 5 In situ mRNA accumulation of *LOFSEP* genes. (A–E) Expression patterns of *PAP2/OsMADS34*. A, stage 1; B, stage 3; C, stage 4; close-up views of a spikelet meristem and a floret meristem are shown in D and E, respectively. (F–J) Expression patterns of *LHS1/OsMADS1*. F, stage 2; G, stage 3; H, stage 5; close-up views of a spikelet meristem and a floret meristem are shown in I and J, respectively. (K–O) Expression patterns of *OsMADS5*. K, stage 1; L, stage 3; M, stage 4; close-up views of floret meristems are shown in N and O. Scale bar = 100 μm. im, inflorescence meristem; pbm, primary branch meristem; sbm, secondary branch meristem; sp, spikelet meristem; fm, floret meristem; rg, rudimentary glume; sl, sterile lemma; le, lemma; pa, palea.

near the origin of grasses to produce *PAP2/OsMADS34*, *LHS1/OsMADS1* and *OsMADS5* clades (Malcomber and Kellogg 2005, Zahn et al. 2005). The early diversification of monocot *LOFSEP* genes and their distinct expression patterns suggests that they might have contributed to generate diversity in the reproductive development among monocots. Notably, *PAP2/OsMADS34* is required for spikelet meristem identity while *LHS1/OsMADS1* is essential for normal development of the lemma and palea. Lemma- and palea-like organs are reiteratively generated in *lhs1* loss-of-function mutants, and glumes are transformed into lemma- and palea-like organs by constitutive expression of *LHS1/OsMADS1* (Prasad et al. 2001, Agrawal et al. 2005, Chen et al. 2006). There is a clear distinction between the expression patterns of *PAP2/OsMADS34* and *LHS1/OsMADS1* (Prasad et al. 2001, Arora et al. 2007, this study). Based on these observations, we postulate that grass-specific *LOFSEP* genes sequentially control spikelet development, in particular leafy organs in the spikelet. First, *PAP2/OsMADS34* works to initiate spikelet meristem identity and to control the development of rudimentary glumes and sterile lemmas. Then, *LHS1/OsMADS1* acts to maintain the spikelet meristem identity and controls palea/lemma organ identity, which form inside of the rudimentary glumes and sterile lemmas. No obvious defect was observed in the loss-of-function mutants of *OsMADS5*, the other member of the *LOFSEP* subgroup (Agrawal et al. 2005). High sequence similarity between *OsMADS1* and *OsMADS5* suggests that they may have redundant functions. Although loss-of-function phenotypes of *OsMADS7* and *OsMADS8*, members of the *SEP3* subgroup in the *SEP* subfamily, are yet to be described, the spatial distribution of their mRNA suggests that they probably control the identity of the inner three floral organs in a similar fashion to the Arabidopsis *SEP3* subgroup genes (Pelucchi et al. 2002, this study). The five rice MADS-box genes are expressed sequentially from the initiation of spikelet meristem to floral organ

development, so that they may control the entire process of spikelet development. This is in contrast to the four *SEP* genes of Arabidopsis, which redundantly act to regulate floral meristem identity and organ identities of all four whorls (Pelaz et al. 2000, Ditta et al. 2004).

Previous analysis of *LHS1/OsMADS1* mRNA expression patterns and spikelet development in grass species has suggested that changes in the expression pattern of *LHS1/OsMADS1* contributed to the diversification of spikelets (Malcomber and Kellogg 2004, Reinheimer et al. 2006). Our study also supports the idea that the *LOFSEP* subgroup of *SEP* genes, a class of genes specific to grasses, plays a crucial role in the control of spikelets that are unique to grasses. Analysis of the function of *PAP2* orthologs in other grasses will facilitate our understanding of the molecular basis of the evolution of spikelet development.

Molecular network controlling rice spikelet development

SEP proteins function as transcription factors in the form of a tetrameric complex (Theißen 2001, Kaufmann et al. 2005). Specific functions of *SEP* genes depend on the combination of MADS-box proteins in the complex. For instance, the combination of class-E SEP and class-A AP1 determines sepal formation in whorl 1 (Pelaz et al. 2001). Similarly, rice SEP proteins could form a tetrameric complex to regulate spikelet and floret development. Indeed, interactions between LHS1/OsMADS1 and RAP1A/OsMADS15 or RAP1B/OsMADS14, both orthologs of Arabidopsis AP1, were reported (Lim et al. 2000). The significance of an AP1 and LHS1 combination for the determination of glumes and florets in grasses has been demonstrated (Preston and Kellogg 2007). Identifying partners of PAP2 in the transcription factor complex will be a prerequisite for further elucidation of the role of *PAP2*. Moreover, isolation of the *PAP1* gene and determination of genetic and molecular interactions between *PAP1* and *PAP2* will lead to a more comprehensive understanding of rice spikelet development.

Materials and methods

Plant materials

Plants grown in a glasshouse under natural conditions were used for the analysis of *pap2-1* phenotypes. For mRNA extraction and in situ hybridization analysis, plants (cv. Nipponbare) were grown in a growth chamber under short day conditions (12 h light 28°C, 12 h dark 24°C).

Scanning electron microscope analysis

Spikelets in wild-type (cv. Nipponbare) and *pap2-1* were fixed in 2.5% glutaraldehyde overnight at 4°C, dehydrated in a series of ethanol solutions and substituted with 3-methylbuthyl acetate. Subsequently, samples were dried at critical point, sputter-coated with platinum and observed under a scanning electron microscope (S-4000; Hitachi, Japan) at an accelerating voltage of 10 kV.

Molecular cloning of the PAP2 gene

Rough mapping of the *PAP2* locus was performed with simple sequence repeat (SSR) markers using the mutant F_2 plants obtained from a cross between *pap2-1* and wild-type (cv. Kasalath) plants. For fine mapping, cleaved amplified polymorphic sequence (CAPS) markers were generated based on single nucleotide polymorphisms identified in nucleotide sequences between Nipponbare and Kasalath.

For the complementation test, a genomic fragment of 6,420 bp containing the *OsMADS34* gene was generated by *Bgl*II and *Eco*RV digestions from a bacterial artificial chromosome (BAC) clone OSJNBa0047E24, and subcloned into pBluescript to generate pBSMADS34. A 1,330 bp fragment containing the promoter region of *OsMADS34* was amplified by PCR using TArget Clone-Plus- (TOYOBO, Japan) polymerase with the following set of primers: 5′-CACCATTGCAGCTACAGTA CACCC-3′ and 5′-CTTGTTCTCGATCCGCTGAA-3′. Another genomic fragment of 2,504 bp containing the *OsMADS34* 3′-untranslated region and a terminator was generated by *Hin*dIII digestion of BAC clone OSJNBa0047E24, and cloned into pBluescript to generate pBSMADS34Term. The amplified fragment of the *OsMADS34* promoter was cloned into pBSMADS34Term to generate pBSPMADS34. The fragment containing the whole region of the *OsMADS34* gene including the promoter, the coding region and the terminator was cloned into the binary vector pPZP (Fuse et al. 2001).

Suppression of OsMADS34 expression by RNAi

To suppress the expression of *OsMADS34*, a part of the coding region amplified with the set of primers 5′-CACCTTATGCTTCG CAAGATGCTG-3′ and 5′-AGGTCGCAGAGTTCATCAAG-3′ was cloned into pANDA (Miki and Shimamoto 2004, Miki et al. 2005) and transferred into wild-type rice (cv. Nipponabare).

Real-time reverse transcription–PCR (RT–PCR) analysis

Total RNA was extracted from very young panicles by using a Plant RNA Isolation Mini Kit (Agilent) according to the manufacture's protocol. After the DNase I treatment, the first-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, USA). RT–PCR was performed using the following primer sets: *PAP2/OsMADS34*, 5′-TTGATGAA CTCTGCGACCTAAA-3′ and 5′-TGCTGCAGTTTCCGTTCC-3′; *LHS1/OsMADS1*, 5′-GTGACCATTCCCTGCAGATT-3′ and 5′-GTCTGCTGCTTCATTGCTCA-3′; *OsMADS5*, 5′-TTGCAACT ACAACCTTAACTCATGT-3′ and 5′-GCAGCATTCATCAATC AAACC-3′; *OsMADS7*, 5′-GTGGCAACGGATTCTTCC-3′ and 5′-CGCATGAGTTGTTCATCTGC-3′; *OsMADS8*, 5′-ACGGAGC TTCAGAGAAAGGA-3′ and 5′-CCTGCTCCCACACTTGCT-3′; and *ACTIN*, 5′-GCCGTCCTCTCTCTGTATGC-3′ and 5′-GG GGACAGTGTGGCTGAC-3′. The relative abundances of mRNAs were measured with a LightCycler 480 system (Roche Applied Science, Germany) using *ACTIN* gene expression as a reference for normalization.

In situ hybridizations

In situ hybridizations were carried out as previously described (Kyozuka et al. 1998). To synthesize a digoxigenin-labeled antisense probe of *PAP2/OsMADS34*, a fragment containing the C-terminal region was amplified by PCR, cloned into pGEMT-easy vector (Promega, USA) and used as the template. The primer set used to amplify *PAP2/OsMADS34* was 5′-GTAGA GGCAGCTCCCCCAC-3′ and 5′-GCTAGGCCATCCACTCAGG AGG-3′. Digoxigenin-labeled antisense probes of *LHS1/OsMADS1* and *OsMADS5* were synthesized by using full-length cDNAs, AK070981 and AK064184, respectively, as templates.

Supplementary data

Supplementary data are available at PCP online.

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