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STAMENLESS 1, encoding a single C2H2 zinc finger protein, regulates floral organ identity in rice

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SUMMARY

Floral organ identity is defined by organ homoetic genes whose coordinated expression is crucial with respect to the time and place of floral organ formation. Here, we report molecular cloning and characterization of the rice *STAMENLESS 1* (*SL1*) gene that is involved in floral development. The *sl1* mutant largely resembles the rice B-class gene mutant *spw1*; both exhibit homeotic conversions of lodicules and stamens to palea/lemma-like organs and carpels. Additionally, *sl1* produces flowers with varied numbers of inner floral organs, and amorphous tissues without floral organ identity were frequently formed in whorls 3 and 4. We also show that *SL1* specifies lodicule and stamen identities through positive transcriptional regulation of *SPW1*/*OsMADS16* expression. *SL1* encodes a member of the C2H2 family of zinc finger proteins, closely related to *JAG* of Arabidopsis. The functional divergence between *SL1* and *JAG* implies that *SL1* was co-opted for its distinctive roles in specification of floral organ identity in rice after the lineage split from Arabidopsis.

Keywords: rice (*Oryza sativa* L.), flower development, floral organ identity, zinc finger protein, functional divergence.

INTRODUCTION

Flowering plants display diverse floral structures. A typical core eudicot flower, for example, is composed of four whorls: sepal, petal, stamen and carpel. The ABC model, mainly based on genetic and molecular studies on *Arabidopsis thaliana* and *Antirrhinum majus*, has been proposed to explain how floral organ identity is defined (Coen and Meyerowitz, 1991). According to this model, three classes of homeotic genes control the floral organ formation, i.e. A-class genes alone specify sepal formation, A-class genes in combination with B-class genes determine petal identity, B- and C-class genes together regulate stamen development, and C-class genes alone specify the innermost whorl, the carpel.

The time and place of floral organ formation, however, require coordinated regulation of these homeotic genes. To achieve this, the activities of floral organ identity genes must be maintained within specific whorls by cadastral genes, which positively or negatively regulate expression of these homeotic genes. Several genes, in addition to A- and C-class genes, are involved in defining floral boundaries in Arabidopsis, including *STERILE APETALA* (*SAP*), *LEUNIG* (*LUG*), *SEUSS* (*SEU*), *RABBIT EAR* (*RBE*) and *SUPERMAN* (*SUP*)

(Sakai *et al.*, 1995; Byzova *et al.*, 1999; Sridhar *et al.*, 2004; Krizek *et al.*, 2006). For example, *SUP* maintains floral organ boundaries by preventing B-class functions from expanding into carpels (Sakai *et al.*, 1995), while *RBE* acts as a repressor of the C-class gene *AGAMOUS* (*AG*) (Krizek *et al.*, 2006). Both *SUP* and *RBE* encode zinc finger transcription factors of the C2H2 type (Sakai *et al.*, 1995; Takeda *et al.*, 2004).

Once floral organ primordia have been initiated, the diverse shapes of individual floral whorls are largely defined by genes responsible for lateral growth. In Arabidopsis, *JAGGED* (*JAG*) and *NUBBIN* (*NUB*) are involved in this lateral organ formation (Dinneny *et al.*, 2004; Ohno *et al.*, 2004). The *jag* mutant produces unenclosed flowers with narrow curling petals, shorter stamens and protruding gynecia (Dinneny *et al.*, 2004; Ohno *et al.*, 2004). The *JAG* paralog, *NUB*, functions redundantly with *JAG* in regulating the lateral growth of stamens and carpels in Arabidopsis (Dinneny *et al.*, 2006). Although *JAG* and *SUP* are expressed in overlapping domains, they show no interaction in floral development, as indicated by the fact that the double mutant *jag-3 sup-5* shows completely additive phenotypes in floral development (Dinneny *et al.*, 2004). A rice ortholog of *JAG* has been identified in rice, but its functions remain unknown (Dinneny *et al.*, 2004).

Rice (*Oryza sativa* L.) has a floral structure that is distinctive from that of Arabidopsis, having a pair of palea and lemma, two small oval-shaped lodicules, six stamens and a pistil. Various lines of evidence support the possibility of functional conservation of some floral organ identity genes between monocots and eudicots. *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are the two B-class genes that specifying petal and stamen identities, respectively, in Arabidopsis (Bowman *et al.*, 1989; Goto and Meyerowitz, 1994; Jack *et al.*, 1994), and *SPW1*/*OsMADS16* and *OsMADS4* have been found to be their orthologs, with conserved functions in rice (Kang *et al.*, 1998; Lee *et al.*, 2003; Nagasawa *et al.*, 2003; Xiao *et al.*, 2003). Similar to loss-of-function mutants of Arabidopsis *AP3* and *PI* genes, lodicules (whorl 2) and stamens (whorl 3) are transformed to palea/lemma-like organs (whorl 1) and carpels (whorl 4) in mutants or transgenic lines with repressed expression of *SPW1*/*OsMADS16* or *OsMADS4* (Kang *et al.*, 1998; Nagasawa *et al.*, 2003; Xiao *et al.*, 2003). Further, *OsMADS3* and *OsMADS58*, the two rice MADS genes that are most closely related to *AG*, are also involved in formation of the two inner whorls and in floral determinacy (Yamaguchi *et al.*, 2006). However, *DROOPING LEAF* (*DL*) mainly provides C-class functions for carpel development in rice (Yamaguchi *et al.*, 2004), alhough it is orthologous to the Arabidopsis *YABBY* gene *CRABS CLAW* (*CRC*) that is involved in nectary development (Bowman and Smyth, 1999). It has been speculated that *DL* was co-opted for its novel functions in specifying carpel identity and floral determinacy after the lineage split between rice and Arabidopsis (Yamaguchi *et al.*, 2004). Thus, although the ABC model is applicable to rice to some extent, it is not well understood how floral organ identity genes are regulated in rice.

We previously identified a rice floral mutant, *stamenless 1* (*sl1*), also named *pistilloid-stamen* (*ps*), that show abnormal floral phenotypes (Wang *et al.*, 2000; Luo *et al.*, 2006). *ps* and *sl1* were later determined to be the same mutant allele. As *sl1* has already been registered (Wang and Zhu, 2000) in the rice database, we use the original name here. *SL1* was fine-mapped to a small region on chromosome 1 (Luo *et al.*, 2006). However, a detailed phenotypic analysis of the *sl1* mutant has not been described. Here, we report molecular cloning of *SL1* and phenotypic analysis of the *sl1* mutant. In addition, we further demonstrated that, although *SL1* shares a similar expression pattern with its Arabidopsis ortholog *JAG*, it exhibits novel functions in specifying the identities of the rice floral organ lodicule and stamen through positive regulation of *SPW1*/*OsMADS16* expression.

RESULTS

The *sl1* mutant produces very large numbers of carpels

A wild-type rice flower comprises two oval-shaped lodicules, six stamens and one carpel, and one pair of palea and lemma, which are enclosed before anthesis and after pollination (Figure 1a,b). *sl1* flowers, however, were not completely enclosed after heading, probably due to their distorted paleae/lemmas (Figure 1c–e) (Wang *et al.*, 2000; Luo *et al.*, 2006). Close examination revealed that varied, sometimes very large, numbers of carpels or carpel-like organs with various shapes were formed inside *sl1* flowers (Figure 1d–g), and palea/lemma-shaped organs of various lengths were found between these carpels and the distorted paleae/lemmas (Figure 1f–h). Although the severity of the *sl1* floral phenotypes varied between flowers, we could not identify apparently normal stamens in the 175 flowers examined; instead there were degenerate stamens, chimeras of stamen and carpel, and carpels with various numbers of stigmas (Table 1 and Figure 1i). Moreover, more than half of these organs that formed in whorl 3 of the *sl1* flowers developed amorphous tissues, i.e. cell masses without any floral organ identity (Table 1 and Figure 1f, g). The innermost whorl was less affected and retained carpel identity, but 44% of the carpels examined had more than two stigmas (Table 1). We also noticed that, in some *sl1* flowers, ovaries with more than two stigmas in whorl 3 were slightly larger than those in the innermost whorl with two branches (Figure 1g). Further, when compared to wild-type (Figure 1j–l), extensive cell proliferation was observed in the inner three whorls of transversely sectioned mature *sl1* flowers (Figure 1m–o). In the basal section of a wild-type flower, six filaments were recognized around the central ovary and the two lodicules were found at the lemma side (Figure 1j),

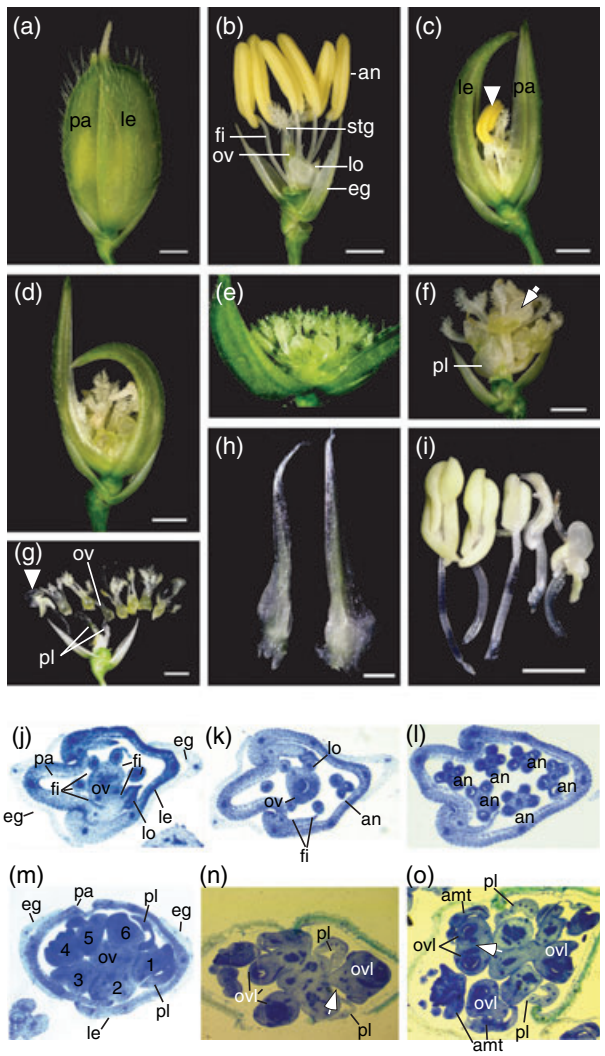

Figure 1. Floral phenotype of the *sl1* mutant.
(a, b) Mature wild-type flower. In (b), the lemma and palea have been removed to reveal the inner organs.
(c–f) Representative *sl1* flowers showing varied numbers of floral organs. Occasionally *sl1* flowers developed stamens as indicated by the arrowhead in (c). Amorphous tissues were also frequently observed as indicated by arrows in (f). In (f), the palea and lemma have been removed.
(g) A mature *sl1* flower with its two inner organs detached. The filament was still recognizable, and anther feature remained at the basal part connected to the filament (indicated by arrowhead).
(h) Two completely transformed palea-shaped organs from an *sl1* flower.
(i) Incomplete transformation of *sl1* stamens with abnormal anthers and apparently normal filaments.
(j–o) Light microscopic images showing transverse sections of *sl1* flowers (m–o) and wild-type flowers (j–l): (j, m) basal, (k, n) central, (l, o) apical parts of the flowers. Note that these palea-like organs contained vascular bundles (stained dark blue). The arrows in (n) and (o) indicate ovary-like organs connected with the innermost organ and two ovary-like organs fused together. The numbers in (m) indicate the six organs residing in whorl 3 where filaments are usually formed in the wild-type.
amt, amorphous tissue; an, anther; ca, carpel; eg, extra glume; fi, filament; le, lemma; lo, lodicule; ov, ovary; ovl, ovary-like tissue; pa, palea; pl, palea/lemma-like; stg, stigma. Scale bars = 1 mm.

Table 1 Floral organ morphology of the *sl1* mutant

	Organ number	Percentage
Whorl 3		
Degenerate stamens[a]	647	28.12
Stamen with amorphous tissues	209	9.08
Chimera of stamen and carpel without amorphous tissues	79	3.43
Chimera of stamen and carpel with amorphous tissues	468	20.34
Carpel with amorphous tissues	590	25.64
Carpel with three or more stigmas	297	12.91
Carpel with two stigmas	11	0.48
Mean organ number per flower in whorl 3	6.38	
Total	2301	100
Whorl 4		
Carpel with two stigmas	210	56.00
Carpel with three or more stigmas	165	44.00
Averaged organ number per flower in whorl 4	1	
Total	375	100

[a]No apparently normal stamens were observed in the 375 flowers, and all paleae and lemmas were unenclosed. All lodicules were elongated to some extent.

whereas in a similarly staged *sl1* flower section, six enlarged organs (ovary-like) and two elongated organs (palea/lemma-like) had formed at the places where filaments and lodicules are usually found (Figure 1m). In sections from the central and apical parts of *sl1* flowers, we often observed larger numbers of carpel-like and palea/lemma-shaped organs [compare Figure 1k, l (wild-type) with Figure 1n, o (*sl1*), respectively]. These observations indicate that, in addition to its roles in specification of lodicule and stamen identities, *SL1* may also regulate cell proliferation in the floral meristem.

SL1 is involved in specifying lodicule and stamen identities

To examine when *SL1* exerts its effects on floral organ identity, we compared floral development between *sl1* and wild-type by SEM analysis. In the *sl1* flower, initiation of floral organ primordia was apparently normal (Figure 2a,b). The earliest abnormality was observed at the time when stamen primordia started to differentiate, and palea and lemma began to close together. Unlike wild-type (Figure 2c,g), stamen primordia in *sl1* flowers failed to differentiate into filaments and anthers (Figure 2d–f). Also, floral meristems in *sl1* flowers lost their determinacy to some extent (Figure 2d–f), resulting in some amorphous tissues being formed in these carpels (Figure 2h–j). The two lodicule primordia were initiated in the *sl1* flower (Figure 2j), but later were much elongated and became palea/lemma-shaped (Figure 2l) compared to the two small wild-type oval-shaped

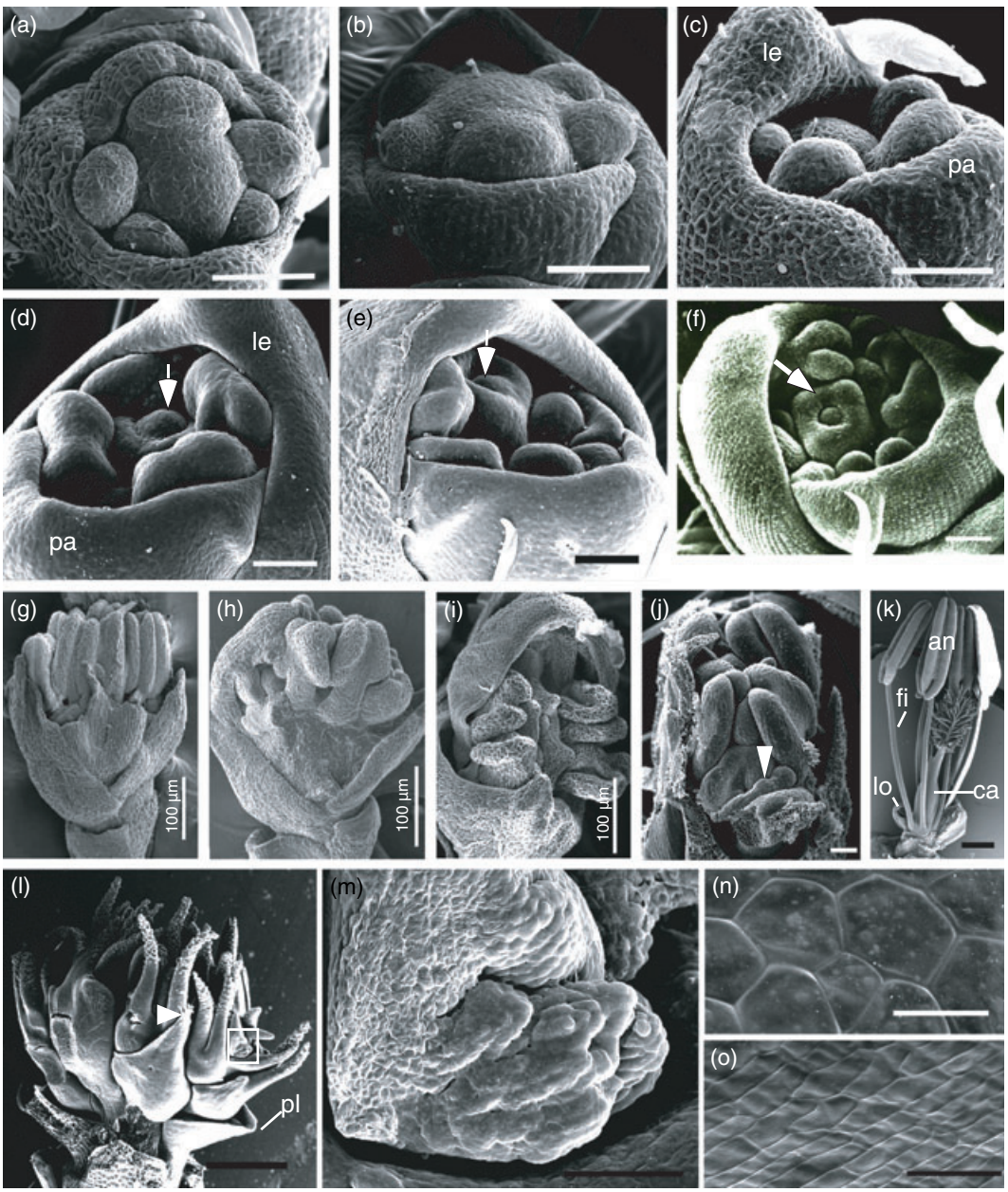

Figure 2. Scanning electron micrographs of *sl1* flowers.
(a) Wild-type flower at an early developmental stage.
(b) *sl1* flower at a similar stage as in (a).
(c) Wild-type flower at the stage when stamen primordia start to differentiate into anthers and filaments.
(d–f) *sl1* flowers at a similar stage as in (c). Arrows indicate indeterminate carpel primordia, especially in (f). Note the cell proliferation around the central primordia (d–f) compared to the wild-type carpel in (c).
(g) Wild-type flower at a mid-development stage.
(h–j) *sl1* flowers at a similar stage as in (g). The arrowhead in (j) indicates an emerging palea/lemma-like organ.
(k) Wild-type flower at the anthesis stage.
(l) Mature *sl1* flower. The arrowhead indicates stigmatic papillae-like tissues formed on palea-shaped organs.
(m) Close-up of the boxed area in (l), showing amorphous tissue of unknown identity.
(n) Epidermis cells of wild-type lodicules.
(o) Epidermis cells of palea/lemma-like organs in (l).
an, anther; ca, carpel; fi, filament; le, lemma; lo, lodicule; pa, palea; pl, palea/lemma-like. Scale bars = 20 μm in (o, p), 50 μm (a–f, p, q), 100 μm (g–i) and 500 μm (k–l). The palea and lemma were removed or partially removed in (g–l).

lodicules (Figure 2k). We also observed stigmatic papillae-like tissues formed on the tips of some palea/lemma-shaped organs in whorl 2 (Figure 2l). The amorphous tissues within *sl1* flowers showed no typical cell features of any floral organ (Figure 2m), suggesting that they were as yet undifferentiated. The epidermis cells of whorl 2 of *sl1* flowers were small and elongated (Figure 2o) compared to wild-type ones (Figure 2n).

Molecular cloning of *SL1*

SL1 was previously localized to a small interval (Luo *et al.*, 2006). In the present study, fine mapping with 1264 recombinants further narrowed the *SL1* locus to a 40 kb interval between markers 39K and 79K (Figure 3a), in which seven coding sequences are predicted (Table S1). After sequencing these coding sequences, we identified a 1 bp insertion in the second exon of the predicted gene *P0408F06.18* from *sl1* (Figure 3b). The insertion was further verified by degenerate cleaved amplified polymorphic sequence (dCAPS) analysis using genomic DNAs and reverse-transcribed total RNA as templates (Figures 3c and S1a). *P0408F06.18* was supported by an EST (C26936), and was previously named *OsJAG* due to its high similarity to the Arabidopsis gene *JAG* (Dinneny *et al.*, 2004).

Because *sl1* is male-sterile (Wang *et al.*, 2000), it failed to produce homozygous seeds for a complementation test. In addition, the original *sl1* mutant was resistant to tissue culture due to its *indica* background. Therefore, to generate *sl1* homozygous cells for transformation, we backcrossed several times the *sl1* progeny derived from an F_2 mapping population to their parent Taipei 309, a *japonica* variety that has been widely used for rice transformation (Chen *et al.*, 1998). Then we introduced the p1301ZF11K vector containing the wild-type genomic sequences for the *OsJAG*/*P0408F06.18* gene into calli induced from immature panicles of these *sl1* plants (Figure 3b). By this approach, we were able to obtained ten independent lines from pZ1301ZF11K via *Agrobacterium*-mediated transformation, and eight out of the ten lines rescued the mutant phenotypes (Figures 3d and S1b,c), demonstrating that *OsJAG*/*P0408F06.18* underlies *SL1*. We therefore renamed *OsJAG*/*P0408F06.18* as *SL1*.

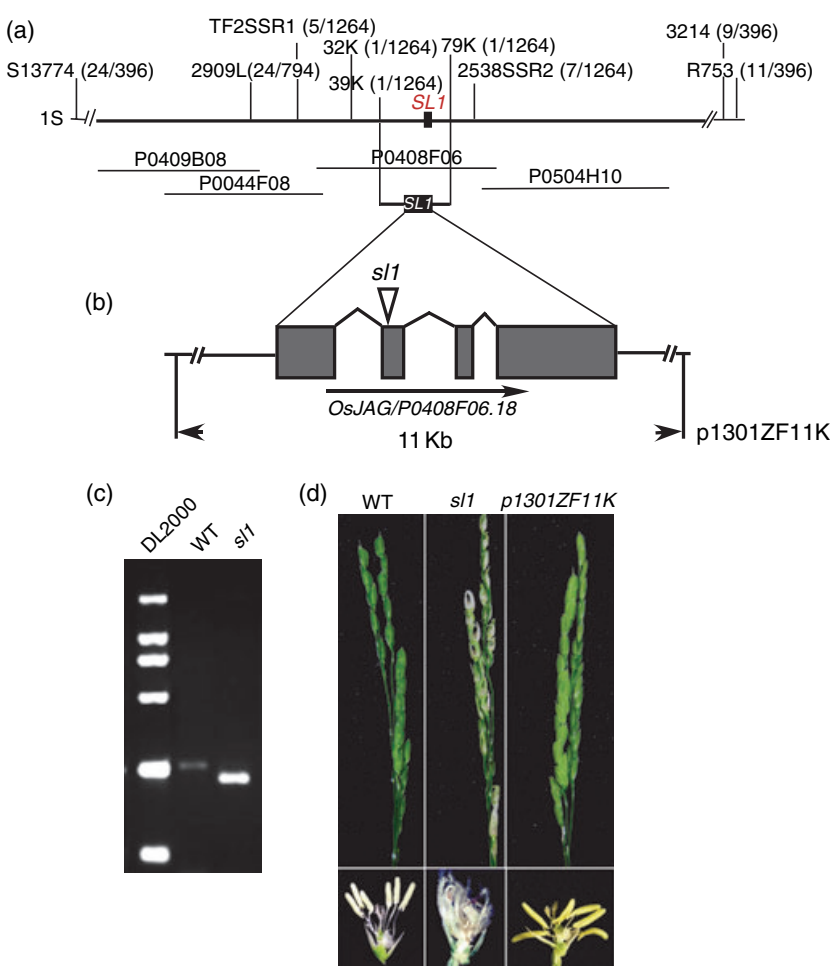

Figure 3. Molecular cloning of *SL1*.
(a) Fine mapping of *SL1* on chromosome 1. The numbers in parentheses indicate the number of recombinants in the segregating chromosomes investigated. The PAC clones covering the region are shown below.
(b) A 1 bp insertion in the second exon of *OsJAG*/*P0408F06.18* caused the mutation in *sl1*. p1301ZF11K for the complementation test contains the 11 kb wild-type genomic fragment.
(c) Verification of the *sl1* mutation by dCAPS. RT-PCR was performed on total RNA isolated from the young panicles of *sl1* and wild-type using primers ZFdCAPSF and ZFdCAPSR2.
(d) Complementation of *sl1* by p1301ZF11K. The upper panel shows representative panicles from wild-type, *sl1* and a representative recapitulation line from p1301ZF11K. The lower panel shows corresponding flowers from the above panicles. The paleae/lemmas were removed from the flowers in the lower panel.

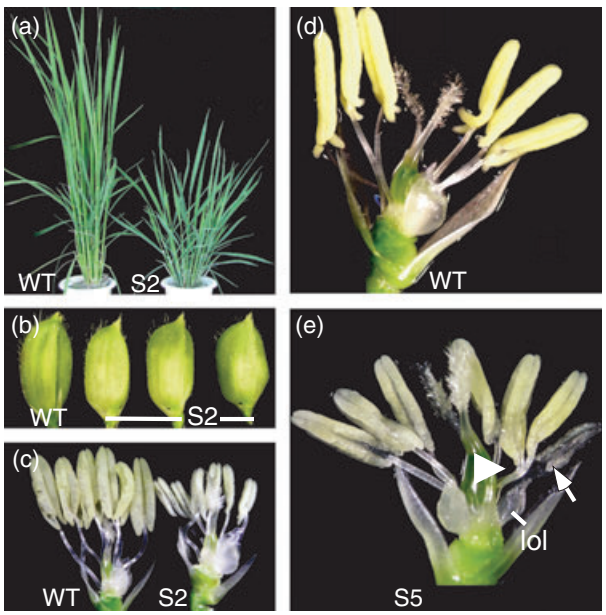

Figure 4. Floral morphology of *SL1* over-expressors.
(a) Representative plants of *SL1* over-expressor line S2 and its non-transgenic control Taipei 309.
(b) Representative flowers of *SL1* over-expressor line S2 and Taipei 309.
(c) Mature flowers of *SL1* over-expressor line S2 and Taipei 309. The palea and lemma were removed to allow the inner whorls to be seen.
(d) Mature flower of Taipei 309.
(e) Mature flower of *SL1* over-expressor line S5. Fused filaments are indicated by the arrowhead, and an extra anther developed from a lodicule-like structure is indicated by an arrow. lol, lodicule-like organs.

To further understand the roles of *SL1* in rice floral development, we also over-expressed *SL1* in Taipei 309 under the control of the maize *Ubi1* promoter. Ten out of thirteen *SL1* over-expressors showed dwarf plant stature with smaller flowers (Figure 4a–c). Some *SL1* over-expressors produced flowers with two stamens fused together at their filaments, and some developed extra lodicules or stamens (Figure 4d,e). Carpel development in these *SL1* over-expressors was apparently not affected (Figure 4d,e). This finding supports the conclusion that *SL1* acts mainly in lodicule and stamen domains during floral development.

SL1 encodes a member of C2H2 zinc finger protein family

Southern blot analysis revealed that *SL1* is a single-copy gene (Figure S1b), and no paralog was identified in the completely sequenced rice genome. *SL1* was deduced to encode a protein of 263 amino acids, sharing similarity to transcription factors with a single C2H2 zinc finger (Figures 5a and S2). A C2H2 zinc finger has been identified in several proteins involved in flower development, such as SUP, JAG and NUB (Sakai *et al.*, 1995; Dinneny *et al.*, 2004; Ohno *et al.*, 2004). SL1 also contains an EAR motif that is predicted to have transcriptional repression activity (Kazan, 2006) (Figure S2). The repressor activity of the EAR motif in SL1 is consistent with the dwarf phenotype obtained by over-expressing *SL1* in Taipei 309 (Figure 4). In addition, there is a glycine-rich stretch following the C2H2 zinc finger (Figure S2). The insertion in the *sl1* allele led to a frame shift and produced a truncated protein (Figure S2).

To further identify motifs shared by SL1 and closely related members from other plant species, including black cottonwood, grape, tomato and maize, together with JAG and NUB of Arabidopsis, we ran the protein motif prediction program MEME using their full-length sequences. SUP was also included due to its shared zinc finger motif and roles in floral development (Sakai *et al.*, 1995). Not surprisingly, SUP only shares motif 1 containing the C2H2 zinc finger with other members (Figure 5a,b), whereas the two monocot members, SL1 and ZmDQ245340 of maize, share very similar motif structures (Figure 5b; consensus sequences of individual motifs are listed in Table S2). Figure 5(a) also shows that, on the basis of motif structure, SL1 is more closely related to JAG than to NUB.

To determine the subcellular localization of the SL1 protein, we generated a construct with the full-length cDNA of *SL1* fused to GFP, and monitored the fluorescence of the transiently expressed SL1–GFP fusion protein and GFP alone in onion epidermis cells. The fluorescence signals of the SL1–GFP fusion protein were observed exclusively in nuclei, while free GFP was diffuse in the cytoplasm and nuclei (Figure 5c), suggesting that SL1 is a nuclear-targeted protein.

SL1 is expressed at early flower developmental stages

To determine the spatial–temporal expression pattern of the *SL1* gene in wild-type plants, we used semi-quantitative RT-PCR and *in situ* hybridization to monitor *SL1* expression in vegetative tissues and panicles at various developmental stages. RT-PCR analysis revealed that *SL1* was highly expressed in young panicles (< 5 cm), weakly in stem, and was not expressed in root and leaf (Figure 6a). During floral development, *SL1* transcripts were first detected in very young panicles of 0.5 cm length, a stage at which organ primordia start to emerge (Ikeda *et al.*, 2004; Itoh *et al.*, 2005), and then higher levels of transcription were found in panicles of length 1 cm and 2–5 cm (Figure 6b). *SL1* expression started to decrease in panicles longer than 5 cm. We also found that *SPW1*/*OsMADS16* shared a very similar temporal expression pattern with *SL1* during floral development – both showed higher expression levels in 1 cm and 2–5 cm panicles (Figure 6b).

Using *in situ* hybridization, *SL1* expression was first detected in the regions where lodicule and stamen primordia were later formed (Figure 7a,b). After carpel primordia were initiated, *SL1* was expressed in all whorls, but stronger signals were detected in lodicules, stamens and carpel (Figure 7c–e). The expression pattern of *SL1* in wild-type flowers is consistent with the observation that the main defects of *sl1* flowers occurred in the inner three whorls.

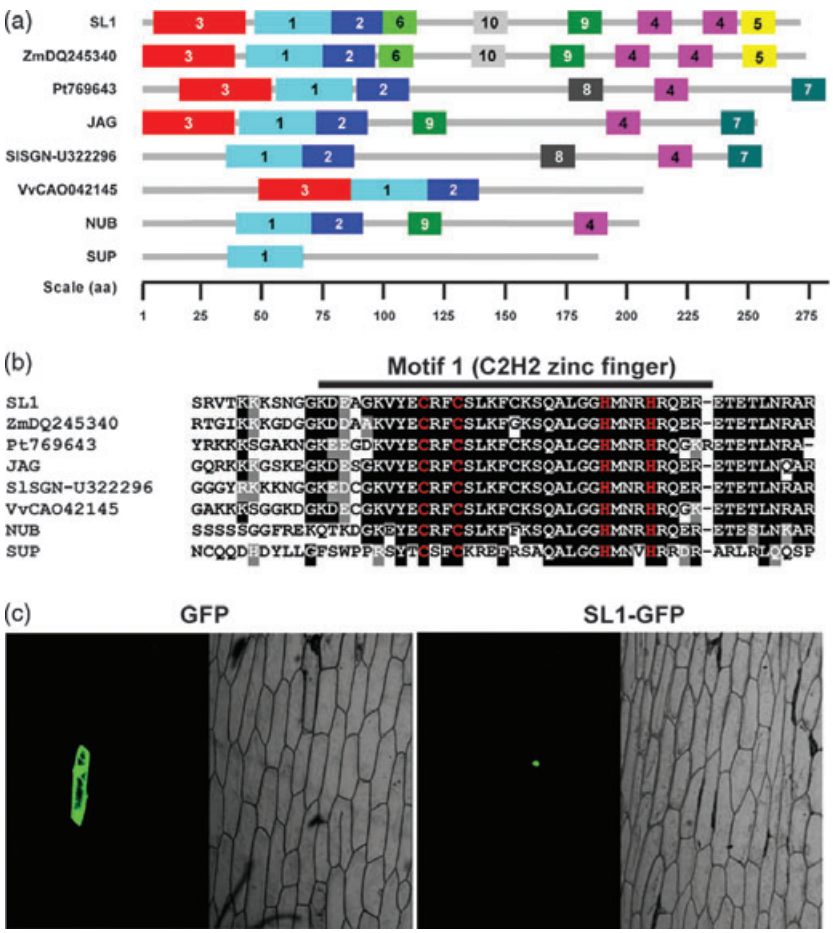

Figure 5. *SL1* encodes a zinc finger protein with a single C2H2 motif.
(a) Protein motifs predicted by MEME. Only motifs with *P* values < 1e-5 are shown. Except for JAG, NUB, SUP and SL1, other proteins are indicated by their accession numbers with a prefix of the first letters of the scientific names for the species: Vv, *Vitis vinifera* (grape); Pt, *Populus trichocarpa* (black cottonwood); Sl, *Solanum lycopersicum* (tomato); Pp, *Physcomitrella patens* (moss); Zm, *Zea mays* L. (maize).
(b) Alignment of amino acid sequences for motif 1 (zinc finger motif).
(c) Subcellular location of the SL1–GFP fusion protein in onion epidermis cells. (Left) A onion epidermal cell expressing free GFP showed fluorescence in the nucleus and the cytoplasm. (Right) An onion epidermal cell expressing SL1–GFP showed fluorescence in the nucleus.

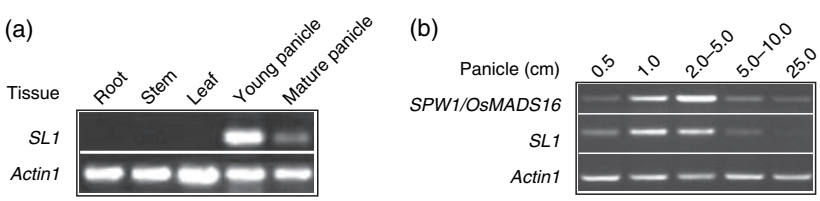

Figure 6. Semi-quantitative RT-PCR analysis of *SL1* and *SPW1*/*OsMADS16* expression.
(a) *SL1* expression in various tissues of wild-type.
(b) Expression of *SL1* and *SPW1*/*OsMADS16* in the developing panicles of wild-type.

SL1 positively regulates *SPW1/OsMADs16*

The *sl1* flower displayed homeotic conversions of lodicules and stamens into palea/lemma-like organs and carpels, which are typical phenotypes of rice B-class mutants (Figures 1 and 2) (Kang *et al.*, 1998; Nagasawa *et al.*, 2003; Xiao *et al.*, 2003). Therefore, we examined *SPW1*/*OsMADS16* expression in the flowers of *sl1* and wild-type by *in situ* hybridization. In wild-type flowers, *SPW1*/*OsMADS16* expression starts in the incipient regions for lodicule and stamen primordia at early floral developmental stages, and continues in these domains afterwards (Nagasawa *et al.*, 2003) (Figure 7f,g), whereas its expression was not observed or very weak in the corresponding domains of *sl1* flowers (Figure 7h–j), suggesting that *SL1* is a positive transcriptional regulator of the rice B-class gene *SPW1*/*OsMADS16*.

We further compared *DL* expression in the developing flowers of *sl1* and wild-type, as *DL* is a major player in specification of carpel identity and carpel determinacy (Yamaguchi *et al.*, 2004). In wild-type flowers, *DL* was first expressed in carpel anlagen (Yamaguchi *et al.*, 2004), and later its expression was restricted to the carpel domain and excluded from the ovule primordia (Yamaguchi *et al.*, 2004)

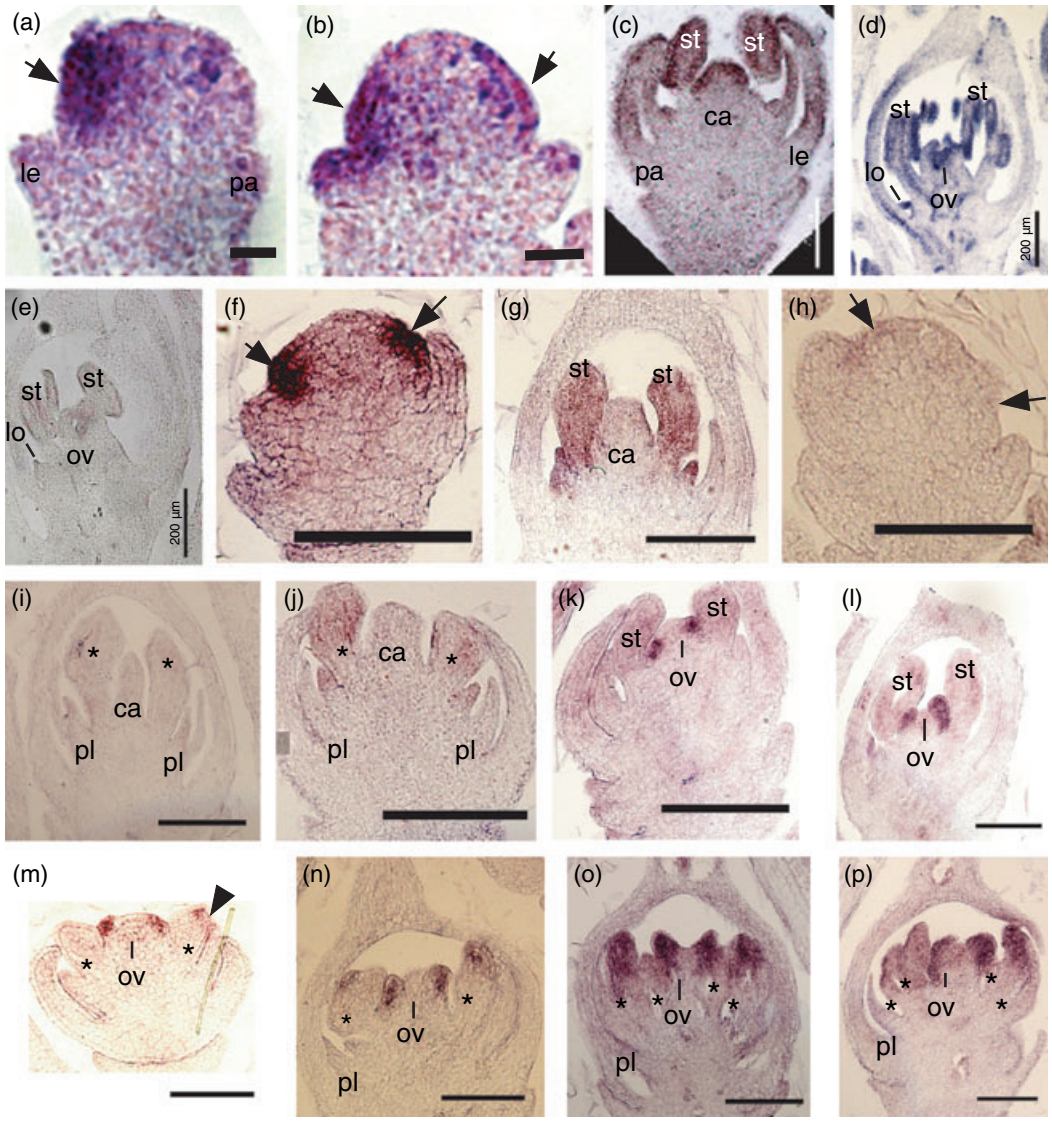

Figure 7. *SL1* expression in *sl1* flowers and wild-type revealed by *in situ* hybridization.
(a–d) *SL1* expression in wild-type flowers as determined by hybridization with an *SL1* antisense probe. Presumptive stamen primordia are indicated by arrows in (a) and (b).
(e) *In situ* hybridizations on a wild-type flower using an *SL1* sense probe.
(f–j) *OsMADS16* expression in wild-type (f, g) and *sl1* (h–j) flowers.
(k–p) *DL* expression in wild-type (k, l) and *sl1* (m–p) flowers.
The arrows in (a), (b), (f) and (h) indicate the regions where lodicule and stamen primordia were formed. Asterisks in (i), (j), (m)–(p) indicate ectopic carpels formed in whorl 3. The arrowhead in (m) indicates *DL* expression at the distal end of a stamen primordium.
ca, carpel; le, lemma; lo, lodicule; ov, ovary; pa, palea; pl, palea-like organs; st, stamen. Scale bars = 20 μm (a, b), 100 μm (c, f–p) and 200 μm (d, e).

(Figure 7k,l). In *sl1* flowers, *DL* expression in the carpel primordia was not affected, but was further extended to whorl 3 (Figure 7m–p). Expression of *DL* was not observed in whorl 2 of *sl1* flowers (Figure 7n–p). In *sl1* flowers, we found that *DL* expression started from the top of whorl 3 (Figure 7m, n), and then extended downwards (Figure 7o,p). Thus, in the *sl1* flower, expression of *SPW1*/*OsMADS16* was repressed in whorls 2 and 3, whereas expression of *DL* was extended to whorl 3.

To assess expression changes of other rice floral homoetic genes in the *sl1* flower, we performed a semi-quantitative RT-PCR analysis on total RNA isolated from young panicles of *sl1* and wild-type. As shown in Figure 8, *SPW1*/*OsMADS16* expression was drastically down-regulated, consistent with the *in situ* hybridization result. *OsMADS4* was slightly repressed in the young panicles of 1 and 2 cm length, but expression of another B-class gene, *OsMADS2*, was apparently not affected (Figure 8). *OsMADS3*, *Os-*

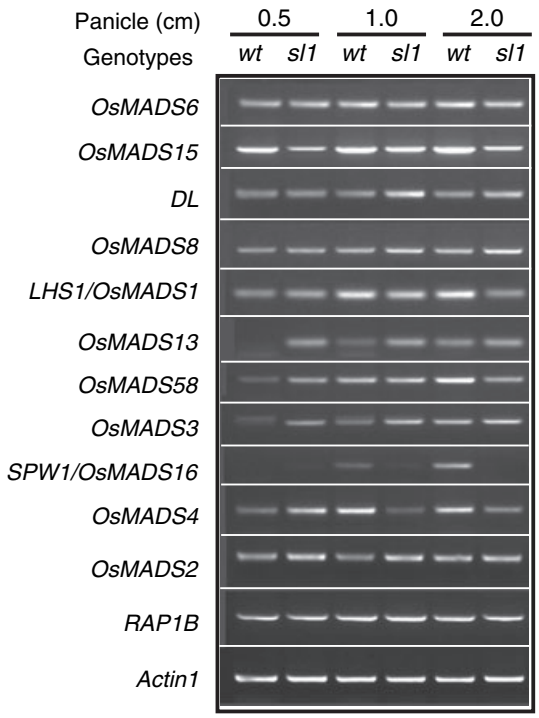

Figure 8. Regulation of rice floral homeotic genes by *SL1*.
Semi-quantitative RT-PCR analysis of the expression of rice floral homeotic genes in the developing panicles of *sl1* and wild-type.

MADS58, *OsMADS13* and *DL*, which are involved in carpel and ovule development (Yamaguchi *et al.*, 2006; Dreni *et al.*, 2007), showed slightly increased expression in *sl1* panicles, whereas expression of two *AP1*/*SQUA* sub-family members, *RAP1B* and *OsMADS15* (Kyozuka *et al.*, 2000; Kater *et al.*, 2006), and three *SEP* sub-family members, *LHS1*/*OsMADS1*, *OsMADS6* and *OsMADS8* (Moon *et al.*, 1999; Jeon *et al.*, 2000; Kater *et al.*, 2006), was not appreciably altered in the *sl1* panicles examined (Figure 8).

DISCUSSION

SL1 is indispensable for specification of floral organ identity

We identified the *SL1* gene by characterizing the *sl1* mutant resulting from a natural mutation, a recessive mutation (Table S3). *sl1* flowers displayed homeotic conversions of varying severity: lodicules were elongated to various extents and became palea/lemma-shaped, and stamens were converted into several types of organs ranging from partially transformed stamens to carpels. Overall, the floral phenotypes observed in *sl1* resemble those of *spw1* – both *sl1* and *spw1* exhibit homeotic conversions in the second and third whorls (Nagasawa *et al.*, 2003), although *sl1* produces a varied numbers of carpels. Thus, *SL1* and *SPW1*/*OsMADS16* may occur in the same pathway to control lodicule and stamen development.

Carpel development was less affected in the *sl1* flower. However, many *sl1* carpels had multiple stigmas, suggesting that *SL1* may also exert weak or indirect effects on carpel formation. With regard to the unenclosed palea and lemma of the *sl1* flower, it is likely that these resulted from the increased numbers of inner organs rather than being a direct effect of the loss-of-function mutation in *SL1*, because the two organs still largely retained their identities. Further, at early floral stages, palea and lemma development was apparently normal in the *sl1* flower. Thus, we reason that *SL1* mainly acts in the second and third whorls.

SL1 regulates cell proliferation in the inner floral whorls

In addition to its roles in specification of lodicule and stamen identities, *SL1* is also involved in regulating cell proliferation during floral development, as amorphous tissues were often formed in the third and fourth whorls of *sl1* flowers. Amorphous tissues have also been observed within flowers of several other rice floral mutants, such as *spw1* and *dl-sup1* (Nagasawa *et al.*, 2003). The double mutant *spw1 dl-sup1* produces an even more severe amorphous tissue phenotype, suggesting that *SPW1* may interact with *DL* in regulation of floral organ determinacy. Loss-of-function mutations in *OsMADS3* and *OsMADS58* cause partial loss of carpel determinacy (Yamaguchi *et al.*, 2006). Further, in addition to these B- and C-class floral homeotic mutants, a mutation in *FON1*, the rice ortholog of Arabidopsis *CLAVATA 1* (*CLV1*), causes undifferentiated cell masses to form in the inner floral whorls (Suzaki *et al.*, 2004). Thus, *SL1*, like *DL*, *FON1*, *OsMADS3* and *OsMADS58*, is necessary for floral determinacy. Nonetheless, it has yet to be determined whether *SL1* is directly involved in regulation of cell proliferation or through the gene(s) mentioned above.

SL1 positively regulates *SPW1/OsMADS16*

Mutation in *SL1* led to weak or no *SPW1*/*OsMADS16* expression in whorls 2 and 3, suggesting that *SL1* may positively regulate *SPW1*/*OsMADS16*. Because the two genes are expressed in the same floral domains and have very similar expression windows, SL1 may be required for proper expression of *SPW1*/*OsMADS16* in its domains of action, i.e. the lodicule and stamen. Thus, the repressed *SPW1*/*OsMADS16* expression may account for most, if not all, of the homeotic conversions that occurred in the *sl1* flower. The slight reduction in *OsMADS4* expression more likely resulted from auto-regulation of the OsMADS4 and SPW1/OsMADS16 complex, because *OsMADS4* expression was less affected in the young panicle of 0.5 cm length, when most flowers started to form organ primordia (Ikeda *et al.*, 2004; Itoh *et al.*, 2005), and our previous study suggested that knockdown of *SPW1*/*OsMADS16* expression could also suppress *OsMADS4* transcription (Xiao *et al.*, 2003). However, we cannot rule out the possibility that *SL1* may directly regulate *OsMADS4* expression. *OsMADS2*

expression was apparently not affected in *sl1* flowers, although *OsMADS2* is also involved in lodicule development (Prasad and Vijayraghavan, 2003; Chen *et al.*, 2006; Yadav *et al.*, 2007; Yao *et al.*, 2008).

The slightly altered expression in *sl1* flowers of other floral homeotic genes, *OsMADS13*, *OsMADS3* and *OsMADS58*, involved in carpel and ovule formation (Yamaguchi *et al.*, 2006; Dreni *et al.*, 2007), may be attributed to their enriched transcripts from carpels that developed in whorl 3. Thus, it is less likely that *SL1* directly represses their expression.

Based on the floral morphology and the altered expression of *SPW1*/*OsMADS16* observed in the *sl1* flower, we propose that *SL1* plays at least two roles in rice floral development (Figure 9a). First, *SL1* is involved in specification of lodicule and stamen identities, probably by maintaining the proper expression of *SPW1*/*OsMADS16*. When SL1 is dysfunctional, *SPW1*/*OsMADS16* expression is substantially suppressed, which leads to insufficient B-class activities in whorls 2 and 3, and increased *DL* expression in whorl 3 as *DL* is negatively regulated by *SPW1*/*OsMADS16* (Yamaguchi *et al.*, 2004). This results in homeotic transformation of lodicules and stamens into palea/lemma-like organs and carpels, respectively (Figure 9b). Second, the formation of amorphous tissues in *sl1* flowers suggests that *SL1* may also regulate floral determinacy. However, these cell masses without particular organ identity in *sl1* flowers could either be a direct effect of the loss-of-function mutation in *SL1* or an indirect effect due to the repressed expression of *SPW1*/*OsMADS16*.

Functional divergence between *SL1* and *JAG*

SL1 is orthologous to the Arabidopsis gene *JAG*, encoding a C2H2-type zinc finger protein.The two genes share some similarities in their expression patterns and gene actions. During early floral development, *JAG* is expressed in the primordia of sepals, stamens and carpels (Dinneny *et al.*, 2004; Ohno *et al.*, 2004). Similarly, *SL1* is also mainly expressed in lodicule and stamen primordia at early floral developmental stages, and later in all four floral whorls. Consistent with their expression patterns, both *sl1* and *jag* mutants show main defects in the second and third floral whorls (Dinneny *et al.*, 2006), suggesting that *SL1* and *JAG* function in similar floral domains. However, despite these similarities, *SL1* and *JAG* play distinctive roles in leaf and flower development. *SL1* regulates floral organ identity and determinacy, and appears not to play a role in leaf development because no obvious defects in leaf morphology were observed in *sl1* (data not shown). In contrast, *JAG*, together with its paralog *NUB*, mainly control leaf and floral organ shapes by promoting cell proliferation on the adaxial sides in Arabidopsis (Dinneny *et al.*, 2004, 2006; Ohno *et al.*, 2004). In addition, *JAG* and *NUB* are not believed to play any role in specification of floral organ identity because even the double mutant *jag nub* shows no homeotic transformation in any floral organ (Dinneny *et al.*, 2006). Further, overexpressing *SL1* in the Arabidopsis *jag2* mutant failed to rescue its defects in leaf and floral organs (Figure S3), reinforcing the view that *SL1* and *JAG* have distinctive functions. This indicates that *SL1* and *JAG* evolved their diversified functions during their respective evolution after the lineage split between rice and Arabidopsis.

In addition to *SL1*, *DL* also exhibits functions that are distinct from those of its Arabidopsis ortholog *CRC*. *DL* is required for specifying carpel identity and determinacy in rice (Yamaguchi *et al.*, 2004). Its loss-of-function mutant *dl* produces extra stamens and shows reduced carpel determinacy, in addition to its defect in leaf morphology (Nagasawa *et al.*, 2003; Yamaguchi *et al.*, 2004). *CRC*, however, mainly regu-

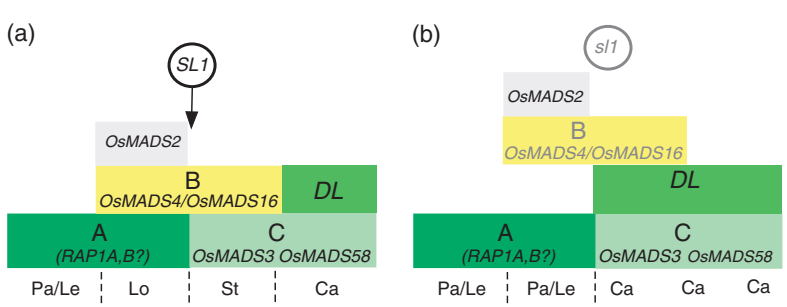

Figure 9. Possible roles for *SL1* in rice floral organ formation.
(a) In wild-type, *OsMADS4* and *SPW1*/*OsMADS16* function as class B genes, together with *AP1* genes (not identified or confirmed yet), to specify lodicule and stamen identities (Kang *et al.*, 1998; Lee *et al.*, 2003; Nagasawa *et al.*, 2003; Xiao *et al.*, 2003). In addition, *OsMADS2* is involved in lodicule development (Prasad and Vijayraghavan, 2003; Chen *et al.*, 2006; Yadav *et al.*, 2007; Yao *et al.*, 2008). Although *OsMADS3* and *OsMADS58* provide some C-class functions (Yamaguchi *et al.*, 2006), *DL* is mainly responsible for specification of carpel identity and determinacy (Yamaguchi *et al.*, 2004). *SL1* is required to maintain the proper expression of *SPW1*/*OsMADS16*, and is also directly or indirectly involved in specifying floral determinacy.
(b) Loss of function of *SL1*, as in the *sl1* mutant, represses expression of *SPW1*/*OsMADS16* in whorls 2 and 3, which causes homeotic conversions of the two whorls into palea/lemma-like and carpels, respectively. *OsMADS4* and *SPW1*/*OsMADS16* are shown in gray to indicate their repressed expression in the *sl1* flower. However, the repression of *OsMADS4* is more likely to be due to auto-regulation of the OsMADS4 and SPW1/OsMADS16 interaction.

lates nectary development and lateral gynecium elongation in Arabidopsis (Bowman *et al.*, 1999). The *crc* mutant shows no floral homeotic alterations (Alvarez and Smyth, 1999). Although *DL* can partially rescue the *crc-1* phenotypes in Arabidopsis, the functions of *DL* in specifying carpel identity and leaf development could not be reproduced in Arabidopsis (Fourquin *et al.*, 2007). Thus, the functional divergence between *SL1* and *JAG*, and *DL* and *CRC*, implies that the mechanisms underlying the regulation of floral organ identity are not strictly conserved between rice and Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant materials

The *sl1* mutant, originally discovered in an *indica* background, is male sterile (Table S3) (Wang *et al.*, 2000). We therefore developed F_2 mapping populations by crossing *sl1* to two *japonica* cultivars, Taipei 309 and Jingxi 17. The homozygous *sl1* plants in the Taipei 309 background for transformation in the complementation test were obtained by back-crossing *sl1* progeny from an *sl1* x Taipei 309 F_2 population to Taipei 309. The *SL1* alleles were genotyped using dCAPS marker ZFdCAPS1/*Dra*I with the *SL1* gene-specific primers ZFdCAPSF1 and ZFdCAPSR1. Primer sequences are listed in Table S4.

Genetic mapping

F_2 progeny with mutant phenotypes were used for genetic mapping, and a few wild-type plants were used for verification. An initial screen for molecular markers linked to *SL1* was performed using genetic markers from public available rice databases, including Gramene (http://www.gramene.org) and Rice Genomic Research Program (http://rgp.dna.affrc.go.jp/publicdata/caps/index.html). Then fine mapping was performed using markers developed from comparisons of genomic sequences from *indica* (http://rise.genomics.org.cn/rice/index2.jsp) and *japonica* (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). Primer sequences for these markers are listed in Table S4.

Generation of transgenic plants

For complementation of the *sl1* phenotype, the 11 kb genomic fragment containing the *OsJAG*/*P0408F06.18* gene (6.7 kb promoter plus 2.5 kb coding sequences and 1.8 kb 3′ sequences) was recovered from *Kpn*I/*Nco*I-digested BAC clone P30 identified from an IRBB56 genomic library (Wang *et al.*, 2001) by PCR using *SL1* gene-specific primers ZF1 and ZF2 (Table S4). Then the fragment was cloned into the pCAMBIA1301 vector to generate plasmid p1301ZF11K. p1301ZF11K was transformed into embryogenic calli derived from immature panicles of *sl1* homozygous plants in the Taipei 309 background via *Agrobacterium*-mediated transformation as described previously (Xiao *et al.*, 2003).

Full-length *SL*1 cDNA was obtained from total RNA isolated from young panicles (< 5 cm) of the wild-type using a BD SMART RACE cDNA amplification kit (BD Biosciences), and cloned into pBluescript II SK(+) (Stratagene, http://www.stratagene.com/) to generate KSSL1FL. Then the full-length *SL1* cDNA fragment was inserted between the maize *Ubi1* promoter and the *nos* terminator of pCTK303 (Wang *et al.*, 2004) to create pCTK303SLFL. pCTK303SLFL was transformed into embryogenic calli derived from Taipei 309 seeds as previously described (Xiao *et al.*, 2003).

RT-PCR analysis

Total RNAs were isolated from various tissues of the *sl1* mutant and the wild-type using an RNeasy plant mini kit and RNase-free DNase (Qiagen, http://www.qiagen.com/) according to the manufacturer's instructions. For each sample, first-strand cDNA was synthesized from 2 μg total RNA using M-MLV reverse transcriptase (Promega, http://www.promega.com/) in a 25 μl reaction volume. Then 0.5 μl of each reverse transcription product was used for PCR with gene-specific primers (Table S4). PCR was performed with 25 cycles (for the loading control *ACTIN 1*) or 30 cycles (for the remaining genes) of 94°C for 30 sec, 58°C for 30 sec and 72°C for 50 sec, followed with a 5 min extension of 72°C.

In situ hybridization

Flowers were collected from 0.2–5 cm long panicles of *sl1* and wild-type, and fixed in FAA (10% formaldehyde, 5% acetic acid, 47.5% ethanol) overnight at 4°C. Samples were then dehydrated through a butanol series, and embedded in Paraplast Plus (Sigma-Aldrich, http://www.sigmaaldrich.com/). Sections 10 μm thick were obtained using a Leica RM2135 microtome (Leica Biosystems, http://www.leica.com). To prepare the *SL1* probe, a 413 bp *SL1* cDNA fragment containing the coding sequence for the last 68 amino acids and 3′ end was first amplified from KSSL1KL using primers SL3iF and SL3iR (Table S4), and then cloned into pGEM-T Easy vector (Promega). The probe was synthesized using a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics Ltd, http://www.roche.com) according to the manufacturer's recommendations. *DL* and *SPW1*/*OSMADS16* probes (Table S4) were prepared in the same way. Pre-treatment of sections, hybridization and immunological detection were performed as previously described (Li *et al.*, 2005), except that the last wash after hybridization was performed in 0.1 × SSC at 55°C for 30 min.

Subcellular localization of SL1 protein

The full-length *SL1* coding sequence was amplified from KSSLFL using primers SalZF and ZFNcoIR (Table S4), and then cloned into vector CaMV35S-sGFP(S65T)-Nos(pUC18) (Niwa *et al.*, 1999) (a gift from Dr Yasuo Niwa, Laboratory of Plant Cell Technology, University of Shizuoka, Japan), to create pSL1-GFP. pSL1-GFP or CaMV35S-sGFP(S65T)-Nos(pUC18) were transformed into onion epidermis by particle bombardment using a PDS-1000/He biolistic particle delivery system (Bio-Rad, http://www.bio-rad.com/). After 24 h, fluorescence was visualized under a Bio-Rad MRC 1024 fluorescence confocal microscope.

Protein motif prediction

Genes closely related to SL1 were identified by BLAST search against various public databases including the Solanaceae Genomics Network for tomato (SGN, http://www.sgn.cornell.edu), the Joint Genome Institute for black cottonwood (http://genome.jgi-psf.org/), Genoscope for grape (http://www.genoscope.cns.fr/externe/English/Projets/Projet_ML/index.html), and NCBI for others (http://www.ncbi.nlm.nih.gov). The protein motif prediction program MEME version 3.5.4 (http://meme.sdsc.edu/meme/meme.html) was used to identify conserved motifs among these proteins (Bailey *et al.*, 2006). The 54 amino acid sequences of motif 1 (zinc finger motif) from these proteins were used for a complete alignment using Clustal X version 1.83.

Microscopy

Flowers at various developmental stages from *sl1* and wild-type were collected and fixed in FAA solution overnight at 4°C, then

dehydrated through an ethanol series from 30–100%. For light microscopy, embedding and sectioning were essentially the same as the preparations for *in situ* hybridization described above. Sections were stained with 0.1% toluidine blue, observed under a Leica DMR light microscope, and images were taken using a Micro Color charge-coupled device (CCD) camera (Apogee Instruments Inc., http://www.ccd.com/). For scanning electron micrograph (SEM) analysis, after a final wash with absolute ethanol, samples were sequentially processed by critical point drying, coating and mounting as previously described (Xiao *et al.*, 2003). Images were obtained using a Quanta200 scanning electron microscope (FEI Co., http://www.fei.com/) at the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China), using an accelerating voltage of 10 or 15 kV.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Southern and dCAPS analysis of the *sl1* mutant and the recapitulation lines.

Figure S2. Deduced amino acid sequence of the *SL1* gene.

Figure S3. Over-expression of *SL1* in the Arabidopsis mutant *jag2*.

Table S1. Predicted coding sequences in the 40 kb interval between markers 39K and 79K encompassing *SL1*.

Table S2. Motif sequences predicted in SL1 and its closely related proteins by MEME.

Table S3. Segregation of the *sl1* allele in three F_2 populations.

Table S4. Primers used in this study.

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The Genbank accession number for the *SL1* sequence is EU443151.

