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SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice

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SUMMARY

We analyzed recessive mutants of two homeotic genes in rice, *SUPERWOMAN1* (*SPW1*) and *DROOPING LEAF* (*DL*). The homeotic mutation *spw1* transforms stamens and lodicules into carpels and palea-like organs, respectively. Two *spw1* alleles, *spw1-1* and *spw1-2*, show the same floral phenotype and did not affect vegetative development. We show that *SPW1* is a rice *APETALA3* homolog, *OsMADS16*. In contrast, two strong alleles of the *dl* locus, *drooping leaf-superman1* (*dl-sup1*) and *drooping leaf-superman2* (*dl-sup2*), cause the complete transformation of the gynoecium into stamens. In these strong mutants, many ectopic stamens are formed in the region where the gynoecium is produced in the wild-type flower and they are arranged in a non-whorled, alternate pattern. The intermediate allele *dl-1 (T65)*, results in an increase in the number of stamens and stigmas, and carpels occasionally show staminoid characteristics. In the weakest mutant, *dl-2*, most of the flowers are normal. All four *dl* alleles cause midrib-less drooping leaves. The flower of the double mutant, *spw1 dl-sup*, produces incompletely differentiated organs indefinitely after palea-like organs are produced in the position where lodicules are formed in the wild-type flower. These incompletely differentiated organs are neither stamens nor carpels, but have partial floral identity. Based on genetic and molecular results, we postulate a model of stamen and carpel specification in rice, with *DL* as a novel gene controlling carpel identity and acting mutually and antagonistically to the class B gene, *SPW1*.

Key words: Rice, *Oryza sativa*, *DROOPING LEAF*, *SUPERWOMAN1*, Floral mutants, Floral organ identity, Homeotic mutations, MADS box

INTRODUCTION

Genetic and molecular mechanisms of flower development have been intensively studied in two dicot species, *Arabidopsis thaliana* and *Antirrhinum majus*. In these species, rapid progress has been made toward the understanding of the genetic regulation of floral organ specification. Genetic analyses of homeotic mutants in two model plants led to a genetic model of flower development (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991). This model postulates that three homeotic activities, designated as A, B and C, specify four different organ identities in a combinatorial manner. Each of these three functions acts in two adjacent whorls, A [*APETALA1* (*AP1*)/*SQUAMOSA* and *APETALA2* (*AP2*)] in whorls 1 and 2, B [*APETALA3* (*AP3*)/*DEFICIENS* and *PISTILLATA* (*PI*)/*GLOBOSA*] in whorls 2 and 3 and C [*AGAMOUS* (*AG*)/*PLENA*] in whorls 3 and 4. It was shown that A and C functions are mutually antagonistic (Drews et al., 1991) and the B function is provided by the interaction of *AP3/DEFICIENS* and *PI/GLOBOSA* (Goto and Meyerowitz, 1994; Tröbner et al., 1992). The ABC model was further supported by molecular data. Cloning of floral organ identity genes from *Arabidopsis* and *Antirrhinum* revealed that all these homeotic genes except *AP2* encode proteins with a MADS box domain (Yanofsy et al., 1990; Sommer et al., 1990; Huijser et al., 1992; Mandel et al., 1992; Tröbner et al., 1992; Jack et al., 1994; Bradley et al., 1993; Joufuku et al., 1994; Goto and Meyerowitz, 1994). Further genetic and molecular analyses indicated that the genetic mechanisms specifying floral organ identities are conserved between the two distantly related dicot plants *Arabidopsis* and *Antirrhinum* (for a review, see Coen and Meyerowitz, 1991). Several mutations affecting the pattern of floral organ formation have also been reported in *Arabidopsis*. One of them, *superman* (*sup*) is characterized as a mutant that produces extra-stamens at the expense of carpels (Schultz et al., 1991; Bowman et al., 1992). Although *SUP* function was once thought to be the negative regulator of B function genes in whorl 4, *SUP* expression pattern in the wild type and *35S-AP3* flower

suggests that the function of *SUP* is rather to regulate cell proliferation in whorls 3 and 4 (Sakai et al., 1995; Sakai et al., 2000).

The floral developmental program in monocots has not been studied to the extent that it has been in dicots, although several homologs of ABC class genes have been isolated from monocot plants. Two genes homologous to *AG* were isolated from maize, one of the extensively studied monocot plants (Schmidt et al., 1993; Mena et al., 1995; Theissen et al., 1995). For instance, the function of one *AG* homologue, *ZAG1*, was determined by analyzing the corresponding knockout line. A loss-of-function mutant of *ZAG1*, *zag1-mum1*, has been isolated which showed loss of determinacy in the central floral whorl, but no alteration of floral organ identity (Mena et al., 1996). These studies suggest that, although the function of floral organ identity genes might have split and diverged, these genes still play important roles in flower development in monocots. As one of the class B homeotic genes, the *silky1* gene of maize was revealed to be homologous to *AP3* (Ambrose et al., 2000). Since the *silky1* mutant shows a homeotic conversion of lodicules and stamens into palea-like organs and carpels, respectively, similar to *ap3*, it may indicate that the class B genes are functionally more conserved than class A and C genes among flowering plants. In rice, several MADS-box genes have also been isolated (Chung et al., 1994; Chung et al., 1995; Kang et al., 1995; Moon et al., 1999; Kyozuka et al., 2000). *OsMADS3* and *OsMADS4* share sequence similarity to *AG* and *PI*, respectively, and their functions were analyzed by transgenic experiments. The transgenic plants expressing antisense *OsMADS3* produced lodicule-like organs in whorl 3 and several abnormal flowers in whorl 4 instead of a carpel, whereas *OsMADS4* antisense plants showed transformation of stamens into carpels (Kang et al., 1998). In contrast, ectopic expression of *OsMADS3* in rice caused a homeotic transformation of lodicules to stamens (Kyozuka and Shimamoto, 2002). These results show that the function of these genes, at least in part, is as predicted from the ABC model. The important role of MADS-box genes in rice flower development is further shown by the report that the *leafy hull sterile* (*lhs1*) mutant, which has a defect in the *OsMADS1* gene and belongs to the *AP1*/*AGL9* group, has abnormalities in meristem identity, organ number and organ identity (Jeon et al., 2000).

The rice flower has an architecture different from those of the two model dicot species. In general, it is believed to consist of three distinct floral organs, one gynoecium with two stigmas, six stamens, and two lodicules (Hutchinson, 1934). In order to be able to compare rice, *Arabidopsis* and *Antirrhinum*, we designate the lodicule region as whorl 2, the stamen region as whorl 3, and the carpel region as whorl 4 in this study. In rice, two bract-like organs, the palea and lemma, subtend these floral organs in an alternate arrangement. The palea is regarded as homologous to the prophyll [the first leaf produced by the axillary meristem (Arber, 1934; Dahlgren et al., 1985)], is smaller than the lemma and has three vascular bundles while the lemma has five. A floret consists of one gynoecium, six stamens, two lodicules, one palea and one lemma. Two empty glumes that are regarded as vestigial organs of two lower florets subtend the apical floret in an alternate arrangement. Two rudimentary glumes (the first two glumes) subtend the empty glumes. These organs form a spikelet of rice.

Several floral mutants have been described in grasses. The *midribless* (*mbl*) mutant, which has midrib-less leaves and the homeotic conversion of the gynoecium into stamens, has been reported in *Panicum maximum* (Fladung et al., 1991). Although the photosynthetic ability of these midrib-less leaves was analyzed in detail, the morphology of floral organs was not fully described. Similar mutants have been described in barley, *ovaryless* (*ovl*) (Tsuchiya, 1962; Tsuchiya, 1969), and in pearl millet, *midribless-1* (*mrl-1*) and *mrl-2* (Rao et al., 1988), in which both the midrib and carpel differentiation are affected. The detailed floral morphology has not been described for either mutant. In rice, a similar mutation, *drooping leaf* (*dl*), was identified previously (Iwata and Omura, 1971), but the floral abnormalities have not been reported. Recently, we identified new *dl* alleles affecting both flower development and midrib formation. We also identified other homeotic mutants, including *superwoman 1* (*spw1*). In this study, we describe the morphological and genetic analysis of these homeotic mutants, which led us to propose a model in which *DL* specifies a novel function in reproductive floral organs. The model was further supported by our molecular characterization of the *SPW1* gene and expression studies.

MATERIALS AND METHODS

Plant materials

Two kinds of homeotic mutants of rice (*Oryza sativa* L.) were used in this study. We identified two recessive alleles, *superwoman1-1* (*spw1-1*) and *superwoman1-2* (*spw1-2*), from M_2 populations of cv. Kinmaze and Taichung 65, respectively. Four recessive alleles of another homeotic gene, *DROOPING LEAF* (*DL*), which was mapped to chromosome 3, were analyzed in this study. The *dl-1* allele was first reported as a spontaneous mutation that originated in the 'Tareba Ine' (HO788) background (Iwata and Omura, 1971), which is designated *dl-1*(HO788) here. The *dl-1* mutation was further introgressed into the background of Taichung 65 through several backcrosses and designated *dl-1*(T65) in this study. The origin of the *dl-2* mutation is unknown. This mutant has been maintained at the University of Tokyo's Experimental Farm. We identified two new mutations, *dl-superman1* (*dl-sup1*) and *dl-superman2* (*dl-sup2*), from MNU (N-methyl-N-nitrosourea)-treated M_2 populations of cv. Taichung 65 and Kinmaze, respectively. For the investigation on the genetic interaction of two floral homeotic genes, *SPW1* and *DL*, the double mutant was constructed by crossing *SPW1*/*spw1-1* heterozygotes as the female parent with *dl-sup1* homozygote as the male parent. Resulting F_1 plants were allowed to self-pollinate and the double mutant plants were selected from the F_2 population.

Morphological analysis

For scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for about 16 hours at 4°C. After they were rinsed with 0.1 M sodium phosphate buffer (pH 7.2), the samples were post-fixed in 1% osmium tetroxide for 3 hours at 4°C. Subsequently, they were rinsed with the buffer, dehydrated through a graded ethanol series, and substituted with 3-methyl-butyl-acetate. Samples were critical-point-dried, sputter-coated with platinum, and observed under the scanning electron microscope (Hitachi S-4000, Tokyo) at an accelerating voltage of 15 kV.

For light microscopic observations, flowers were fixed in 2.5% glutaraldehyde for at least 16 hours at 4°C. They were dehydrated through a graded ethanol series, and then embedded in Technovit 7100 resin (Kulzer, Germany). Samples were sectioned in 4 μm and stained with 0.05% Toluidine Blue-O and observed under the light microscope (Olympus AX-80, Tokyo).

Linkage analysis and sequencing

F_2 populations were generated by the self-pollination of F_1 plants derived from the cross between *SPW1/spw1-1* (Japonica cv. Kinmaze) and Kasalath (Indica). Plants homozygous for *spw1-1* were selected from F_2 populations and used for linkage analysis of *OsMADS16*. The genomic DNA of these F_2 mutants was extracted by the proteinase K DNA extraction method (http://www.its.caltech.edu/~plantlab/html/ .index.html). Genomic DNA was digested with *Xba*I and subjected to gel electrophoresis. The separated DNA was blotted to Nylon membrane and hybridized with DIG-labeled *OsMADS16* DNA as a probe. The membrane was washed in 1× SSPE 0.1%SDS at 65°C for 1 hour and then in 0.1× SSPE 0.1%SDS at 65°C for 1 hour. Detection was carried out according to the manufacturer's instructions (Roche Molecular Biochemicals).

To obtain genomic clones containing *OsMADS16*, we screened a BAC library, which was constructed in the Japonica YT14 background (Bryan et al., 2000), by PCR using primers, M16F4:ATGTTCTC-CTCCACCGGCAAG and M16R8:GTCCAGATCTTCTCCCATC-CTT. We isolated one BAC clone and subcloned a 7 kb *Bam*HI DNA fragment into pUC18. The subcloned fragment was nebulized and 2 kb-long DNA fragments were cloned into the *Sma*I site of pUC18 and sequenced. For sequencing of mutant alleles, we amplified genomic DNA from *spw1-1* and *spw1-2* by PCR with the following primers: GGTTCCCAACTCATCGATCCATC and AAGCATGAAATATGC-ACGGATCTG for exon 1-4, ACGGTTCATGATCAGATCCGTGCA and GTCAACAGCTTCCAAGGGAAGGA for exon 5, CACACA-TATGCTGGACCCTGTGTC and CATAGCACACATCAAGTGGT-TTGGT for exon 6 and 7. Amplified fragments were cloned into pGEM-T Easy vector (Promega). At least three clones from each PCR-amplified fragment were sequenced to determine mutations.

RNA isolation and analysis

Total RNA was extracted from 500 mg young inflorescence tissues of wild type, *spw1-1* and *spw1-2* as described previously (Naito et al., 1988). Poly(A) RNA was obtained using an mRNA purification kit (Amersham Pharmacia Biotech UK Limited). 1 μg of poly(A) RNA was loaded on a 1% denaturing agarose gel, which contained 1× Mops, 1.85% formaldehyde, and separated for 2 hours at 70 V. RNA was transferred on Hybond-N+ nylon membrane (Amersham Pharmacia Biotech UK Limited). Hybridization was performed at 42°C in the buffer with 5× Denhardt's, 50% formamide, 5× SSC and 100 μg/ml herring sperm DNA. An *SPW1* cDNA fragment without the MADS box domain, i.e. the fragment that covered the region between 102 aa residue and the 3′ end of *SPW1* cDNA, was used to make a randomly primed probe with [α-^{32}P]dCTP. After hybridization, the membrane was washed with 6× SSC for 30min twice at room temperature and then washed with 0.5× SSC at 65°C for 1 hour. Signals were detected on a phosphoimager screen (Molecular Dynamics, Inc.) and visualized on a STORM 820 scanner (Molecular Dynamics, Inc.). As a reference, a DNA fragment of the rice ubiquitin gene was amplified using a set of primers (AGCGTCGACTCCTTCTTGGAT and ATCTTCGTGAAGACGCT-GACG). The fragment was labeled and hybridized on the same blot as described above.

For RT-PCR analysis, 1 μg of total RNA was treated with 1 unit of RNase-free DNase (Promega) and further reverse transcribed by using oligo(dT) primer and Retroscript RT PCR kit (Ambion). One twentieth of transcribed cDNA was subjected to PCR reaction. To amplify the mutant *SPW1* cDNA, we used a primer set consisting of spw1RTF: CAGGTCGCCATCATCATGTTCTC and spw1RTR: GCTCCTGCTGCAGAGTCTCGTACG. The Advantage-GC2 PCR kit (Invitrogen) was used for set up PCR reactions following the manufacture's instructions. PCR was performed for 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 68°C for 30 seconds. Amplified DNA fragments were cloned into p-GEM-T Easy (Promega), transformed into DH10B (Invitrogen) and sequenced.

In situ hybridization

Wild-type and mutant flowers were fixed in 3% paraformaldehyde and 0.3% glutaraldehyde for 16 hours at 4°C. They were dehydrated through a butanol series and embedded in Paraplast and sectioned at 8 μm using a rotary microtome. DIG-labeled RNA probe was synthesized from the 0.6 kb-long, 3′ region of *OsMADS45* and the 0.7 kb-long, 3′ region of *OsMADS16* excluding the MADS-box region, following the manufacturer's instructions (Roche Molecular Biochemicals). Hybridization and immunological detection with alkaline phosphatase were performed according to the method of Kouchi and Hata (Kouchi and Hata, 1993). Radioactive in situ hybridization was performed as described previously (Sakai et al., 1995), with the exception that the exposure length was 6 weeks.

RESULTS

Phenotypes of *superwoman1* mutants

A wild-type rice flower has two white lodicules at the adaxial (lemma) side in whorl 2, six stamens in whorl 3 and one gynoecium with two stigmas in whorl 4 (Fig. 1A,E). Since two allelic mutants, *spw1-1* and *spw1-2*, showed indistinguishable phenotypes, only *spw1-1* was analyzed in this study. In *spw1-1* flowers, the two lodicules in whorl 2 and six stamens in whorl 3 of the wild-type flower were transformed to palea-like organs and carpels, respectively (Fig. 1B,F). The whorl 2 organs were green and elongated without trichomes, resembling paleae. In rice, the palea and lemma can be distinguished by two characters: (1) the palea has three vascular bundles and the lemma has five; (2) the palea has a membranous region of its margin that is not covered with trichomes (Fig. 2A). Although ectopic palea-like organs in *spw1-1* did not develop distinctive vascular bundles, epidermal cells of these palea-like organs were narrow, elongated and rectangular, similar to cells in the marginal region of the wild-type palea (Fig. 2B,C). Therefore, lodicules appeared to be transformed into palea-like organs rather than lemmas in *spw1-1*. The number of ectopic palea-like organs varied: two (76%), three (16%) or four (8%) (Fig. 2D). The palea-like organs that were occasionally formed on the palea side often exhibited a carpelloid nature with frequently formed stigmatic papillae in the apical portion (Fig. 2E).

Although the carpels in whorls 3 and 4 of *spw1* often produced ovaries and stigmas, they failed to produce fused and functional ovaries (Fig. 2F). The number of stigmas for each carpel varied from four to none. Frequently, nucellar tissues were formed in carpels, including whorl 3 carpels, and protruded from the ovary (Fig. 2G-I). When the nucellar tissue remained in the ovary, an ovule-like structure was often formed without producing a fully differentiated embryo sac (Fig. 2H). Occasionally, when the nucellar tissue completely protruded from the underdeveloped ovary, the ovule-like structure was not formed (Fig. 2I). When the *spw1-1* carpels were crossed with wild-type pollen, there was no seed set, showing that *spw1-1* carpels, including the one in whorl 4, were sterile.

Early development of wild-type and *spw1* mutant flowers

In the wild-type flower, six stamen primordia start to develop just after the palea primordium has established (Fig. 3A). After stamen primordia are produced, the carpel primordium becomes enlarged (Fig. 3B). The gynoecial ridge starts to

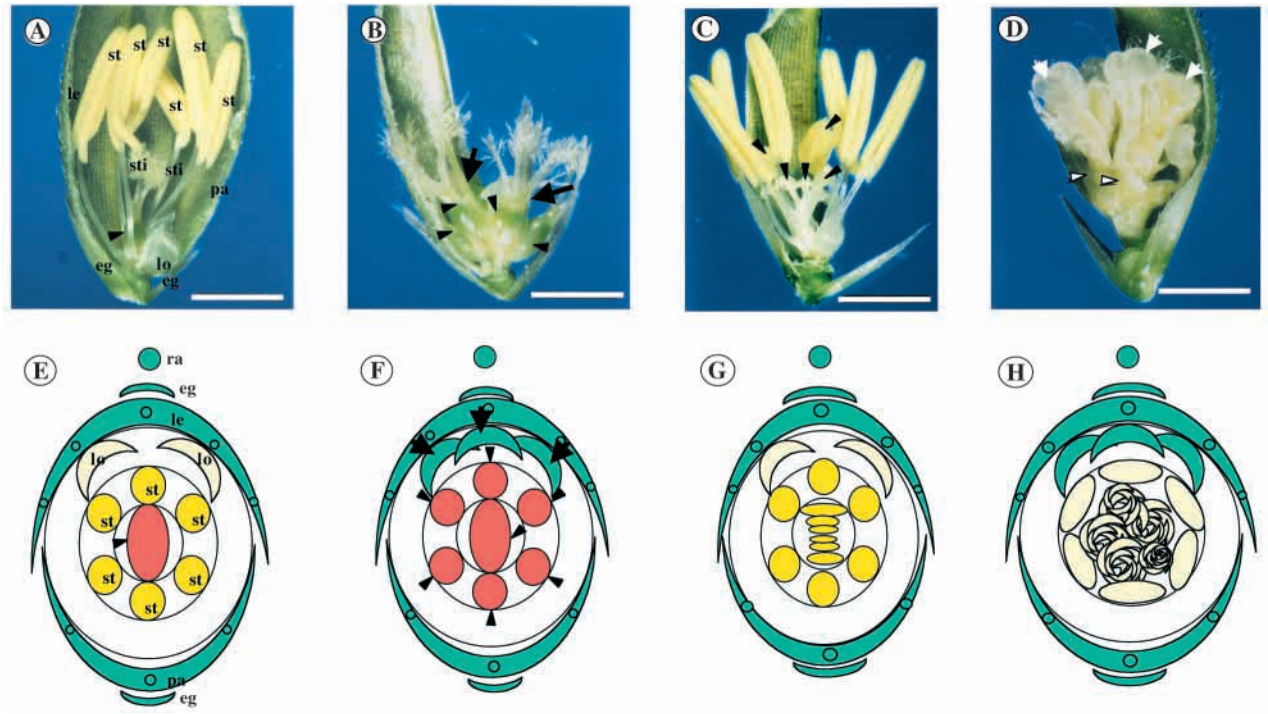

Fig. 1. Phenotypes of wild-type, *spw1-1*, *dl-sup1* and *dl-sup1 spw1-1* flowers. (A) A wild-type flower with two empty glumes (eg), lemma (le), palea (pa), two lodicules (lo), six stamens (st) and one gynoecium (arrowhead) with two stigmas (sti). A half of the lemma and palea were removed to show the inside of the flower. (B) A *spw1-1* flower. Stamens and lodicules are homeotically transformed into carpels (arrowheads) and palea-like organs (arrows), respectively. (C) A *dl-sup1* flower. The carpel is homeotically transformed into ectopic stamens (arrowheads). (D) A *spw1-1 dl-sup1* flower with ectopic palea-like organs (arrowheads) and organs of unknown identity (arrows). The lemma and a half of palea were removed in B-D. (E-H) Floral diagram of (E) the wild-type flower, (F) the *spw1-1*flower, (G) the *dl-sup1* flower, and (H) the *dl-sup1 spw1-1* flower. ra, rachilla. Bar: 1 mm.

develop on the lemma side of the floral meristem and encloses the ovule primordium. The lemma side of the carpel protrudes to form stigmas (Fig. 3C).

In the *spw1-1* flower, the shape of the six primordia of the ectopic carpels in whorl 3 was similar to that of the wild-type stamen primordia at the initial stage (Fig. 3D). Whereas wild-type stamen primordia became rectangular in shape, the six primordia in whorl 3 of the *spw1-1* flower became broad, and followed the developmental course of the gynoecium (Fig. 3E). When the central gynoecium formed stigma primordia, the ectopic palea-like organs became apparent in the position of wild-type lodicules between the lemma and ectopic carpels (Fig. 3F). These results indicated that the number of organ primordia in whorl 3 was not altered, and the transformation of stamens into carpels occurred at a very early stage of floral organ development.

Phenotypes of *drooping leaf* mutants

Four *dl* mutants, which showed the drooping leaf phenotype (Fig. 4B), produced flowers with varying degrees of abnormalities in carpel formation (Table 1, Fig. 5). The blade and sheath of *dl* mutants failed to form the midrib and fully developed clear cells (Fig. 4D). The *dl* mutants appeared to produce a lateral vein at the position of the midrib of the wild-type leaf. Other leaf structures were not affected.

The *dl-1* (HO788) mutant produced drooping leaves and, frequently, abnormal flowers. Although more than half of *dl-1* (HO788) flowers were normal, about 40% of flowers produced a gynoecium with three or four stigmas (Table 1, Fig. 5B). Very rarely, *dl-1*(HO788) flowers produced staminoid carpels, in which anthers were formed apically, or ectopic stamens originated from the base of carpels. Despite these abnormalities, *dl-1*(HO788) plants exhibited only slightly reduced seed fertility (80.4%) comparable to the wild type (87.5%).

dl-1(T65), the introgressed *dl-1*(HO788) mutation in another Japonica background, affected carpel development more severely than *dl-1*(HO788). In *dl-1*(T65), the number of stigmas was increased in about 60% of flowers, and the transformation of the gynoecium into stamens or the production of ectopic stamens was detected in nearly 10% of flowers (Table 1, Fig. 5C). Occasionally, staminoid carpels, which bore anther tissues on the carpel tissue, were formed between more completely transformed ectopic stamens and the whorl 3 stamens (Fig. 5D). When two carpels were formed in *dl-1* (T65) flowers, they were aligned in the lemma-palea direction. The seed fertility of *dl-1*(T65) (52.7%) was lower than that of *dl-1*(HO788).

Plants harboring the *dl-2* mutation showed drooping leaves, while flowers were normal except for producing carpels with three stigmas at a low frequency (Fig. 5E, Table 1). Thus, *dl-2* affects only the midrib development and rarely the carpel development, and can be considered the weakest of the four *dl* alleles.

The homeotic conversion of the carpel into stamens as well as the formation of drooping leaves was observed in all *dl-sup1*

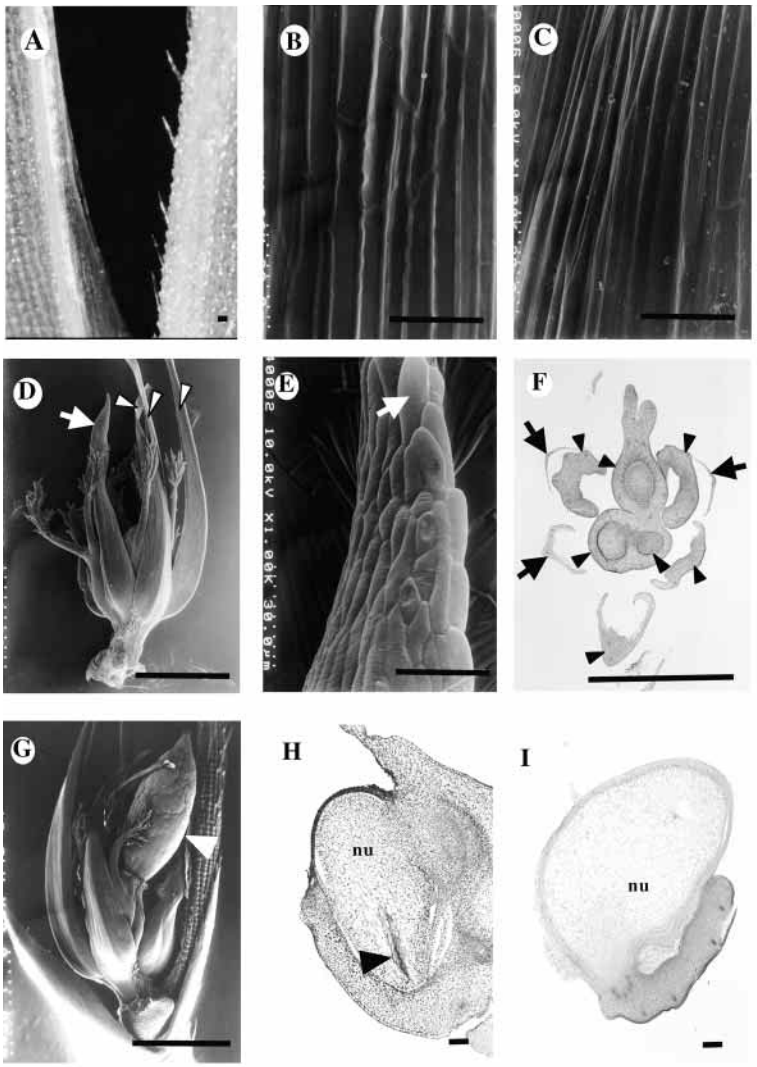

Fig. 2. Micrographs of *spw1-1* flowers. (A) A wild-type palea with a membranous marginal region (left) and the lemma with trichomes on the entire surface (right). (B) Scanning electron micrographs (SEM) of the abaxial, marginal region of the wild-type palea. (C) SEM of the abaxial surface of a palea-like organ of the *spw1-1* flower. (D) The *spw1-1* flower with three palea-like organs (arrowheads) and one carpeloid palea-like organ (arrow). (E) Apical portion of the carpeloid palea-like organ with stigmatic cells (arrow). (F) A transverse section of the *spw1-1* flower with seven carpels (arrowheads) and three palea-like organs (arrows). (G) A side view of the *spw1-1* flower with nucellar tissues protruding from the ovary (arrowhead). (H) A longitudinal section of the *spw1-1* ovary with nucellus and degenerated embryo sac (arrowhead). (I) A transverse section of nucellar tissues of an ectopic carpel formed in whorl 3, which is completely protruding from the *spw1-1* ovary without internal differentiation. nu; nucellar tissue. Bar, 1 mm in (D,F,G) and 30 μm in (A-C,E,H,I).

and *dl-sup2* plants (Fig. 1C,G, Fig. 5F,H, Table 1). The phenotype of *dl-sup2* (Fig. 5H, Table 1) was almost the same as that of *dl-sup1*. In both mutants, the transformation was complete, producing no carpel. The number and position of the original stamens were not affected, and the ectopic stamens were produced in alternate arrangement in the position of the gynoecium (Fig. 5G,I). The number of ectopic stamens in *dl-sup1* varied from three to seven, and the ectopic stamens were produced in the lemma-palea direction (Fig. 5G). As a result, many alternately arranged extra stamens occupied the position of the wild-type gynoecium. The ectopic stamens often had broad filaments (Fig. 5H).

We analyzed the development of *dl-sup1* and *dl-sup2*. The primordia of six stamens in whorl 3 were normally produced in *dl-sup1* flowers (Fig. 3G,J). The primordium of the first ectopic stamen emerged as a lateral protrusion on the lemma side of the floral meristem (Fig. 3H,K). When normal anthers became rectangular in shape, the first ectopic stamen primordium became broad and subtended the central apical meristem (Fig. 3K). At the stage when two stigmas were differentiated from the carpel primordium in the wild-type flower, several ectopic stamens were produced alternately, and the floral meristem at the center appeared to remain undifferentiated in the mutant flower (Fig. 3I,L).

Interaction between *SPW1* and *DL*

To elucidate a possible genetic interaction between *SPW1* and *DL*, we constructed the double mutant, *spw1-1 dl-sup1*. The *spw1-1 dl-sup1* flower showed an unexpected phenotype, which could not be explained by additive or epistatic interactions (Fig. 1D,H). Lodicules were homeotically transformed into palea-like organs in whorl 2 as in *spw1-1*. Interior to whorl 2, yellowish and soft organs were produced

Table 1. Frequency of pistil abnormalities in *dl* mutants

Genotype	No. of flowers examined	Normal	More than two stigmas	Two pistils*	Extra stamens with normal pistil†	Staminoid pistil‡	Extra stamens with no pistil§
Wild type	50	50	0	0	0	0	0
dl-1(HO788)	65	36	27	0	1	1	0
dl-1(T65)	82	20	51	2	6	1	2
dl-2	43	40	3	0	0	0	0
dl-sup1	50	0	0	0	0	0	50
dl-sup2	39	0	0	0	0	0	39

*Two pistils were formed without altering the stamen number.
†These extra stamens were produced around normal pistil without staminoid characteristics.
‡Staminoid pistil was produced instead of or in the vicinity of the normal pistil. Extra stamens were frequently produced.
§The pistil was completely transformed to stamens.
See above for labeling shifts.

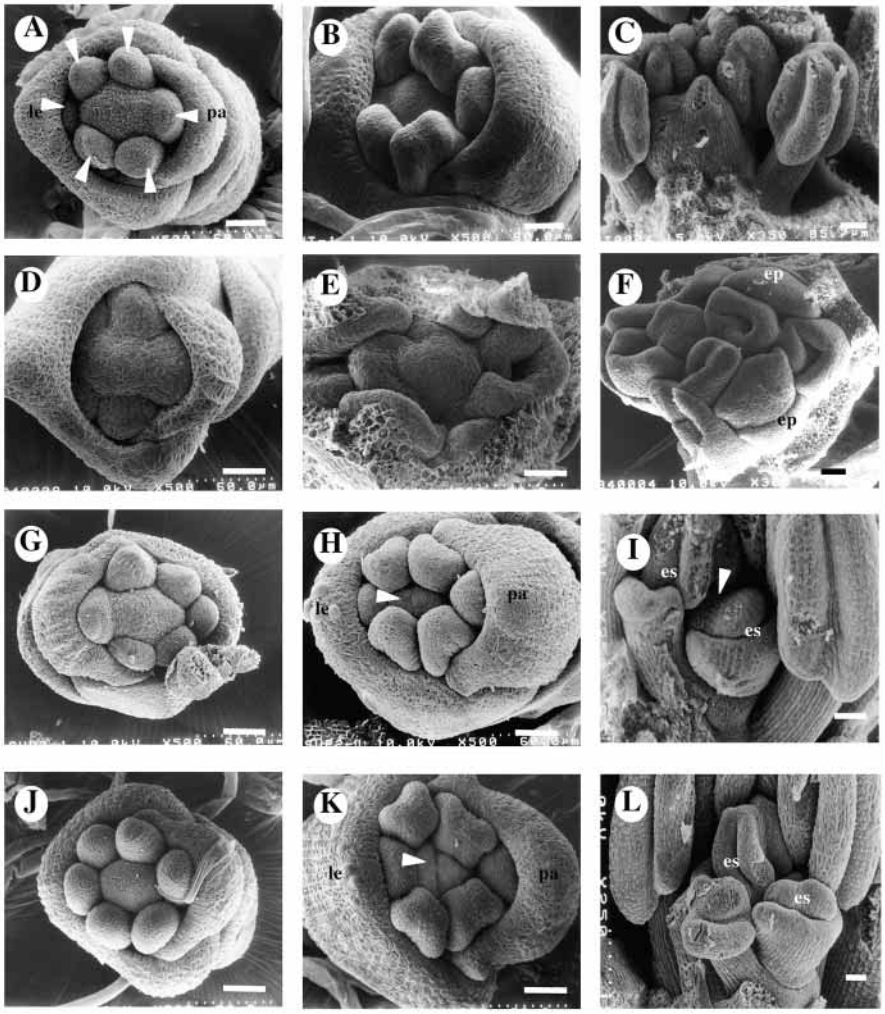

Fig. 3. Scanning electron micrographs of early developmental stages of *spw1-1*, *dl-sup1* and *dl-sup2* flowers. (A-C) Wild-type; (D-F) *spw1-1*; (G-I) *dl-sup1*; (J-L*) dl-sup2*. (A,D,G,J) Young flowers at the stage when organ primordia were formed in whorl 3. (B,E,H,K) Young flowers at the stage when stamen primordia have started to form the anther structure in wild type. (C,F,I,L) Flowers at the later stage when the carpel primordia start to form stigmas in wild type. (D,G,J) In *spw1-1*, *dl-sup1* and *dl-sup2* flowers, six organ primordia were formed at the positions where stamen primordia were formed in the wild type. (E) In *spw1-1*, six organ primordia in whorl 3 broaden to form carpels. (F) Emergence of ectopic palea-like organ primordia in *spw1-1*. (H,K) *dl-sup1 and dl-sup2* flowers. The primordium of the first ectopic stamen (arrowhead) is developing from the lemma side of whorl 4. (I,L) At the later stage, the floral apical meristem (arrowhead) has continued to produce many ectopic stamens. le, lemma; pa, palea; ep, ectopic palea-like organ; es, ectopic stamen. Bars: 30 μm.

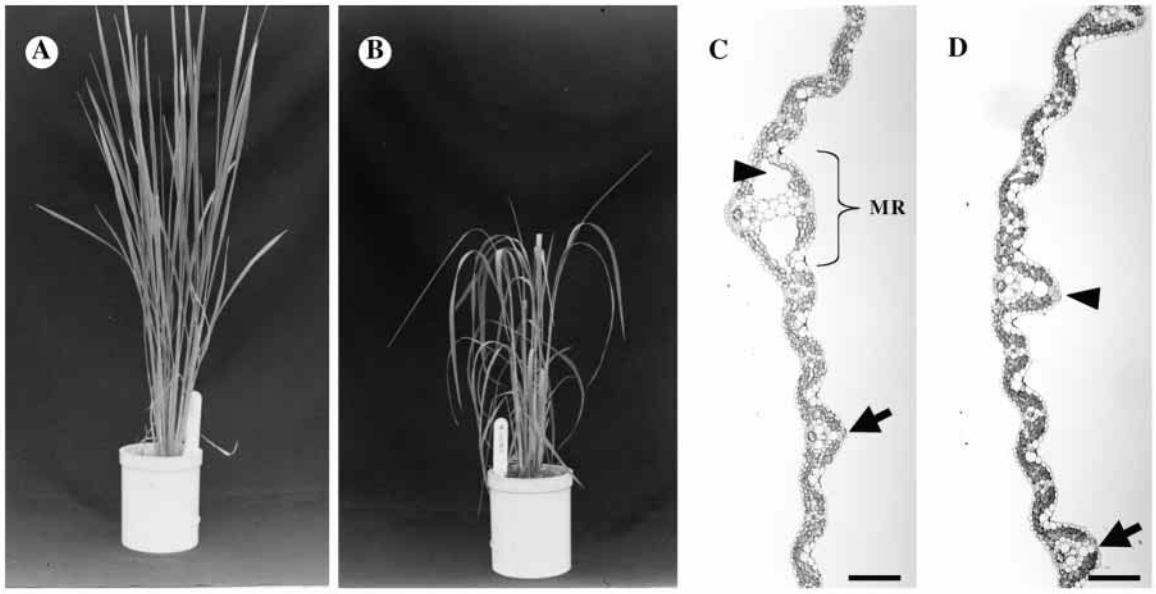

Fig. 4. Phenotype of *dl-1(T65)* plants in the vegetative phase. (A) Wild-type plant in the vegetative phase. (B) *dl-1(T65)* plant at the same stage with drooping leaves. (C) Transverse section of the wild-type leaf blade. The midrib (MR) in the middle of the leaf blade has large clear cells (arrowhead), and an overall structure that is distinct from that of the lateral veins (arrow). (D) Transverse section of the *dl-1(T65)* leaf blade that lacks a midrib. The vein in the middle of the leaf blade (arrowhead) resembles the lateral veins (arrow). Bars (C,D), 200 μm.

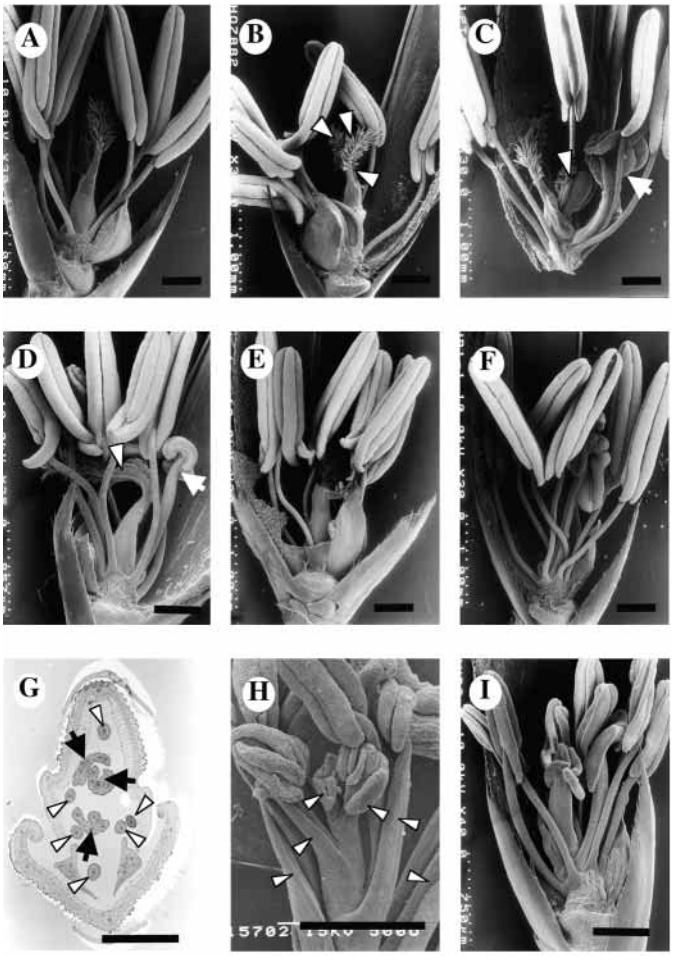

Fig. 5. Micrographs of wild-type and *dl* flowers. The lemma and a half of palea were removed in all flowers. (A) A wild-type flower with two empty glumes, two lodicules, six stamens and one carpel with two stigmas. (B) A *dl-1(HO788)* flower forming the carpel with three stigmas (arrowheads). (C) A *dl-1(T65)* flower with an abnormal cell mass produced from the carpel (arrowhead) and an ectopic stamen (arrow) emerging from the base of the carpel. (D) A *dl-1(T65)* flower producing an extra stamen (arrow) and a mosaic organ, staminoid carpel (arrowhead), in which an anther is formed on the ovary. (E) A *dl-2* flower without any apparent abnormality. (F) A *dl-sup1* flower with a homeotic conversion of the carpel to stamens. (G) A cross section of the *dl-sup1* flower. In addition to the six normal stamens (arrowheads) and two lodicules, ectopic stamens (arrows) are formed. (H) Extra stamens (arrowheads) are alternately produced in the position of the carpel in *dl-sup1* flowers. (I) A *dl-sup2* flower with homeotic conversion of the carpel into stamens. Bars: 500 μm.

indeterminately which were neither stamens nor carpels (Fig. 1D). The upper part of these organs was often enlarged. The abaxial side of the most outer organ in whorl 3 was occasionally covered with trichomes similar to those of paleae or lemmas (Fig. 6A). Also, at the top of these organs, large and long trichomes were formed, which resembled hairs of the bracts subtending primary inflorescence branches (Fig. 6B,C). Trichomes were not observed in more inner organs. These characteristics suggest that the organs in whorl 3 have partial inflorescence-like identity. Longitudinal sections of these organs indicated that the inside of the enlarged part had no pollen or ovule (Fig. 6E).

The ontogeny of double mutant flowers showed that the primordia in whorl 2, corresponding to lodicules in the wild type, developed into palea-like organs as in *spw1-1*. The six primordia corresponding to wild-type stamens in whorl 3 seemed to be normal at the beginning (Fig. 6F). However, the primordia in whorl 3 successively became broader similar to those seen in the development of *spw1-1* ectopic carpels (Fig. 6G), but the subsequent development of the *spw1-1 dl-sup1* flower deviated from the *spw1-1* flower. In the region interior to whorl 3, the central meristem became enlarged toward the palea and lemma (Fig. 6G), and continuously produced new meristems in a medial plane (Fig. 6H), forming a number of apices (Fig. 6H). Unidentifiable organs were formed indeterminately from each meristematic apex (Fig. 6D,I).

To elucidate whether these organs were floral in nature, we analyzed *OsMADS45* expression by means of in situ hybridization. *OsMADS45* shares homology with *Arabidopsis SEP1* (*AGL2*) and *SEP2* (*AGL4*) (Pelaz et al., 2000) and was shown to be expressed in floral organs in whorl 2, 3 and 4 of wild-type flowers (Greco et al., 1997) (Fig. 7C). In order to see whether *OsMADS45* is expressed specifically in flowers, we examined expression in various tissues. The expression of *OsMADS45* appeared to be floral specific and was not detected in the vegetative shoot or inflorescence meristems (Fig. 7A,B). After stamen primordia started to develop, *OsMADS45* was expressed in developing stamen and lodicule primordia (Fig. 7C). *OsMADS45* RNA was further detected in the developing carpel (Fig. 7C) and in integuments (data not shown). In *spw1 dl-sup1* flowers, the expression of *OsMADS45* was not altered (Fig. 7D). *OsMADS45* was expressed in organ primordia formed in whorl 2,3 and 4. This result suggested that these organs still retain partial floral organ identity.

Identification of the *SPW1* gene

The phenotype of *spw1* mutants is similar to the *ap3* and *pi* mutants of *Arabidopsis*. It has been reported that there are three homologs of B function genes in rice, *OsMADS2* and *OsMADS4*, which are genes homologous to *PI*, and *OsMADS16*, which is homologous to *AP3* (Chung et al., 1995; Moon et al., 1999). In order to examine a possible linkage of *SPW1* to one of these homologs, we attempted to analyze the segregation of the *spw1* phenotype with these genes, using the F_2 *spw1* mutants derived from the cross between *spw1-1* and Indica cv. Kasalath. By analyzing RFLP associated with *OsMADS16* in 30 F_2 *spw1-1* plants, the Japonica genotype was found to completely co-segregate with the *spw1-1* allele (data not shown). Based on the tight linkage of *SPW1* and *OsMADS16*, we examined the *OsMADS16* genomic sequence of the wild type and mutants. The 4.3 kb-long *OsMADS16* genomic region consisted of seven exons and six introns (Fig. 8A,B). By sequencing *spw1* mutants, we identified a G to A base change at the 3′ end of the third intron in *spw1-1* and a G to A base change at the 5′ end of the fifth intron in *spw1-2* (Fig. 8A).

The effect of *spw1* mutations on the *SPW1* transcript was analyzed by RNA blot hybridization. Total RNA from young panicles of *spw1-1, spw1-2* and the wild type was separated for the purpose, which was subsequently hybridized with a probe specific to *SPW1*. In the two mutants, the accumulation of *SPW1* RNA was significantly reduced. Furthermore, the size

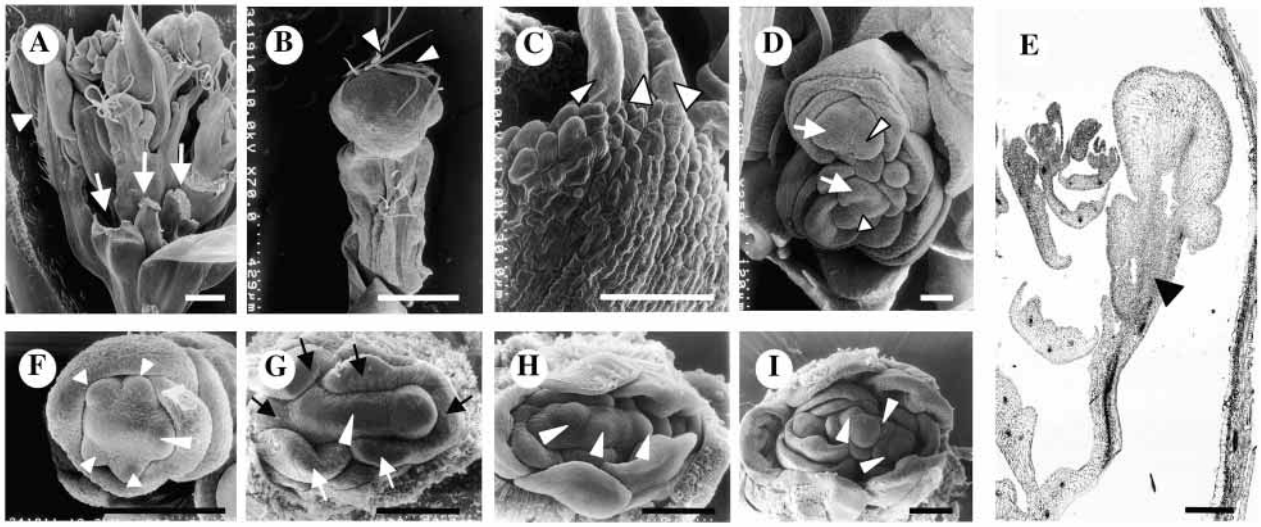

Fig. 6. Micrographs of the *spw1-1 dl-sup1* double mutant. (A) A *spw1 dl-sup1* flower. Palea-like organs with trichomes are formed in whorl 2 (arrowhead). Three ectopic palea-like organs (arrows) were removed to show the inside of the flower. (B) An organ of undefined identity. Long hairs are indicated by arrowheads. (C) Enlargement of the hairs (arrowheads) formed at the margin of the organ in B. (D) A vertical view of the flower. Bifurcated floral meristems (arrowheads), producing organ primordia (arrows) indeterminately. (E) A longitudinal section of an organ of unknown identity (arrowhead) showing no apparent differentiation of gametophytic tissues inside. (F-I) Scanning electron micrographs showing early development of *spw1-1 dl-sup1* flowers. (F) A *spw1-1 dl-sup1* flower producing six organ primordia (one primordium is covered by the lemma) in whorl 3 (arrowheads). (G) Six primordia in whorl 3 develop into structures that are not found in the wild type (arrows), and the floral meristem becomes enlarged in the lemma-palea direction (arrowhead). (H) The central floral meristem proliferates to form a number of apices (arrowheads). (I) Additional organs (arrowheads) are formed in an irregular arrangement from each meristematic apex. Bars: 500 μm (A,B,E), and 50 μm (C,D,F-I).

of *SPW1* RNA in *spw1-2* appeared to be shorter than the wild-type RNA (Fig. 8C). In order to analyze the effect of *spw1* mutations more precisely, we further analyzed *SPW1* RNA by using RT-PCR. The sequence of amplified DNA fragments revealed that the splicing of *SPW1* RNA was affected by the mutations (Fig. 8E). In *spw1-1*, the mutation occurred at the acceptor site of the third intron, leading to cryptic splicing at a position six bases downstream of the acceptor site and causing the deletion of two conserved amino acid residues in the K box (Fig. 8B). In *spw1-2*, the mutation occurred at the donor site of the sixth intron and resulted in deletion of the entire fifth exon (Fig. 8B).

Expression of the *SPW1* gene

SPW1 expression in wild-type flowers was analyzed by means of in situ hybridization using *SPW1* antisense RNA as probe. *SPW1* RNA started to accumulate in incipient primordia of lodicules and stamens in wild-type flowers (Fig. 9A). The strong expression of *SPW1* RNA continued to be seen in stamen and lodicule primordia (Fig. 9B,C) and also in mature tissues of filaments and anthers except developing microsporophylls (Fig. 9D). No signal was detected in the gynoecium or in the lemma and the palea.

SPW1 expression was also examined in *spw1* mutants. We failed in several attempts to detect reproducible signals in mutants by using the non-radioactive method, and therefore we performed in situ hybridization with a radioactive probe. After exposing the hybridized tissues for 6 weeks, we detected weak signals of *SPW1* RNA in incipient organ primordia in the region of whorls 2 and 3 of *spw1-1* developing flowers (Fig. 9M,N). However, shortly after organ primordia were formed in whorls 2 and 3, *SPW1* RNA was not observed above the limits of detection (Fig. 9O,P).

In order to examine the relationship between *DL* and *SPW1* at the transcriptional level, we further analyzed the accumulation of *SPW1* RNA in *dl-sup1* mutant flowers. The expression pattern of *SPW1* RNA in *dl-sup* flowers was indistinguishable from the wild-type expression until the stage when whorl 4 organ primordia emerged from the floral meristem (Fig. 9E). When the gynoecial ridge began to rise in the wild-type flower, *SPW1* RNA was ectopically expressed in the lemma side of the whorl 4 floral meristem in *dl-sup1* flowers where the first ectopic stamen primoridum would arise (Fig. 9F). *SPW1* RNA was further detected in the region where the second ectopic stamen arises (Fig. 9H). *SPW1* expression in the whorl 4 area appears to be limited to the developing primordia, often leaving several cell layers that do not accumulate *SPW1* RNA. Also, throughout the floral development, *SPW1* expression was not detected in the very central region of the floral meristem of *dl-sup1* (Fig. 9F-H).

DISCUSSION

SPW1 is a rice B function gene

We identified the *SPW1* gene by mapping *spw1* mutations to the *AP3* homolog MADS-box gene, *OsMADS16*. Both *spw1-1* and *spw1-2* carry mutations in the splicing acceptor-donor junctions, which cause abnormal splicing reactions and result in the deletion of part of the protein sequence. In *spw1-1*, the mutation at the acceptor site of the third intron leads to cryptic splicing six bases downstream, which causes the deletion of

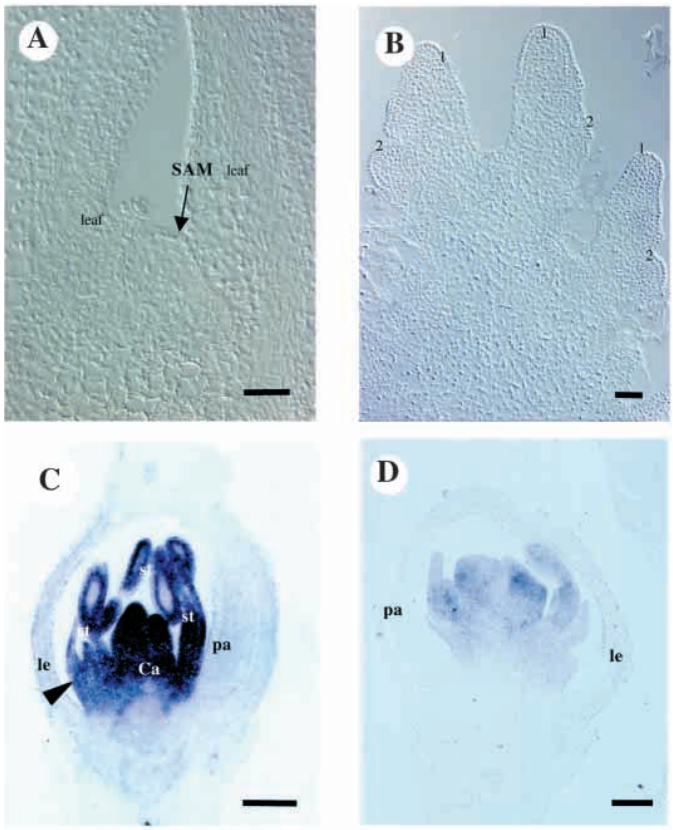

Fig. 7. *OsMADS45* expression in the wild type and *spw1 dl-sup* double mutant. (A) Wild-type vegetative shoot, (B) wild-type inflorescence shoots, (C) wild-type flower and (D) *spw1-1 dl-sup1* flower (longitudinal sections). In wild type, the expression was detected in lodicules (arrowhead), stamens (st) and the carpel (ca), but not in vegetative or inflorescence shoots. In the mutant flower, the expression was detected in organs formed in whorl 2 and inner regions SAM, shoot apical meristem; le, lemma; pa, palea; st, stamen primordia. The numbers, 1 and 2, indicate the primary and secondary inflorescence meristems, respectively. Bar, 50 μm.

two amino acid residues, QR, in the K box (Jacks et al., 1992; Sommer et al., 1990). The residues are preserved in AP3 and DEF, indicating their importance in the AP3 family proteins. In *spw1-2*, the deletion caused by the splicing defect extends to the entire fifth exon, which encodes 14 aa residues of the C-terminal region. There are several reports of the same type of mutations at the splice donor site (GU to AU). However, in all cases, the splicing was blocked to accumulate unprocessed RNA (Orozco et al., 1993; Bradley et al., 1995; Liu and Filipowicz, 1996). To this extent, it is interesting to see whether or not *spw1-2* represents a rather unique case, which might indicate that splicing proceeds from the 3′ to the 5′ end of pre mRNA. Regardless of how the deletions are produced, *spw1-1* phenotypically appears to be as strong as *spw1-2*.

The functional correlation between *SPW1* and *AP3* is shown not only by the sequence similarity but also by loss-of-function phenotypes. The recessive *spw1* mutations transform whorl 3 organs (stamens) to whorl 4 organs (carpels) and whorl 2 organs (lodicules) to organs that resemble the palea, which is normally formed exterior to whorl 2 in the wild-type flower. Also, the maize *silky1* mutant that carries a lesion in the gene homologous to *AP3* (Ambrose et al., 2000) exhibits a phenotype very similar to rice *spw1*. This also supports the notion that class B floral organ identity genes are present in monocots. Although these class B homeotic genes appear to have conserved functions in organ identity specification, other functions of these genes in flower development appear to be diverse. In *spw1* mutants, the number of whorl 2 organs is more than that of the wild type. Furthermore, the *spw1* mutants do not form any functional carpels even in whorl 4, resulting in complete sterility, whereas *ap3* or *pi* mutants are female fertile. The sterility of *spw1* seems to correlate with overproduction of undifferentiated nucellar tissue. It is noteworthy to mention that the *Arabidopsis* class B mutants, *ap3* and *pi*, also affect cell proliferation; however, in these mutants there is a reduction of organ number in whorl 3, while whorl 2 organ number is not altered (Bowman et al., 1991; Jack et al., 1992; Sakai et al., 2000). To this extent, *SPW1* appears to have a specific function in the regulation of the whorl-specific proliferation, which is distinct from *AP3* and *PI*.

Implication of *spw1* homeotic transformation in monocot flower evolution

The identification of *SPW1* as a class B gene in rice provides insight into the floral structure of rice and other monocots. The comparison of *spw1* loss-of-function phenotypes with the class B mutant phenotypes in dicots strongly suggests that lodicules are equivalent to petals and the palea-like organs formed in *spw1* whorl 2 correspond to sepals, whorl 1 floral organs in dicots. This finding is very similar to what was described for the maize *silky1* mutant (Ambrose et al., 2000). Our observation of *spw1* whorl 2 organs being morphologically not identical to the wild-type palea might lead to two diverse interpretations. (1) The transformation of lodicules to paleae is incomplete because of the presence of residual lodicule identity in *spw1*, or (2) organs formed in *spw1* whorl 2 are indeed the sepal-equivalent organs, which are, however, distinct from the wild-type palea and usually not formed in the wild-type floret. These organs are evident only when the class B function is missing in whorl 2. In the latter case, the palea could be considered a bract-like organ, in agreement with the general hypothesis that grass flowers lack sepals (Hackel, 1887; Arber, 1934; Dahlgren et al., 1985). The evolutionary relevance of the homology between paleae and sepals remains to be explored.

DL plays an important role in vegetative and reproductive development

Based on the loss-of-function phenotype, *DL* has functions in two distinct developmental pathways, midrib and carpel development. Mutants similar to *dl* have been identified in other grass species, barley (Tsuchiya, 1962), pearl millet (Rao et al., 1988), and *Panicum maximum* (Fladung et al., 1991). These mutations are single and recessive, and affect both the midrib formation and carpel development. In *P. maximum*, the *mbl* mutation causes midrib-less leaves and the conversion of the gynoecium into stamens (Fladung et al., 1991). In pearl millet, at least two loci, *MRL-1* and *MRL-2*, have been identified, both affecting midrib and carpel development (Rao et al., 1988). In barley, *ovl* causes the loss of midrib and the degeneration of ovary (Tsuchiya, 1962). Although the flower phenotype of these mutants has not been fully characterized except in the case of *mbl* of *P. maximum*, *mrl-1*, *mrl-2* and *ovl* do not show any

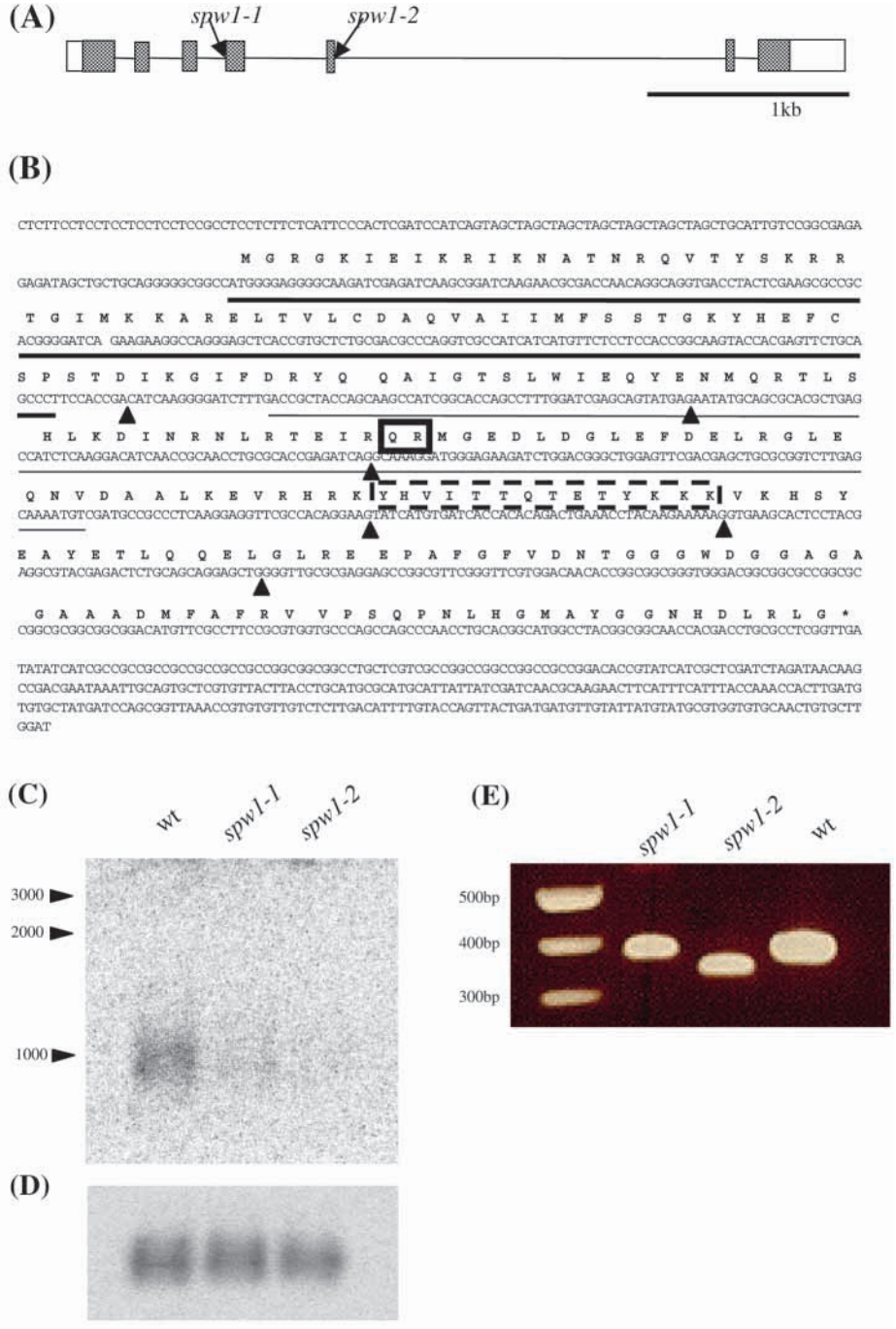

Fig. 8. Structure of *SPW1* gene and the molecular analysis of *spw1* mutations. (A) Genomic structure of the *SPW1* gene. The *SPW1* gene consists of seven exons and six introns. The length of introns are 101, 204, 173, 448, 2168 and 124 bp, respectively. In the *spw1-1* mutant, the last base of third intron of G is mutated to A. In *spw1-2*, the first base of the fifth intron of G is changed to A. (B) Deduced cDNA and protein sequence of *SPW1*. Arrowheads indicate the position of introns. The MADS box is indicated by the thick line and the K-box by the thin line. The solid box indicates the two amino acid residues deleted in *spw1-1*. The dashed box shows the exon that is deleted in *spw1-2*. The genomic sequence is available at the GenBank (accession no. AF424549). (C) RNA blot analysis of the *SPW1* transcript in *spw1* mutants. 1 μg of poly(A) RNA from *spw1* mutants and wild-type was subjected to gel electrophoresis and hybridization with an *SPW1*-specific probe. 1 kb-long *SPW1* transcript was detected in the wild type as well as mutants, but with a lesser amount in mutants. Unspliced RNA was not detected in mutants. (D) The same blot was hybridized with the probe for ubiquitin genes (accession number AC103891). (E) *SPW1* RT-PCR analysis of *spw1* mutants. The amplification of the region between exon 1 and 6 showed that the *spw1-2* mutation resulted in the deletion of an approximately 100 bp-long sequence from *SPW1* mRNA.

homeotic conversion of carpel into stamens. However, midrib-less leaves and occasional abnormal carpel development are common to all the above mutants. Since similar mutations pleiotropically affecting both leaf midrib and carpel development have not been reported in dicots, these genes appear to have a unique combination of functions in monocots. The phenotypic characteristics of four *dl* alleles, particularly floral phenotypes, indicate that *dl-2* is the weakest allele, which does not affect floral organ development, and *dl-sup1* and *dl-sup2* are the strong alleles converting the carpel into stamens. No mutants have been identified which produce normal leaves but cause homeotic conversion of the carpel into stamens. This suggests that the formation of the midrib requires more complete activity of the *DL* gene product than the carpel development does.

The floral phenotype of strong *dl* mutants (*dl-sup1* and *dl-sup2*) is similar to that of *superman* (*sup*) mutants in *Arabidopsis*, which form extra stamens interior to the stamen whorl (Schultz et al., 1991; Bowman et al., 1992). It is reported that *SUP* encodes a C2H2-type zinc-finger protein, and is expressed in the adaxial region of the whorl 3 floral meristem (Sakai et al., 1995). The function of *SUP* is shown to be involved in co-ordinated proliferation control of whorl 3 and 4 floral meristems. However, the *dl* flower exhibits

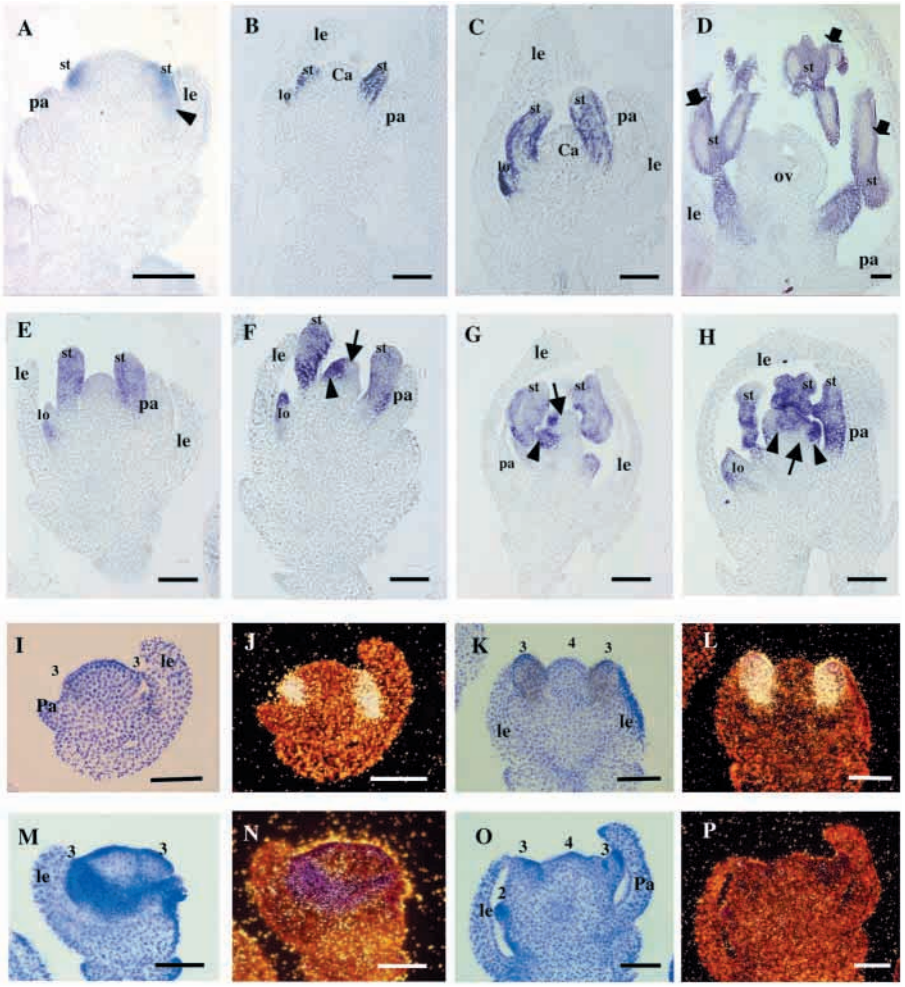

Fig. 9. *SPW1* expression in wild-type, *dl-sup1* and *spw1-1*flowers. (A-D, I-L) Wild-type, (E-H) *dl-sup1* and (M-P) *spw1-1* flowers. (A-H) Non-radioactive, and (I-P) radioactive in situ hybridization. (A) *SPW1* RNA was detected in the floral meristem where incipient lodicule (arrowhead) and stamen primordia were formed. (B-D) *SPW1* expression was detected in developing stamen and lodicule primordia but not in the carpel primordium (B,C) and (D) in the anthers (arrows) and filaments but not in the young ovary (ov) with developing stigma. (E) The expression pattern of *SPW1* in *dl-sup1* background was indistinguishable from the wild type at the stage when whorl 4 showed no sign of differentiation. (F,G,H) Ectopic expression of *SPW1* was detected in the region where ectopic stamens are formed (arrowheads). Ectopic expression of *SPW1* is not detected in the center of the floral meristem throughout flower development (arrows). (I,K,M,O) Bright-field, and (J,L,N,P) dark-field micrographs. (I,J) Floral meristem of a developing wild-type flower. (K,L) A young wild-type flower with developing floral organ primordia. (M,N) Floral meristem of a developing *spw1-1* flower. (O,P) A young *spw1-1* flower with developing floral organ primordia. le: lemma, pa: palea, lo: lodicule, st: stamen, ca: carpel, ov: ovule. The numbers 2, 3 and 4, indicate the position of whorls 2, 3 and 4, respectively. Bars. 30 μm.

several phenotypes seemingly distinct from *sup*. Firstly, *SUP* does not affect vegetative development (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). Secondly, ectopically formed stamens are arranged in a different pattern. In *sup* mutants, the extra stamens are formed in extra whorls that exhibit a duplicated pattern of the stamen whorl (whorl 3) (Schultz et al., 1991; Bowman et al., 1992). In contrast, these extra stamens in *dl-sup* mutants are formed not as a duplication of whorl 3 but as a branch structure along the axis of the palea and lemma. These differences could be explained by assuming that *DL* acquired several new functions during its divergence away from the *DL/SUP* ancestral gene. Nevertheless, taking into account the interaction between *DL* and *SPW1* as discussed below, we are tempted to consider that *DL* is distinct from *SUP* in specification of carpel identity.

Genetic interaction between *SPW1* and *DL*

In order to examine potential genetic interactions between *dl* and *spw1*, we constructed the double mutant. The double mutant showed a phenotype distinct from that seen in *Arabidopsis ap3 sup*. The *ap3 sup* double mutant has a phenotype similar to *ap3* (Schultz et al., 1991; Bowman et al., 1992), but the development of whorl 4 organs is suppressed, which is largely explained by an additive interaction of two mutations (Sakai et al., 2000). In the *spw1-1 dl-sup1* double mutant flower, however, organs whose identities are neither carpel nor stamen are indeterminately produced in whorls 3 and 4. This phenotype again indicates that *DL* function is not to regulate the boundary between whorl 3 and whorl 4 but rather to provide the carpel organ identity and the whorl 4 determinacy. Furthermore, the expression pattern of *OsMADS45*, which we showed to be flower specific, suggests that the organs formed whorl 3 and 4 of double mutant flowers have floral organ identity.

A model of rice flower development

According to previous data derived from cDNA sequences and expression patterns, genes corresponding to the ABC floral homeotic genes in *Arabidopsis* and *Antirrhinum* appear to be present in rice, and their corresponding functions were partly proved by antisense experiments (Chung et al., 1994; Chung et al., 1995; Kang et al., 1995; Kang et al., 1998; Moon et al., 1999; Kyozuka et al., 2000). Two genes sharing homology to *AG*, *OsMADS3* (also referred to as *RAG*) and *OsMADS13*, have been cloned in rice (Lopez-Dee et al., 1999; Kang et al., 1998). Their expression patterns and transgenic experiments suggested that *OsMADS3* is the C function gene in rice (Kang et al., 1998; Kyozuka et al., 2000; Kyozuka and Shimamoto, 2002). Also, two genes sharing homology to *PI*, *OsMADS2* and *OsMADS4*, have

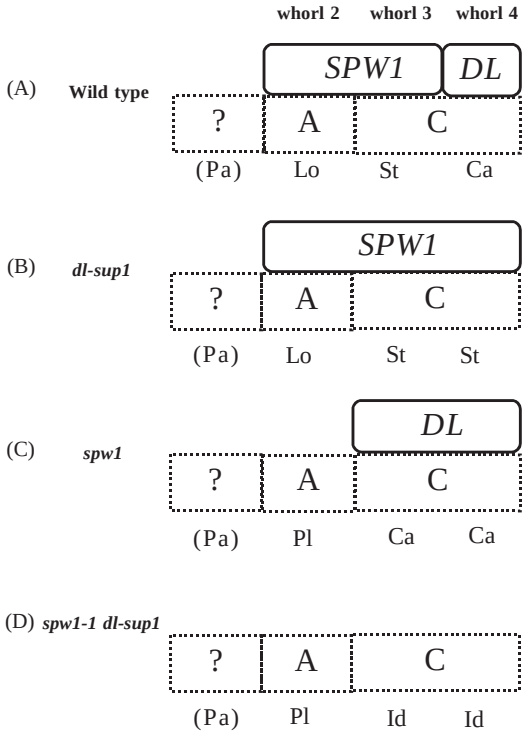

Fig. 10. A model of *DL* and *SPW1* functions in floral organ specification. The model is also based on data that indicate the presence of class A and C gene(s) in rice (for a review, see Goto et al., 2001). (A) In the wild-type flower, *DL* acts in whorl 4 and specifies carpel identity (Ca) together with class C gene(s). *SPW1* acts in whorl 2 and 3 as one of the B function genes and specifies stamen identity (St) in whorl 3 together with class C gene(s) and lodicule identity (Lo) in whorl 2 together with class A gene(s). (B) In *dl* mutants, *SPW1* function expands to whorl 4, which results in the homeotic transformation of the carpel into stamens. (C) In *spw1* mutants, stamens are transformed into carpels by the ectopic activity of *DL* in whorl 3. Whorl 2 organs are specified as palea-like (Pl) through the class A function alone. (D) In the *spw1-1 dl-sup1* double mutant, organs with unknown identity (Id) are formed in whorls 3 and 4 without class B or *DL* functions. Organs in whorl 2 are palea-like, the same as those formed in *spw1* mutants, specified by the class A function alone (see text). Pa, palea.

been cloned in rice. Based on the expression patterns in flowers, *OsMADS2* and *OsMADS4* are believed to be *PI* homologs (Chung et al., 1995; Kyozuka et al., 2000). In the case of *OsMADS4*, the function has further been elucidated by anti-sense experiments (Kang et al., 1998). As for the *AP3* homolog, *OsMADS16* was initially isolated by screening for proteins which could interact with OsMADS4 protein (Moon et al., 1999). We show in this report that the *SPW1* gene is identified as *OsMADS16* and the loss of *SPW1* function causes the phenotype corresponding to the class B mutant phenotype. There are several rice genes sharing sequence homology to the *Arabidopsis* class A gene, *AP1*. Among them, the *LHS1* gene (*OsMADS1*) was shown to carry *AP1* function at least in part (Jeon et al., 2000).

As discussed above, our data show that *DL* has a novel function that has not yet been described in other flowering plants. With regard to its function in floral organ specification, *DL* appears to act in whorl 4 to specify carpel identity, presumably in conjunction with other genes including class C genes (Fig. 10A). The transformation of floral organs in *dl* as well as *spw1* mutants can be explained by the mutually exclusive interaction of both genes: the loss of *DL* function results in the spatial expansion of *SPW1* function in whorl 4, which leads to the transformation of the gynoecium into stamens (Fig. 10B). Likewise, the loss of *SPW1* function causes spatial expansion of *DL* function in whorl 3, which leads to the transformation of stamens into carpels (Fig. 10C). Such mutually antagonistic interaction of *DL* and *SPW1* resembles the one between class A and C activities in *Arabidopsis* (Bowman et al., 1991; Drews et al., 1991). The loss of both *SPW1* and *DL* causes the transformation of whorl 3 and 4 organs into indeterminate structures that produce organs with no apparent identity (Fig. 10D). This indicates that these two rice genes, *SPW1* and *DL*, play an essential role in specifying floral organ identity. In *Arabidopsis*, genes that specify carpel identity were isolated and their interactions with ABC homeotic genes studied (Alvarez and Smyth, 1997; Alvarez and Smyth, 1999; Liu et al., 2000). These genes include *LEUNIG* (*LUG*), *AINTEGUMENTA* (*ANT*), *CRABS CLAW* (*CRC*) and *SPATULA* (*SPT*). Among them, *crc* and *spt* mutants show a significant reduction in carpelloidy in combination with the *pi-1* mutation (Alvarez and Smyth, 1999). None of the loss-of-function mutants of these genes, however, exhibits a phenotype similar to *dl-sup*. Nevertheless, it would be interesting to see whether there is any common genetic pathways controlled by *CRC/SPT* in *Arabidopsis* and *DL* in rice.

Although data on *DL* expression is currently not available, the studies on *SPW1* expression in mutants provide additional data that are consistent with the model. *SPW1* expression was significantly downregulated in the *spw1* mutant. Although this transcriptional repression could be due to a splicing defect, resulting in increased RNA turnover, or possible autoregulation similar to the one reported for *AP3* (Jack et al., 1994), it could also be due to ectopic *DL* activity in the *spw1* mutant background. However, ectopic *SPW1* expression was detected in whorl 4 of the strong *dl* mutants. Ectopic expression was not detected at the stage of the initial *SPW1* expression. Rather, it was seen at the later stage when whorl 3 stamen primordia started to differentiate into filament and anther structures and the whorl 4 floral meristem proliferated to form a mound. The pattern of ectopic expression again differed from what has been observed in *sup* mutants in *Arabidopsis*, where *AP3* expression gradually spread into the whorl 4 area (Sakai et al., 1995). In *dl-sup*, ectopic expression of *SPW1* occurred abruptly in part of the whorl 4 floral meristem. This also suggests that the organ identities of whorls 3 and 4 are not specified at the same stage in rice as they are in *Arabidopsis*. In rice, the specification of whorl 4 appears to occur after that of whorl 3. A model of sequential specification of floral organs has been discussed in other species (Hicks and Sussex, 1971). In *Arabidopsis* and tobacco, floral organ specification was shown not to require signals from outer whorls (Day et al., 1995). It remains to be explored whether floral whorls in rice are also autonomous and independent from each other with regard to organ specification.

According to recent report, the *DL* gene has been identified by implementing a map-based cloning strategy (T. Yamaguchi, Y. Nagato and H. Hirano, personal communication). Further analyses of the *DL* gene at the molecular level could shed more light on the unique function of *DL* and its interaction with other floral homeotic genes.

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