Regulation of Inflorescence Branch Development in Rice Through a Novel Pathway Involving the Pentatricopeptide Repeat Protein sped1-D

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ABSTRACT Panicle type has a direct bearing on rice yield. Here, we characterized a rice clustered-spikelet mutant, sped1-D, with shortened pedicels and/or secondary branches, which exhibits decreased pollen fertility. We cloned *sped1-D* and found that it encodes a pentatricopeptide repeat protein. We investigated the global expression profiles of wild-type, 9311, and sped1-D plants using Illumina RNA sequencing. The expression of several GID1L2 family members was downregulated in the sped1-D mutant, suggesting that the gibberellin (GA) pathway is involved in the elongation of pedicels and/or secondary branches. When we overexpressed one GID1L2, AK070299, in sped1-D plants, the panicle phenotype was restored to varying degrees. In addition, we analyzed the expression of genes that function in floral meristems and found that *RFL* and *WOX3* were severely downregulated in sped1-D. These results suggest that sped1-D may prompt the shortening of pedicels and secondary branches by blocking the action of GID1L2, RFL, and Wox3. Moreover, overexpression of *sped1-D* in *Arabidopsis* resulted in the shortening of pedicels and clusters of siliques, which indicates that the function of *sped1-D* is highly conserved in monocotyledonous and dicotyledonous plants.

PEDICEL length is one of the most important properties of rice yield. However, little is known about the mechanisms that control pedicel elongation. Here, we report a novel rice panicle mutant with shortened pedicels (sped1-D), which results in the formation of spikelet clusters on secondary branches, and we present evidence that the *sped1-D* gene encodes a pentatricopeptide repeat protein involved in the gibberellin (GA)-signaling pathway. In addition, RFL and WOX3, which play important roles in inflorescence branch elongation, are downregulated in sped1-D.

Inflorescence architecture is an important evolutionary characteristic in the reproductive processes of flowering plants. Many studies have focused on inflorescence development, and many mutants and corresponding genes related to flowering have been isolated in model dicotyledonous plants, such as *LEAFY* (*LFY*) (Schultz and Haughn 1991), *APETALA1* (*AP1*) (Mandel *et al.* 1992), *CLV1*, *CLV2*, *CLV3* (Clark *et al.* 1993, 1995), *WUSCHEL (WUS)* (Laux *et al.* 1996; Mayer *et al.* 1998), *BREVIPEDICELLUS* (*BP*) (Venglat *et al.* 2002), and *BELLRINGER* (*BLR*) (Byrne *et al.* 2003) in *Arabidopsis*. In addition, the well-known WUS–CLV feedback loop was proposed, which regulates inflorescence structures generated from inflorescence meristems (Brand *et al.* 2000; Schoof *et al.* 2000; Nardmann and Werr 2006). In monocotyledonous plants, inflorescences are commonly referred to as panicles, and studies about these inflorescences have mainly focused on maize and rice. To date, a dozen mutants have been identified from maize, and their corresponding genes have been cloned, such as *KNOTTED1* (*Kn1*) (Greene *et al.* 1994; Jackson *et al.* 1994), *INDETERMINATE SPIKELET1* (*IDS1*) (Chuck *et al.* 1998), *FASCIATED EAR2* (*FEA2*) (Taguchi-Shiobara *et al.* 2001), *BRANCHED SILKLESS1* (*BD1*) (Chuck *et al.* 2002), and *ZFL1*, *ZFL2* (Bomblies *et al.* 2003). In addition, more than 20 panicle-type mutants have been identified in rice. However, only *lax* (Komatsu *et al.* 2002), *fzp9* (Yi *et al.* 2005), *sp1* (Li *et al.* 2009), aberrant panicle organization 1 (*APO1*), (Ikeda-Kawakatsu *et al.* 2012), and *TAWAWA1* (Yoshida *et al.* 2012) have been isolated. Cloning of additional genes that control different stages

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of inflorescence growth will provide further insights into the molecular mechanisms underlying rice panicle development.

The pentatricopeptide repeat (PPR) protein family is one of the largest and most perplexing families in plants (Small and Peeters 2000). There are 450 PPR proteins in *Arabidopsis thaliana* (Lurin *et al.* 2004) and 477 in rice (*Oryza sativa*) (O'Toole *et al.* 2008). The large number of PPR proteins in these plants is consistent with the role of these proteins in organelle gene expression and transcriptional regulation in plants (Delannoy *et al.* 2007; Schmitz-Linneweber and Small 2008). PPR proteins govern various steps in RNA metabolism, such as cleavage, splicing, stability, editing, and translation, by forming sequence-specific associations with RNA (Schmitz-Linneweber and Small 2008; Fujii and Small 2011). Increasing evidence indicates that PPR proteins play important roles in plant development (Ding *et al.* 2006; Sung *et al.* 2010; Sosso *et al.* 2012; Yuan and Liu 2012).

In this study, we characterized the rice mutant sped1-D, a clustered-spikelet dominant mutant with shortened pedicels and secondary branches and demonstrated that the mutant phenotype of sped1-D is due to two nucleotide substitutions in *SPED1*. The *sped1-D* gene encodes a mitochondrion-localized PPR-like protein, which indicates that this gene may regulate the development of inflorescence branches through a novel pathway.

Materials and Methods

Plant material and culture

The rice spontaneous mutant sped1-D, which contains clustered spikelets, was isolated in the breeding line 9311. A F2 mapping population with 1929 wild-type and 5598 mutant-type plants (χ^2 test value is 1.576, $<\chi^2_{0.95} = 3.84$, indicating that the segregation ratio is 1:3) was generated from crosses between the sped1-D mutant and the *japonica* variety TP309. All of these plants, including the parents and their offspring (Supporting Information, Table S5), were grown in the fields or greenhouse.

Scanning electron microscopy analysis

Samples were fixed overnight in FAA at 4°. After dehydration in a graded ethanol series and substitution with 3-methylbutyl-acetate, the samples were critical-point dried, sputter-coated with gold, and observed under a scanning electron microscope (S-3000N; Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV.

Positional cloning and complementation test of sped1-D

The *sped1-D* locus was mapped to a 19.2-kb DNA fragment between two closely linked sequence-tagged site (*STS*) markers, csp30 and csp25, on chromosome 6 using an F2 population of *sped1-D* and TP309 (*spp. japonica*). Three putative genes were identified in this region using a gene prediction program, Rice Automated Annotation System (http://RiceGAAS.dna.affrc.go.jp). The genomic sequences of the *sped1-D* candidate gene in the sped1-D mutant were determined by performing direct sequencing after PCR amplification. For complementation, a 2855-bp fragment, which included 775 bp upstream of the initiation codon and 567 bp downstream of the stop codon of *sped1-D*, was cloned into the binary vector pCAMBIA1301 and introduced into wild-type, TP309, and 9311 rice by *Agrobacterium*-mediated transformation (Hiei *et al.* 1994). The full-length cDNA of *sped1-D* was prepared and cloned into pCAMBIA1300 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The overexpression construct was also transformed into TP309 and 9311 using *Agrobacterium*-mediated transformation.

DGE analysis

Immature wild-type and mutant panicles were harvested at heading time, and their total RNAs were extracted using Trizol (Invitrogen). DGE (digital gene expression profiles) library construction and sequencing were carried out at Beijing Genomics Institute (BGI-Shenzhen, Shenzhen, China). Raw sequences were transformed into clean tags by removing dirty raw reads. The total numbers of clean tags from the wild-type and mutant library were 5,678,386 and 5,737,757, respectively. All clean tags were mapped to the reference sequences and only 1-bp mismatch was considered. Clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designed as unambiguous clean tags. The number of unambiguous clean tags for each gene was calculated and normalized to TPM (number of transcripts per million clean tags) ('t Hoen *et al.* 2008; Morrissy *et al.* 2009). The rigorous algorithm $p(\chi) = e^{-\lambda}\lambda^{\chi}/\chi!$ (λ and χ represent the real transcripts and clean tags of the gene, respectively) was used to identify differentially expressed genes (DEGs) between two samples (Audic and Claverie 1997). *P*-value corresponds to the differential gene expression test, and the false discovery rate (FDR) method was used to determine the threshold of *P*-values. The DEGs were identified using the criteria FDR ≤ 0.001 and absolute value of the $\log^2$ ratio ≥ 1. Cluster analysis of gene expression patterns was performed with Cluster (Eisen *et al.* 1998) and Java Treeview (Saldanha 2004) software. Significantly enriched metabolic pathways and signal transduction pathways were identified using the KEGG database to perform pathway enrichment analysis of DGEs compared with the whole genome background.

Bioinformatics analysis

The full-length cDNA of *SPED1* was obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/). Domain prediction for SPED1 was performed using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). A search for SPED1 homologs in plants was performed using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was performed using Multalin (http://multalin.toulouse.inra.fr/multalin/). Unrooted neighbor-joining trees of SPED1 homologs were generated using MEGA5. Bootstrap values of >50% are shown. Prediction of the 3-D structure of SPED1 and sped1-D was carried out using phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/). The template

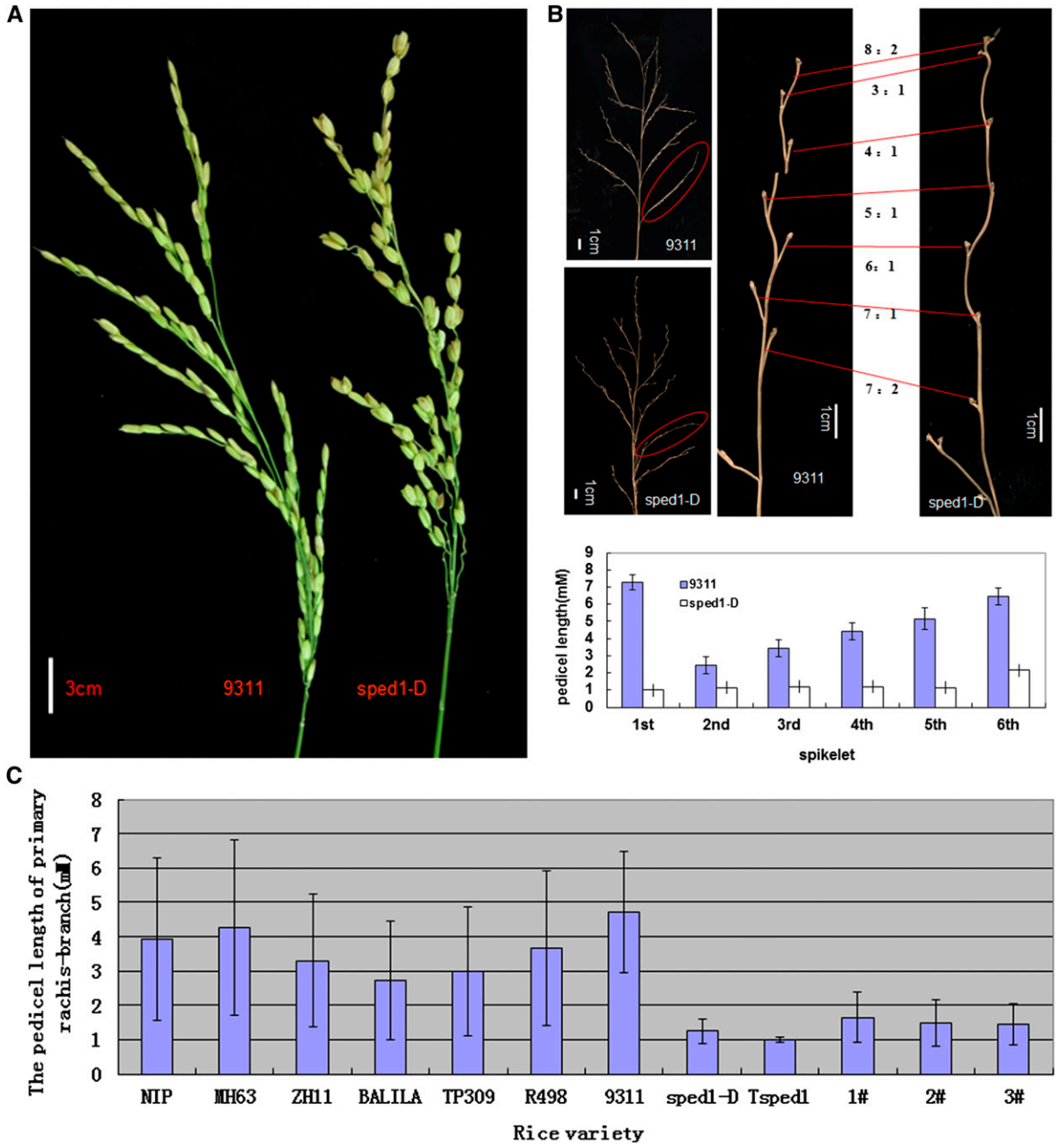

Figure 1 The phenotype of the panicles of the sped1-D mutant. (A) Cluster spikelets of sped1-D (right) *vs.* the wild type, 9311 (left). (B) Comparison of pedicel lengths in secondary branches between sped1-D and 9311. The ratio of the lengths of pedicels 1–6 on one secondary branch from the same position in TP309 and sped1-D (left); statistical analysis of the length of six pedicels on secondary branches (right). Error bars indicate standard error (SE) for sevenreplicate experiments. (C) Pedicel lengths of secondary branches in seven rice varieties, Tsped1, sped1-D, and three hygromycin-positive T0 complementation transgenic plants (1–3). Error bars indicate SE for seven replicate experiments. (D) The degree of abortion and thousand-grain weight of sped1-D seeds are negatively correlated with the degree of clustering in spikelets. (E) Five types of seeds are present in the mutant Tsped1, normal glumes, and paleas but without kernels; 2, abnormal paleas and without kernels; 3–5, seeds with normal kernels but altered grain filling.

for homology modeling was the crystal structure of the pentatricopeptide repeat protein ppr10 from maize.

Subcellular localization

The *SPED1* and *sped1-D* coding regions were amplified without their stop codons and inserted into the multiple cloning site (MCS) of the CaMV 35S promoter–MCS–YFP coding sequence-NOS terminator cassette of the pSAT6-EYFP-C1 vector (Tzfira *et al.* 2005). This construct generated SPED1::YFP and sped1-D::YFP fusion proteins. MitoTracker Red (Invitrogen, http://www.invitrogen.com/), a mitochondrion-specific dye, was used to label the mitochondria. Protoplast preparation and transformation procedures were performed as previously described (Bart *et al.* 2006).

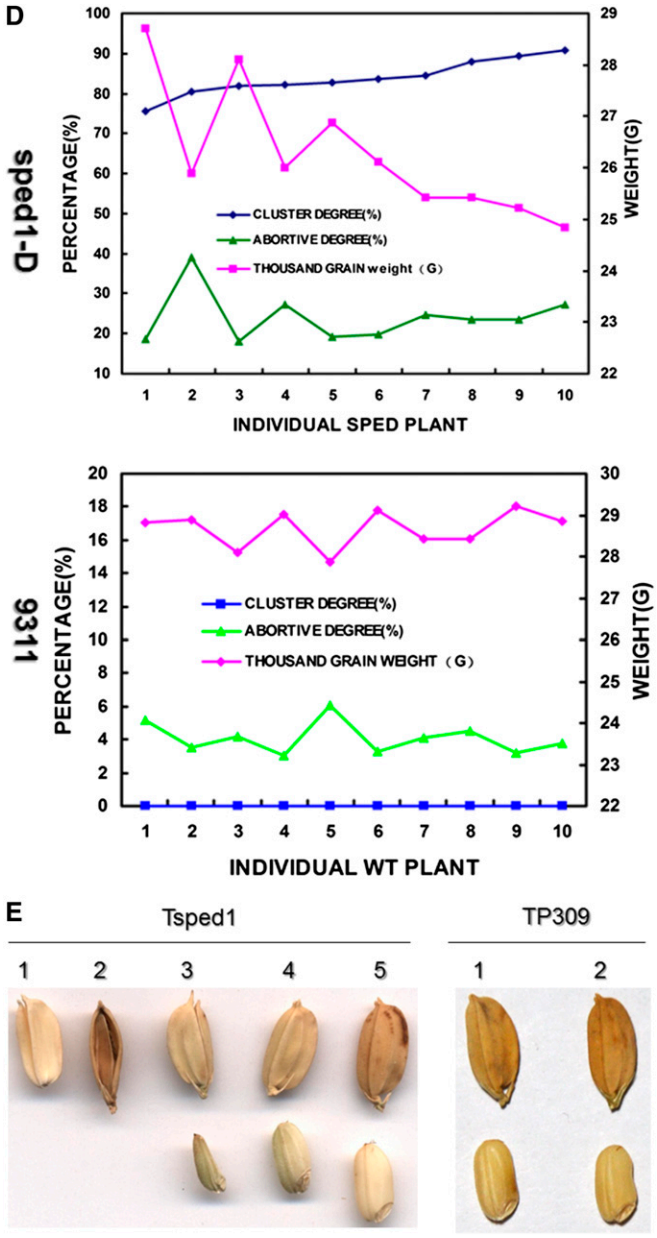

Figure 1 (*Continued*)

RT–PCR analysis

Total RNA was extracted from leaf tissue using Trizol (Invitrogen), and first-strand cDNA was synthesized using MMLV reverse transcriptase (Promega) and the oligo(dT) 15 primer. RT–PCR was performed using the following conditions: an initial 5-min denaturation at 95° followed by 95° for 30 sec, 56° for 30 sec, and 72° for 40 sec (35 cycles for *SPED1* and the other genes, 26 cycles for *Actin1*).

Design and construction of artificial miRNA

WMD (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was used to predict artificial miRNAs targeting *SPED1*. Two 21-nt miRNA sequences were selected to construct the artificial miRNA (SPED-1, GTGAGCAAGATTCCCAAATTA; SPED-2, TGACGTTCCAATTAACAAGGA). Artificial miRNA precursors of SPED-1 and SPED-2 were then amplified and subcloned into the plant binary vector pCambia2300 under the control of the maize ubiquitin promoter, using the pNW55 plasmid (which contains a natural osa-MIR528 precursor) as the template, to simultaneously replace the 21 bases of the natural osa-MIR528 miRNA and miRNA*, as described previously (Warthmann *et al.* 2008). The primers used in this experiment include the following: KPN1-OSMIR528-F, 5′-TCGGTACCCAGCAGCAGCCACAG CAAA-3′; BAMH1-OSMIR528-R, 5′-TCGGATCCGCTGCTG ATGCTGATGCCAT-3′; osMi528-SPED-1-F, 5′-TTGGCTGTAG CAGCAGCAGTAATTTGGGAATCTTGCTCACCAGGAGATT CAGTTTGAAG-3′; osMi528-SPED-1-R, 5′-AACAGCCTAGCAG CAGGAATAATTTAGGAAACTTCCTCACAGAGAGGCAAAAG TGAAGT-3′; osMi528-SPED-2-F,5′-TTGGCTGTAGCAGCAG CAGTCCTTGTTAATTGGAACGTCACAGGAGATTCAGTTTG AAG-3′; osMi528-SPED-2-R, 5′-AACAGCCTAGCAGCAGGAAT CCTTGCTAATAGGATCGTCAAGAGAGGCAAAAGTGAAGT-3′.

Analysis of RNA editing

Total RNAs extracted from seedling leaves were treated with RQ1 DNase (Promega). Then, cDNAs were synthesized using MMLV reverse transcriptase. These cDNAs were used as templates for PCR amplification of mitochondrial genes. Information about sequences and editing sites was obtained from the RNA Editing Database (REDIdb; http://bio-logia.unical.it/py_script/search.html) (Picardi *et al.* 2007). Primers were designed to cover all 491 mitochondrial editing sites, and PCR was performed using Taq polymerase. The RT–PCR products were directly sequenced. Sequencing chromatograms were manually compared between the wild type and mutant.

To analyze RNA editing efficiency, PCR was performed with Pfu DNA polymerase (Promega) using the Nad9 primer set. The product was recovered from the agarose gel and ligated into the pGEMTeasy vector (Promega). A total of 100 independent positive clones per sample were sequenced.

Results

Shortened pedicels or secondary branches in rice sped1-D mutant

In this study, we identified a rice mutant of the *O. sativa ssp. indica* cultivar 9311. All of its pedicels and some secondary branches were significantly shortened, which caused varying degrees of spikelet clustering (Figure 1A). All of the pedicels in the mutant were ~1 mm long, regardless of whether they were clustered (Figure 1B). The degree of spikelet clustering mainly depended on the extent to which the secondary branches were shortened. Namely, when the secondary branches were shortened in one to three segments from the top of the panicle, a two- to four-spikelet cluster was produced. The pedicel lengths of the first to sixth spikelet of a primary branch of the mutant were much shorter than those of the wild-type rice varieties (Figure 1C). A decrease in pollen fertility was

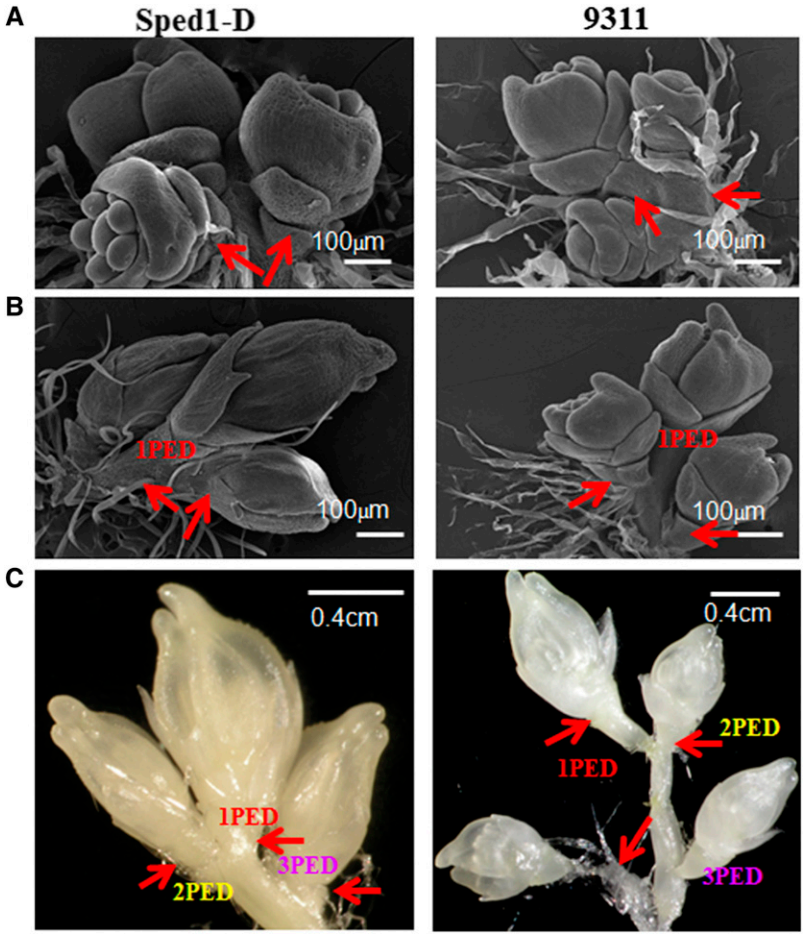

Figure 2 Morphology of sped1-D and 9311 panicles, as observed by light and scanning electron microscopy (SEM). (A) SEM images showing that there was no difference in the development of spikelets between sped1-D and 9311 during the early stages of floral organ differentiation.Bars, 100 μm. (B) SEM images showing that the pedicels and top peduncles did not elongateduring the advanced stage of floral organ differentiation in sped1-D and 9311. Bars, 100 μm. (C) The pedicels and top peduncles of 9311 rapidly elongated during the time of rapid elongation of rachis and branches, while those of sped1-Ddid not elongate. Bars, 120 μm; red arrows indicate the pedicels.

also found in the mutant compared with the wild type (Figure S1). Moreover, as the degree of clustering increased in a panicle, the degree of abortion increased, while the thousand-grain weight of the rice decreased (Figure 1, D and E). In light of this discovery, we name the mutant sped1 (*S*HORTENED *PED*ICELS AND/OR SECONDARY BRANCHES 1).

Rice inflorescences normally go through nine stages of development, from the establishment of the rachis meristem to heading and flowering (Ikeda *et al.* 2004). SEM and light microscopy revealed that there was no difference in panicle development between wild-type and sped1 plants from stage 1 to stage 6 (data not shown); the spikelet pedicels and secondary branches were not elongated in either plant (Figure 2, A and B). However, after stage 7, the pedicels of the spikelets and secondary branches of the wild type elongated quickly, while those of sped1 did not (Figure 2C). We applied nine hormones including GA3 to sped1 during panicle initation once a week for a month. However, none of the shortened pedicels or secondary branches in the mutant plants was affected (Table S1).

To study the heredity of the mutant sped1, we crossed the mutant with dozens of rice varieties, including TP309, R549, R498, R527, and Balila. The F1 plants all exhibited clustered spikelets to varying degrees, and three-quarters of the F2 plants exhibited two- to four-spikelet clusters, depending on the genetic background. These results indicate that the clustered spikelet phenotype is controlled by an incompletely dominant gene in the *indica* background (Table S2). Therefore, the sped1 mutant was formally designated sped1-D (dominant) relative to wild type (SPED1, recessive), and a series of sped1-D identical mutants in different genetic backgrounds were also designated, such as TP309-sped1-D (Tsped1), Kitaake-sped1-D (Ksped1), R549-sped1-D (R549sped1), R498-sped1-D (R498sped1), R527-sped1-D (R527sped1), and Balila-sped1-D (Balilasped1; Figure S2 and Table S5). The grain plumpness and thousand-grain weight were affected in each of these mutants (Figure 1, D and E).

Isolating the sped1-D gene by map-based cloning and identical-gene mutant analysis

Genetic analysis showed that the sped1-D phenotype is controlled by a dominant gene. We isolated the *sped1-D* gene using map-based cloning. First, a primary mapping experiment, which employed 349 F2 wild-type plants obtained by crossing sped1-D with TP309, revealed that the *sped1-D* gene was anchored to a 0.3-cM interval between two SSLP markers, RM5957 and RM3827, on chromosome 6. These two flanking markers were used to screen 1580 F2 wild-type individuals to detect recombination in the regions flanking

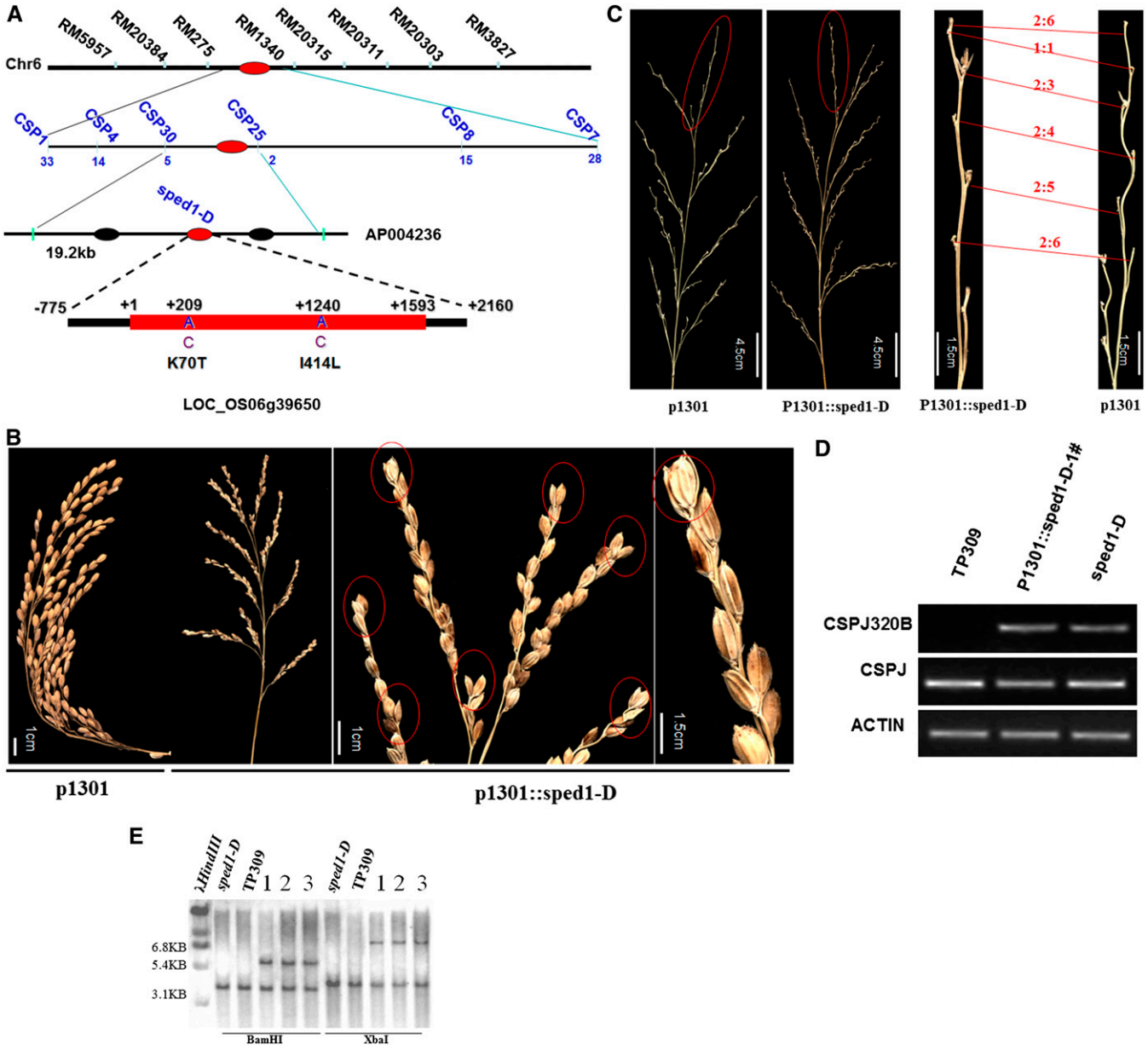

Figure 3 Map-based cloning of*sped1-D*. (A) After the initial mapping of *sped1-D* on chromosome 6 using SSLP markers, the *sped1-D*locus was further narrowed down and limited to a 19.2-kb region of the PAC clone AP004236, between the *STS* marker CSP30 and CSP25, by analyzing 1580 F2 plants; three genes were predicted to be located in this region. The number of recombination events between the markers and the *sped1-D* locus is shown under the horizontal line; sequence analysis of the three genes between *sped1-D* and wild type showing that the second gene, *LOC_OS06g39650*, with two nucleotide substitutions, is the candidate gene, which was again confirmed by analyzing the sequence of the other seven isogenic sped1-D mutants. The three nucleotide substitution loci are marked in this figure, and below these, the corresponding amino acid substitutions are indicated. (B) Functional complementation of the wild type, TP309, with the candidate mutant transgene. The hygromycin-positive transgenic plants containing p1301-sped1-D exhibited the two- to three-spikelet cluster phenotype, while those with p1301(CK) showed the normal panicle phenotype I. Scale bar, 1.5 cM. (C) The pedicels of transgenic plants with p1301-sped1-D were shortened compared with those of control plants; the ratios of the lengths of pedicels 1–6 on one secondary branch from the same position in p1301 (right) and p1301::sped1-D (left)are indicated. (D) RT–PCR analysis with *SPED1* or *sped1-D*-specific primers, CSPJF (5-GAAGCAATTCCATGCAATGAGG-3), CSPJR (5-GGTCCAGTCAAACTAATGG-3′), and the discriminating STS markerprimers, CSPJ320BF (5′-CAATGAGGGAGGTTTATCAGC-3′), CSPJ320BR (5′-CCAAACAGTGGCATAGCATCCTTT-3′). All plants produced a 400-bp band; the 1 T0 transgenic plant and sped1-D mutant both produced a 144-bp band, while no band was seen in the control plants (TP309). *Actin* was used as a control. (E) Three T2 plants of the 1 transgenic line were analyzed by Southern analysis. Genomic DNA was digested with restriction enzyme *Bam*HI and *Xba*I, respectively, and hybridized with the sped1-D gene probe. Two bands were observed in transgenic plants, and only one band was present in the control plants.

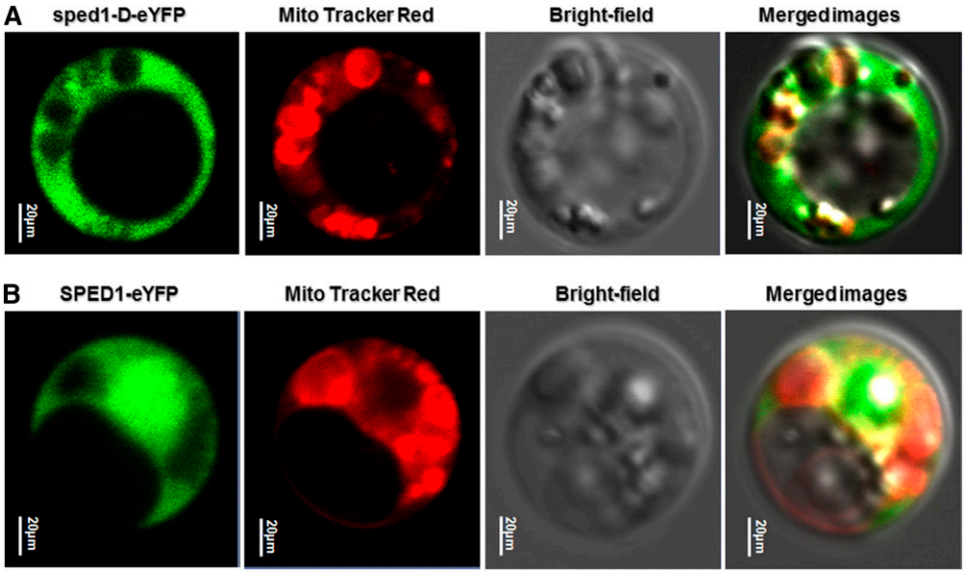

Figure 4 Subcellular localization of SPED 1 and sped1-D. Transient expression of sped1-D::eYFP (A) and SPED1::eYFP (B) in rice protoplasts.

sped1-D. RM5957 identified 30 recombinants, and RM3827 identified 19 recombinants. Next, six SSLP markers and six *STS* markers (Table S3) were further analyzed in the 49 recombinants to narrow down the region containing *sped1-D*. Two recombinants between *sped1-D* and CSP25 and 4 recombinants between *sped1-D* and CSP30 were detected. Thus, *sped1-D* was fine-mapped to a 19.2-kb region on the P1-derived artificial chromosome clone (PAC), AP004236. There were three open reading frames located in this region, namely *LOC_Os06g39640*, *LOC_Os06g39650*, and *LOC_Os06g39660*. Next, we sequenced all three genes from six identical *sped1-D* mutants (Balilasped1, Tsped1, Ksped1, R549sped1, R498sped1, and R527sped1) and the corresponding wild-type plants (Balila, TP309, Kitaake, R549, R498, and R527). We found that there were two nucleotide substitutions, namely, A209C and A1240C, in the coding region of *LOC_Os06g39650* in all six allelic mutants, which resulted in changes in two amino acids (K70T and I414L). These results strongly suggest that *LOC_Os06g39650* is the best candidate for the *sped1-D* gene (Figure 3A).

The candidate gene was further confirmed by genetic complementation. A 2855-bp fragment containing the entire *LOC_Os06g39650* gene from sped1-D plants was cloned into pCAMBIA1301 and introduced into rice cultivars 9311 (*O. Sativa ssp. indica*) and TP309 (*O. Sativa ssp. Japonica*) via *Agrobacterium tumefaciens*-mediated transformation. A total of 12 hygromycin-positive T_0 transgenic plants from TP309 were obtained, while none was obtained from 9311. Of these TP309 transgenic plants, 5 plants showed the clustered spikelet phenotype, with two or three spikelets clustering on secondary branches due to the shortening of their pedicels and secondary branches (Figure 3, B and C and Figure S3). All of the T_1 plants were further examined by performing PCR amplification of the hygromycin phosphotransferase gene and Southern blot analysis using the candidate gene as a probe (Figure 3, D and E). The results confirmed that we indeed cloned the rice *sped1-D* gene.

sped1-D encodes an uncharacterized protein containing five PPR groups and a truncated E-motif

The *sped1-D* gene, which is intronless, contains one 1593 nucleotide coding sequence and encodes a 530-amino-acid protein. The middle region of sped1-D contains five groups of PPR motifs, which demonstrates that sped1-D encodes a PPR-like protein. Phylogenetic analysis among sped1-D and other PPR proteins showed that sped1-D shares the highest sequence similarity with *Arabidopsis* proteins such as at1g31430 and SLO1 (Sung *et al.* 2010). Sequence alignment showed that sped1-D protein contains a truncated E-motif and two conserved altered amino acids, 70K and 414I (Figure S4). Secondary and 3-D structural analysis with Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) showed that the two point mutations in sped1-D resulted in an increase in the proportion of alpha helices, which altered the conformation of this protein (Figure S4, File S3, and File S4). These results suggest that the alteration of two amino acids affects the organelle targeting or nucleotide binding of sped1-D.

SPED1/sped1-D is predicated to be targeted to the mitochondria, with a cleavage site located at the 24th-amino-acid residue (probability is 0.874 and 0.9136, respectively) by TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/) and MitoProtP (http://ihg.gsf.de/ihg/mitoprot.html). SPED1::YFP and sped1-D::YFP plasmid were then transferred into protoplasts of the rice variety TP309, and the transformed protoplasts were treated with Mito Tracker Red and examined by confocal laser scanning microscopy. The yellow fluorescent signal was mainly localized to the cytoplasm, indicating that the SPED 1-YFP and sped1-D-YFP fusion proteins are mainly targeted to the cytoplasm (Figure 4).

The expression patterns of sped1-D and inflorescence development-related genes

SPED 1 or *sped1-D* was constitutively expressed in young inflorescences and other tissues or organs (Figure 5A).

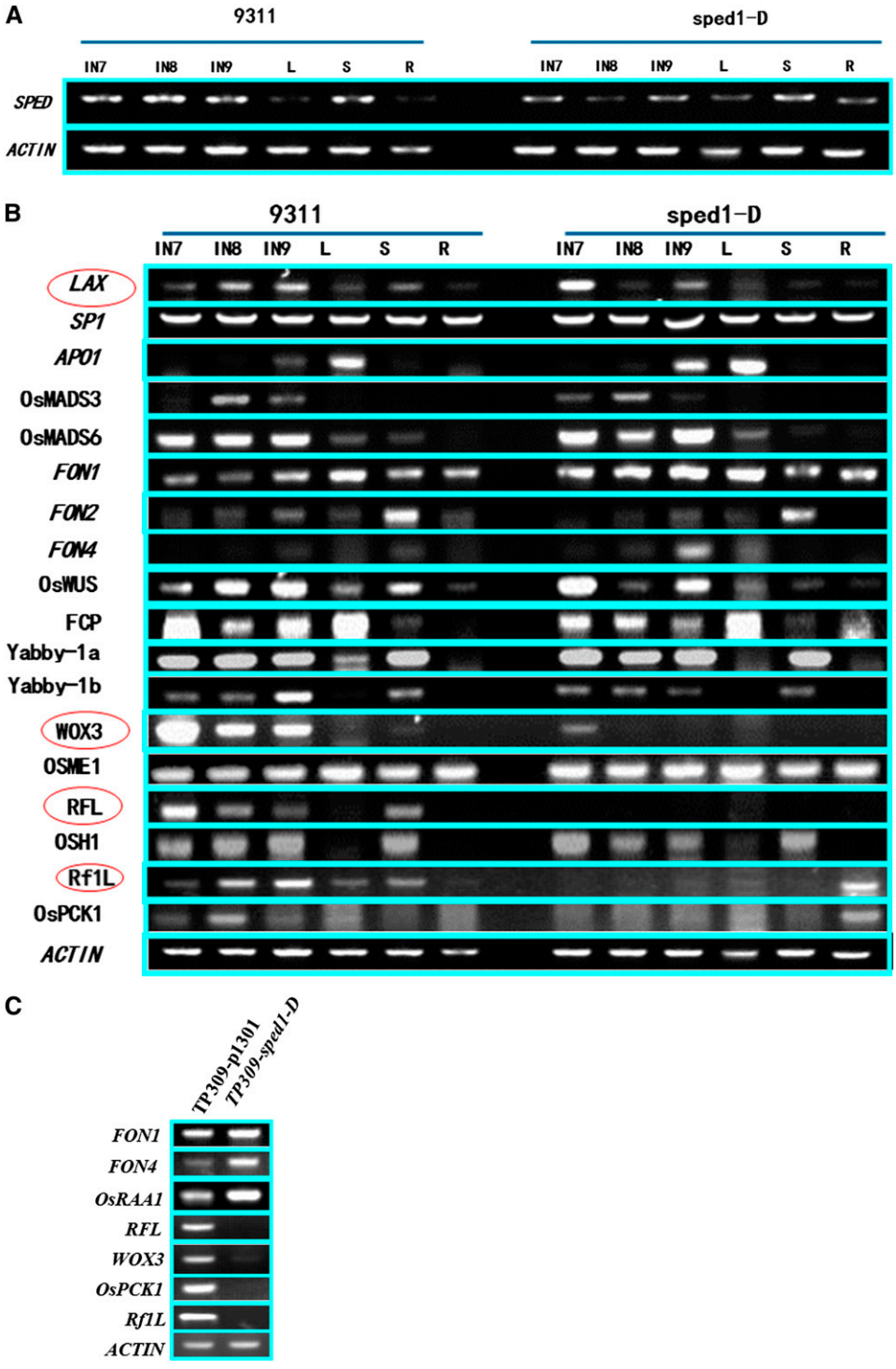

Figure 5 Expression analysis of flowering-related genes in wild-type, sped1-D, and transgenic plants containing p1301-sped1-D. (A) RT–PCR analysis shows that *SPED1* or *sped1-D* constitutivelyexpressed in young inflorescences from stage 7 to stage 9 (IN7 to IN9) and in the shoot (S), with slight downregulation in leaves (L) and roots (R). (B) RT–PCR analysis of 17 flowering-related genes. *RFL*, *WOX3*, and *Rf-1L* were strongly downregulated or entirely silenced in young inflorescences. (C) The expression pattern of these flowering-related genes in young, stage 9 TP309 panicles containing p1301-sped1-D was the same as that of the sped1-D mutant.

However, *sped1-D* had some effects on the expression of other genes associated with flower development (Figure 5B and Table S4). For example, *OsWUS* (Nagasaki *et al.* 2005), *FON1*(Suzaki *et al.* 2004), and *FON4* (Chu *et al.* 2006) were slightly upregulated in the mutant, while *WOX3* (Dai *et al.* 2007) and *Rf-1*-like (*Rf-1L*, *LOC_Os01g42620*) (Komori *et al.* 2004) were strongly downregulated; *RFL* (Kyozuka *et al.* 1998) was entirely silenced. *OsWUS*, *FON1*, *and FON4* are involved in the determination of meristem size, while *WOX3* is involved in meristem activation, *Rf1L* is involved in fertility and *RFL* plays an important role in inflorescence branch elongation. These results indicate that *sped1-D* promotes the shortening of pedicels and secondary branches, perhaps through the CLV-WUS pathway, and influences pollen fertility through the action of *Rf1L*. Other genes, such as *OsME1* (Nomura *et al.* 2005) in stress–response pathways and *OsPCK1* (Nomura *et al.* 2005) in photosynthetic pathways, were also regulated by *sped1-D*. The similar expression

Figure 6 The function of sped1-D is conserved between *Arabidopsis* and rice. (A) Overexpression of *sped1-D* in *Arabidopsis* Col-0 results in the defective elongation of terminal pedicels and pods (II and III, wild type; I, IV–VII, transgenic plant and its pod). (B) PCR check of *sped1-D* (CSPJ320B)and *hygromycin*-resistance gene (HPT) in T0 transgenic *Arabidopsis* plants.

patterns were observed in transgenic TP309 plants containing the mutant gene *LOC_Os06g39650* (Figure 5C), which confirm that the mutant *LOC_Os06g39650* is *sped1-D*.

Transgenic rice and Arabidopsis overexpressing sped1-D have clustered spikelets and siliques, respectively

To investigate how the transcription of *sped1-D* affects panicle development, a binary plasmid with *sped1-D* driven by the CaMV 35S promoter (sped1-D-OX) was introduced into wild-type TP309 rice via *Agrobacterium*-mediated transformation. Twenty independent transgenic lines showed varying degrees of spikelet clustering; RT–PCR analysis revealed that the levels of *sped1-D* transcript increased accordingly (Figure S5). These results indicate that *sped1-D* is a dominant gene, and the function of *SPED1* is inhibited in these transgenic plants.

SPED 1 is highly homologous to some *Arabidopsis* proteins, such as SLO1, CRR4, CRR2, and especially At1g31430 (Figure S4). We produced transgenic *Arabidopsis* Col-0 plants that overexpressed *sped1-D*, and observed a series of novel phenotypes, including impaired meristem activation and defective elongation of terminal pod pedicels in T_0 transgenic plants (Figure 6, A and B). These results indicate that the function of *sped1-D* is highly conserved in monocotyledonous and dicotyledonous plants.

SPED 1 and sped1-D also affect pollen fertility in rice spikelets

Because the fertility of the *sped1-D* mutant was negatively correlated with the presence of spikelet clusters (Figure 1) and *sped1-D* encodes a PPR-like protein, we speculated that *sped1-D* is somehow related to rice spikelet fertility. To verify this notion, we first searched international rice mutant resources for suitable TOS17 and T-DNA insertion lines for *SPED1*, but no such line was available. Therefore, we generated *SPED1* knockdown transgenic lines by cleaving *SPED1* transcripts using an artificial miRNA method (Warthmann *et al.* 2008). The precursor of osa-MIR528 (accession no. MI0003201) was selected as the endogenous stemloop backbone for the artificial miRNA transgenes. Then, two artificial miRNA-directed *sped1-D* RNA silencing binary vectors, miR528-sped-1 and miR528-sped-2, were constructed and introduced into TP309 via *Agrobacterium*-mediation transformation. A total of five and nine G418-positive transgenic T0 lines were obtained, respectively. All five transgenic T0 lines of miR528-sped-1 exhibited a normal phenotype during both the vegetative and reproductive stages, while six miR528-sped-2 lines showed poor spikelet fertility or even sterility (Figure S1). In addition, the six miR528-sped-2 lines exhibited a high level of miR528-sped-2 expression but no *SPED 1* expression (Figure S5).

SPED 1 and sped1-D have no significant effect on RNA editing

Growing research suggests that PPR proteins with E motifs may participate in RNA editing in chloroplasts and mitochondria (Okuda *et al.* 2007, 2009; Sung *et al.* 2010; Takenaka 2010; Yuan and Liu 2012). *SLO1*, a homologous gene of *SPED1*, participates in two sites' editing of mitochondrial RNA (Sung *et al.* 2010), suggesting that SPED1 protein is involved in mitochondrial RNA editing. In rice mitochondria, there are ~491 editing sites for 33 ORFs and one pseudogene (Notsu *et al.* 2002). We first analyzed the editing sites of the largest membrane-bound protein assemblies in rice mitochondria, complex I, because it covers the most editing sites. Complex I has more than 30 subunits, but only 9 (nad1–4, 4L, 5–7, and 9) are usually encoded by the mitochondrial genome. Therefore, we carried out bulk sequencing of the nine genes using the RT–PCR products from sped1-D and wild-type 9311. This analysis revealed that the editing efficiency of only three C residues (C92, C298, and C328) out of the 11 editing sites in Nad9 changed (from

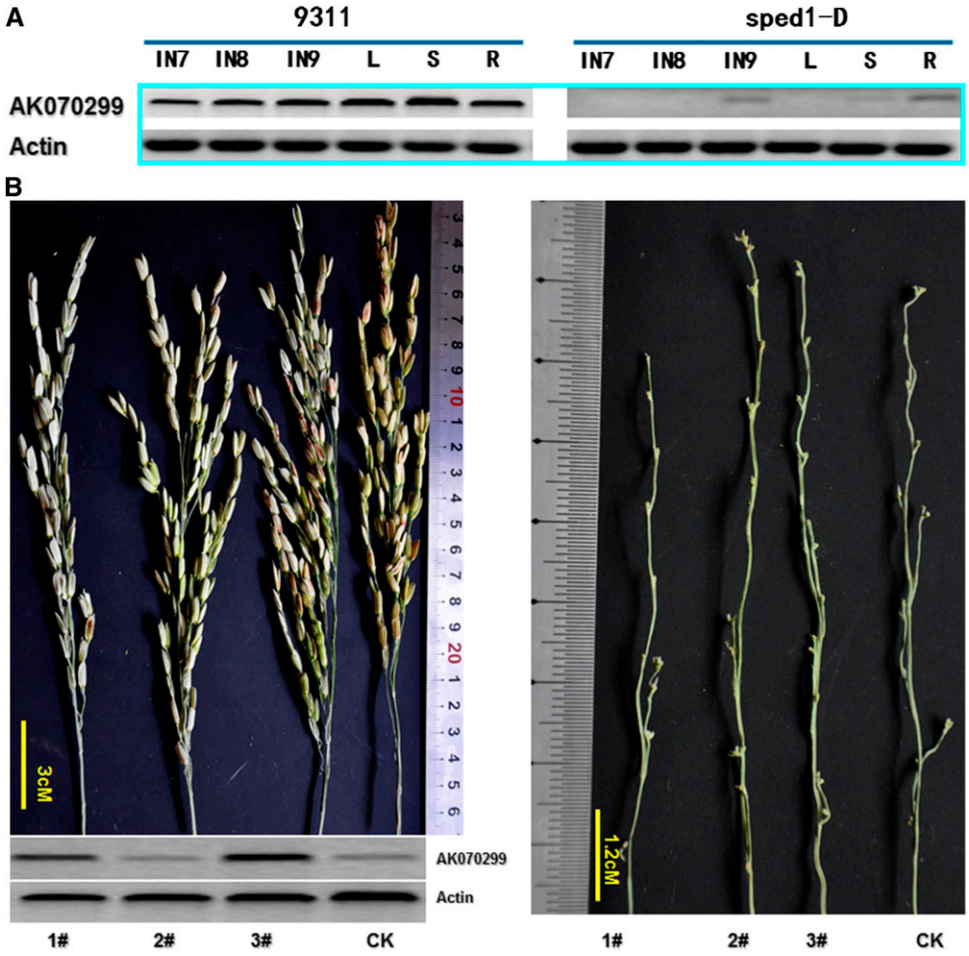

Figure 7 GA signal pathway involoves in the elongation of pedicels and secondary branches. (A) The expression pattern of gibberellin receptor GID1L2-like gene, *AK070299* in sped1-D and wild type. (B) Overexpression of *AK070299* gene in sped1-D mutant can restore its panicle phenotype. The recover level is associated with the expression level of *AK070299* (1–3 refer the T0 sped1-D transgenic plants with overexpression of *AK070299*).

20 to 40%, 25–45%, and 25–45%, respectively),while there was no difference in editing efficiency among the Nad1–4, 4L, or 5–7 genes. We then examined the editing efficiency of all 11 editing sites in SPED-RNAi transgenic plants, which exhibited low pollen fertility but no spikelet clusters, and found that their editing efficiency was increased, with editing events reaching 70–80% (Figure S6). No apparent difference in other editing sites of the mitochondrial genome was detected between sped1-D and wild-type 9311 (data not shown). These findings suggest that *sped1-D* is involved in regulating the editing efficiency of 11 editing sites of the Nad9 gene, but the latter is not involved in the shortening of pedicels and secondary branches.

GA signaling pathway involves in the elongation of pedicels and secondary branches

To elucidate the molecular mechanisms by which *SPED1/sped1-D* regulate the elongation of pedicels and secondary branches, we performed DEG analysis of panicles of sped1-D and wild-type 9311 using RNA-Seq technology. A total of 5,737,757 and 5,678,386 clean tags 21 bp in length were generated, respectively, which represent 52,845 genes, comprising ~78.41% of rice genes. Sequencing saturation analysis indicated that the data were sufficient for quantitative analysis of gene expression. We used a FDR $\leq$ 0.001 and an absolute value of $\log^2$ ratio $\geq$ 1 as the threshold to judge the significance of differential gene expression. Of the 24,221 genes expressed in either sped1-D or 9311 plants, 1247 DEGs were detected, namely 672 genes that were upregulated and 574 that were downregulated in sped1-D relative to wild-type plants. Detailed gene lists (with fold changes, GO, gene descriptions, pathways, and other relevant information) are provided in File S1 and File S2. The expression of genes involved in hormone signaling and metabolism-related genes associated with cell growth and division such as carbohydrate metabolic process, aminoglycan metabolic process and cellulose synthase was primarily altered in the sped1-D mutant. Among these, gibberellin receptor GID1L2 family genes were downregulated in sped1-D, which may account for the mutant phenotype. The transcript level of *LOC_Os06g11130* (*AK070299*) decreased 86.7% in sped1-D, which was further corroborated by RT–PCR analysis. A 450-bp specific band was amplified strongly from wild-type 9311 cDNA at the early panicle differentiation stage, while a very weak band was obtained from that of *sped1-D* (Figure 7A). In fact, *LOC_Os06g11130* (*AK070299*) is expressed constitutively in organs and tissues of 9311. These results indicate that gibberellin receptor GID1L2 family genes, especially

LOC_Os06g11130 (*AK070299*), may be involved in the elongation of pedicels and secondary branches. If the expression of *LOC_Os06g11130* (*AK070299*) is required for the elongation of pedicels and secondary branches, its overexpression in sped1-D plants can restore the clustered spikelet phenotype to the normal phenotype. We used *Agrobacterium*-mediated transformation to produce rice harboring the full-length cDNA of *LOC_Os06g11130* (*AK070299*) in the pZh01 vector (Xiao *et al.* 2003). We generated 55 plants from seven independent transgenic rice lines and confirmed the presence of the hygromycin-resistance gene in these plants by PCR. Of these, 9 plants showed the normal panicle phenotype, while 12 showed varying degrees of alleviation of spikelet clustering, which indicates that *LOC_Os06g11130* (*AK070299*) is involved in the elongation of pedicels and secondary branches (Figure 7B).

Discussion

Identical-gene mutant analysis is an effective strategy for cloning target genes

Map-based cloning has been widely used to help elucidate complex biological processes in plants, such as developmental regulation and gene expression cascades. However, critical bottlenecks of gene functional complementation analysis still exist, including the determination of candidate genes and the relatively low efficiency of genetic transformation. In this study, we succeeded in cloning *sped1-D* using map-based cloning in combination with identical-gene mutant analysis. First of all, we obtained identical-gene mutants of *sped1-D* in different backgrounds (Figure S2). Once the *sped1-D* gene was narrowed down to a limited region, we reliably obtained the target gene by comparing the sequences of this region among the identical-gene mutants. In addition, the functional complementation transformation can be accomplished easily in its identical-gene mutants with *japonica* background.

SPED1 might serve as a regulatory factor to affect genes expression

Most characterized PPR proteins serve as sequence-specific RNA-binding proteins inside organelles, regulating RNA processing, maturation, and translation. However, some PPR proteins are present outside of organelles. The *Arabidopsis* GLUTAMINE-RICH PROTEIN23 (GRP23) might interact physically with subunit III of RNA polymerase II through its C-terminal Gln-rich WQQ domain and bind directly to *cis*-regulatory elements of DNA through its N-terminal basic domain (Ding *et al.* 2006). PNM1 might be involved in the regulation of its own gene expression in the nucleus and play a role in gene expression adjustments between the mitochondria and the nucleus (Hammani *et al.* 2011). The two point mutations in sped1-D resulted in an increase in the proportion of alpha helices, leading to an altered protein conformation. These changes might affect the nucleotide binding abilities of sped1-D and the regulation of gene expression, such as *LAX1*, *WOX3*, *RFL*, *Rf1L*, and *GID1L*.

Pedicel and secondary branch elongation is mediated by gibberellin signaling

GAs regulate many phases of plant development, including seed germination, stem growth, floral induction, pollen development, and fruit growth (Kende and Zeevaart 1997; Olszewski *et al.* 2002). GA biosynthesis is mainly controlled by feedback regulation, which provides an important link between GA metabolism and GA-signaling pathways (Phillips *et al.* 1995; Hedden and Phillips 2000; Olszewski *et al.* 2002; Weston *et al.* 2008). GAs are synthesized from ent-kaurene via geranylgeranyl diphosphate, and all metabolic steps after ent-kaurene are oxidative. Among these oxidases, GA20-oxidase (GA20ox) is responsible for the removal of the C-20 of GA12 in the formation of C19-GAs (Mauriat and Moritz 2009). *Arabidopsis* plants harboring a mutant *AtGA20ox1* (*ga5*) gene exhibit a semi-dwarf phenotype. In this study, gibberellin receptor GID1L2 family genes were downregulated in the sped1-D mutant. Therefore, treatment of sped1-D plants with GA during panicle initiation did not lead to elongation of secondary branches or pedicels, as the mutant meristems failed to respond to GA signaling. These results indicate that GA signaling plays an important role in the elongation of pedicels and secondary branches.

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GENETICS

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Regulation of Inflorescence Branch Development in Rice Through a Novel Pathway Involving the Pentatricopeptide Repeat Protein sped1-D

Guanghuai Jiang, Yanghai Xiang, Jiying Zhao, Dedong Yin, Xianfeng Zhao, Lihuang Zhu, and Wenxue Zhai

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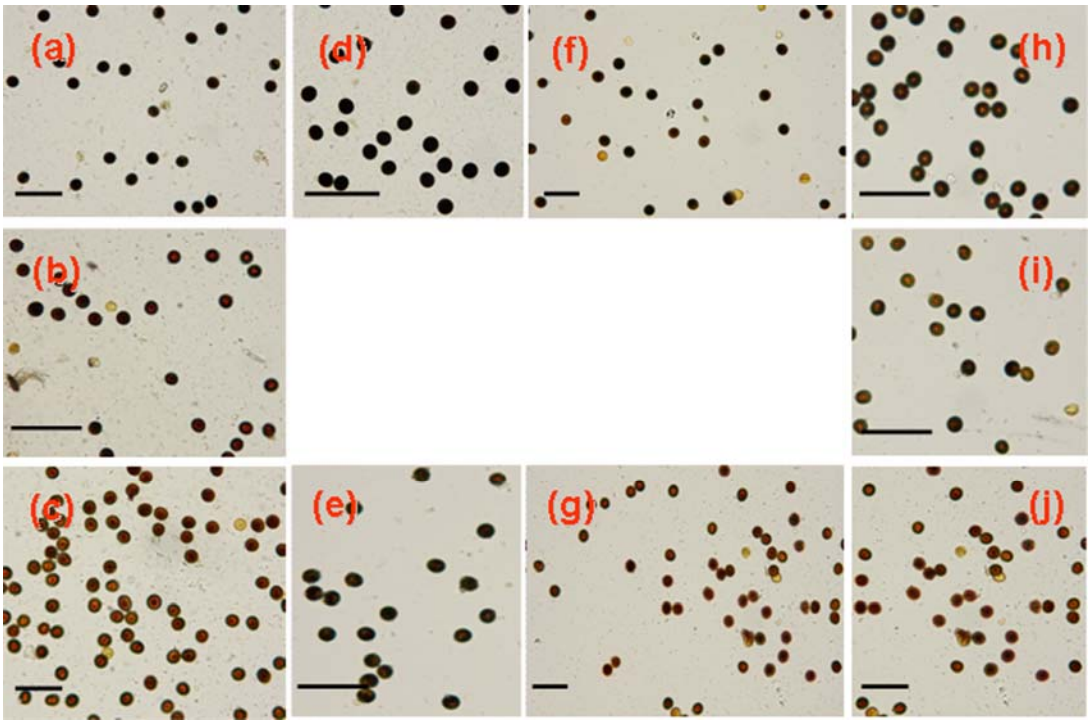


Figure S1 Pollen viability in sped1-D, SPED1-RNAi and control plants. Pollen viability was effected in sped1-D (a, 1 spikelet; b, 2 spikelets; c, 3 spikelets) and in TP309 plants containing *sped1-D-OX* plasmid (h, 1 spikelet; i, 2 spikelets; j, 3 spikelets) compared with that of the wild type (d, TP309 and e, 9311); SPED1-silenced plants had very low pollen viability (f, miR528-sped-1 and g, miR528-sped-2). The pollen had low viability and appeared light brown when assessed by staining the starch in the pollen with a 1% I-KI solution; the mature pollen grains from the control and sped1-D plants were stained dark brown.

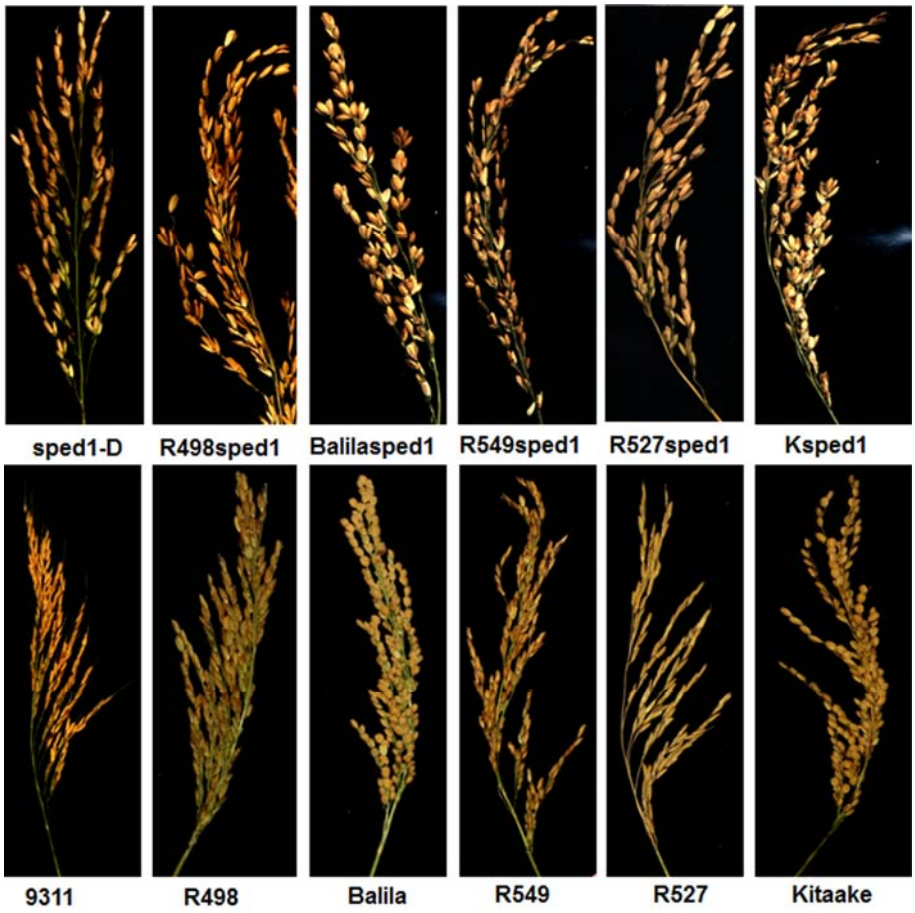


Figure S2 The phenotype of sped1-D isogenic mutants in different genetic backgrounds (Upside is the series isogenic sped1-D mutant, below is the corresponding wild type).

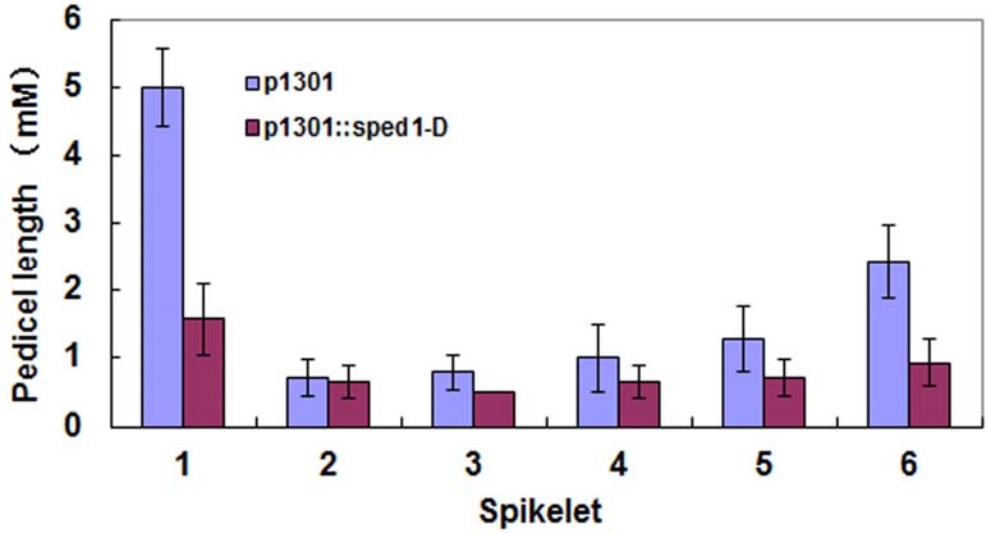


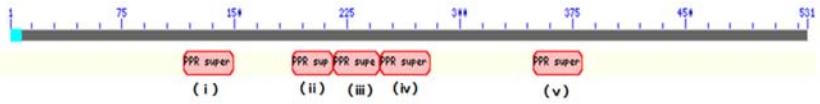
Figure S3 Statistical analysis of the lengths of six pedicels on secondary branches of a TP309 plant containing p1301-*sped1-D*. Error bars indicate standard error (SE) for seven replicate experiments.

Figure S4

A)

Cleaved sequence （34aa）

MAMAAARRGHGMPLWECNVLIRTLARRGSFARVMAVYYDLRARGLVADSFTYPFVLR
AVGVLKLSVEGRKAHAAAVKTGFRWDAYTGSSLMEMYTMLGRVDIARKVFDEMPSRALVLW
NMMVRCYIRCGWYSAAVALSEQMERSGV（i）
TPDRVTLVTAVTACSRARDLSLGRRIHVYMDNVFGFNLPVANALLDMYTKNDCLEEAVKLFEQ
MPARNIISWTILVSGYGLAGQLDKARVLFNQCKEKDLILWTAMINACVQHGCFEEALTLFRDM
QMQRV（ii）
EPDRFTVVTLLTCCANLGALDQGEWIHQYAEQRKMKIDAVLGTALIDMYSKCGHIEKSLEVFW
RMQGRD（iii）
ATAWTAIICGLATNGQAGRALELFQDMQRSKVKPDGVTFIGVLSACCHGGLVDEGRKQFHAM
REV（iv）
YQIEPRVEHYSCLVNLLGRAGLLDEAERLIGDV（v）
PINKDAMPLFGALLTACKAHGNVEMSERLTKRICEQDSQITDVNLLMSNVYATASRWEDVIRV
RGKMAHPTVKKNAGCSLIEVKGY.



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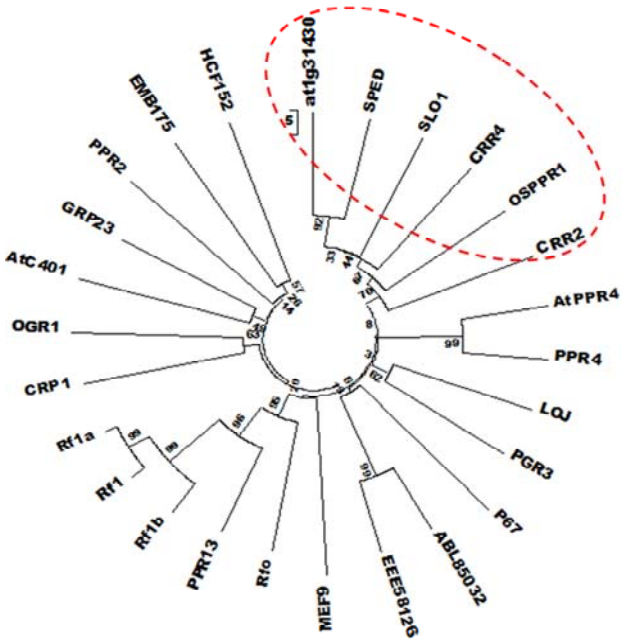
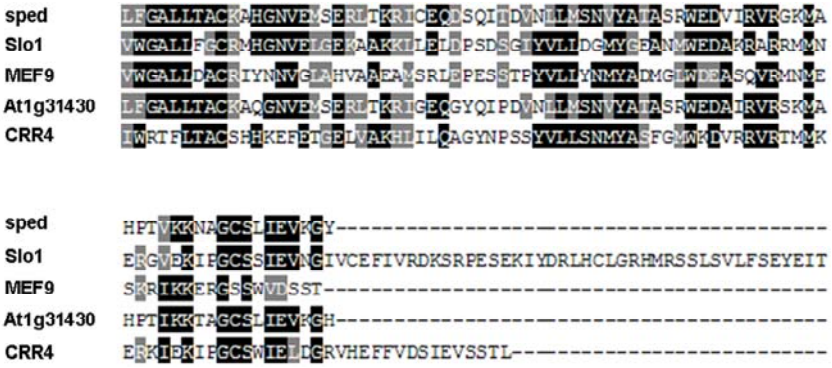


C)



D)

```
                61        70        80        90       100       110       120
                |--------+---------+---------+---------+---------+---------|
OsSPED1         VLKLSVEGRKAHAAAVKTGFRWDAYTGSSLMEMYTMLGRVDIARKVFDEMPSRALVLWNM
BdSPED1         VLKLSVEGRKAHAAAMKTGFWWDAYTASSLMDMYTLLGRADVARKLFDEMPHRALVVWNM
HvSPED1         ALQISVEGRKAHAATLKTGFRWDAYTASSLMDMYTMLGRLEAARKVFDEMPQRAVVVWNM
SiSPED1         VMKLSVEGRKAHAAAVKTGFRWDAYTASSLMDMYAMLGRADLARKVFDEMPRRFLVLWNM
SbSPED1         VMKLSVEGRKVHAAAVKTGFRWDAYTASSLLEMYTMLGRVDFARKVFDEMPQRFLVLWNM
ZmSPED1         TMKLSVEGRKVHAAAVKTGFRWDAYTACSLMEMYTMLGRADSARRVFDEMPQRFLVLWNM
AtSPED1         RLRKVIEGEKVHGYAVKAGLEFDSYVSNSLMGMYASLGKIEITHKVFDEMPQRDVVSWNG
Consensus       .$kls!EGrKaHaaavKtGfrwDaYtasSL$.MYtnLGr.#.arkvFDEMPqR.lV.WNm
                          *
                121       130       140       150       160       170       180
                |--------+---------+---------+---------+---------+---------|
OsSPED1         MVRCYIRCGWYSAAVALSEQM-ERSGVTPDRVTLVTAVTACSRARDLSLGRRIHVYMDNV
BdSPED1         MIRCYVRCGRYTAAIALAEEM-ERSGLTPDKVTLVTSVTVCSRAGDLSLGRRIHAYMDGV
HvSPED1         MLRCYVRCGRNTEAVALAEEM-ERGRLTPDRVTLLTALTACSRAGDLSLGRKIHAYMDGV
SiSPED1         MIRCYVRCGRFTAAVALAEEM-ERSGATPDRVTLVTAVTACSRAGDLSLGRRIHSYMDAV
SbSPED1         MMRCYIRCGRFTAAVALAEEM-ERSGATPDRVTLVTAVTACSRAGDLSLGRRIRAYMDGV
ZmSPED1         MMRCYIRCGRFTAAVALAVQM-ESGGATPDRVTLVTAVTACSRAGDLNLGRRIHSYMDGV
AtSPED1         LISSYVGNGRFEDAIGVFKRMSQESNLKFDEGTIVSTLSACSALKNLEIGERIYRFVVTE
Consensus       $ircY!rcGrftaA!alae.M.#rsgltpDrvTlvtavtaCSrag#LslGrrIh.%md.v
                181       190       200       210       220       230       240
                |--------+---------+---------+---------+---------+---------|
OsSPED1         FGFNLPVANALLDMYTKNDCLEEAVKLFEQMPARNIISWTILVSGYGLAGQLDKARVLFN
BdSPED1         FGFSLPVANALLDMYMKNGCLEEAVKLFEQMPSRNIISWTILVSGYAFAGQLDKARVLFY
HvSPED1         TGFSLPVANALLDMYVKNGCLEEAVNLFEKMPSRNVVSWTTLVSGYAFAGQVDKARLLFH
SiSPED1         FGFNLPVANALLDMYTKNGCLEEAVKMFEQMPERNIISWTILVSGYALAGQLDKARALFY
SbSPED1         FGFSLPVANALLDMYTKNGYLEEAVKMFQQMPERNIISWTILVSGYAVAGQLDKARMLFY
ZmSPED1         FGFSLPVANALLDMYTKNGYLEEAVKMFEQMPERNIISWTILVSGYGIAGQLDKARVFFY
AtSPED1         FEMSVRIGNALVDMFCKCGCLDKARAVFDSMRDKNVKCWTSMVFGYVSTGRIDEARVLFE
Consensus       fgfslp!aNALlDM%.KngcL#eAvk.F#qMp.rN!isWTi$VsGY..aGqlDkARvlF.
                241       250       260       270       280       290       300
                |--------+---------+---------+---------+---------+---------|
OsSPED1         QCKEKDLILWTAMINACVQHGCFEEALTLFRDMQMQRVEPDRFTVVTLLTCCANLGALDQ
BdSPED1         QCSEKDLIMWTAMINACVQHGCFEEALSLFREMQMQRVEPDRFTIVTLLTCCANLGALDQ
HvSPED1         QCTEKDLIMWTAMINAYVQHGCFIEALSLFRDMQMHQIEPDRFTVVTLLTCCANLGALDQ
SiSPED1         QCTEKDLILWTAMINACVQDGSFEEALSLFRDMQLQRVEPDRFTVVTLLTCCANIGTLDQ
SbSPED1         QCTQKDLILWTAMINACVQHGSFEEALTLFRDMQLQRVEPDKFTVVTLLTCCANIGALDQ
ZmSPED1         QCTQKDLILWTSMINACVQHGSFEEALILFRDMQLQRVEPDKFTVVTLLTCCANIGALDQ
AtSPED1         RSPVKDVVLWTAMMNGYVQFNRFDEALELFRCMQTAGIRPDNFVLVSLLTGCAQTGALEQ
Consensus       qc.eKDl!$WTaMiNacVQhg.FeEAL.LFRdMQ.qr!ePDrFtvVtLLTcCA#.GaL#Q
                301       310       320       330       340       350       360
                |--------+---------+---------+---------+---------+---------|
OsSPED1         GEWIHQYAEQRKMKIDAVLGTALIDMYSKCGHIEKSLEVFWRMQGRDATAWTAIICGLAT
BdSPED1         GEWIHQFAVDRKMKVDAVLGTALIDMYAKCGHVKKSMEVFEQMQGRDTTAWTSIICGLAT
HvSPED1         GQLIHQFAEGRNMKLDAVLGTALIDMYAKCGHVEKSVEVFERMEGRDTKAWTAIICGLAT
SiSPED1         GEWIHQYAEGRKMKIDAVLGTALIEMYSKCGHVDKALDVFERMQGRDAAAWTAIICSLAT
SbSPED1         GEWIHQYAEGRNMKIDAVLGTALIEMYSKCGHVDKSLQIFGRMQGRDAAAWTAIICGLAT
ZmSPED1         GEWIHQYAESRNMKIDAVLGTALIEMYSKCGHVDKSLQIFGRMQGKDAAAWTAIICGLAT
AtSPED1         GKWIHGYINENRVTVDKVVGTALVDMYAKCGCIETALEVFYEIKERDTASWTSLIYGLAM
Consensus       GewIHq%ae.r.nk.DaVlGTAL!#MYaKCGh!eksl#!F.rnqgrDtaaWTaiIcgLAt
                361       370       380       390       400       410       420
                |--------+---------+---------+---------+---------+---------|
OsSPED1         NGQAGRALELFQDMQRSKVKPDGVTFIGVLSACCHGGLVDEGRKQFHAMREVYQIEPRVE
BdSPED1         NGQAGRALELFEDMERSKVKPDSITFIGVLSACCHGGLVDEGRKQFHAMKDVYRIPPRIE
HvSPED1         NGQAGRALELFEDMERSEAKPDSVTFIGVLSACCHGGLVDEGRKQFRAMKEVYRIRPRVE
SiSPED1         HGQASRALELFEEMQISKVKPDGITFIGVLSACCHGGLVDEGRRHFQAMKEVYRIEPRIE
SbSPED1         NGQASKALELFEEMQRSKTKPDGITFIGVLNACCHGGLVDEGRRHFQAMKEIYQIEPRIE
ZmSPED1         NGQASKALELFEEMQRSKTKPDGITFIGVLSACCHGGLVDEGQRYFQAMKEVYHIEPRIE
AtSPED1         NGMSGRALDLYYEMENVGVRLDAITFVAVLTACNHGGFVAEGRKIFHSMTERHNVQPKSE
Consensus       nGqagrAL#L%e#M#rskvkpD.!TF!gVLsACcHGGlVdEGrk.FhaMk#vy.!.Pr.E
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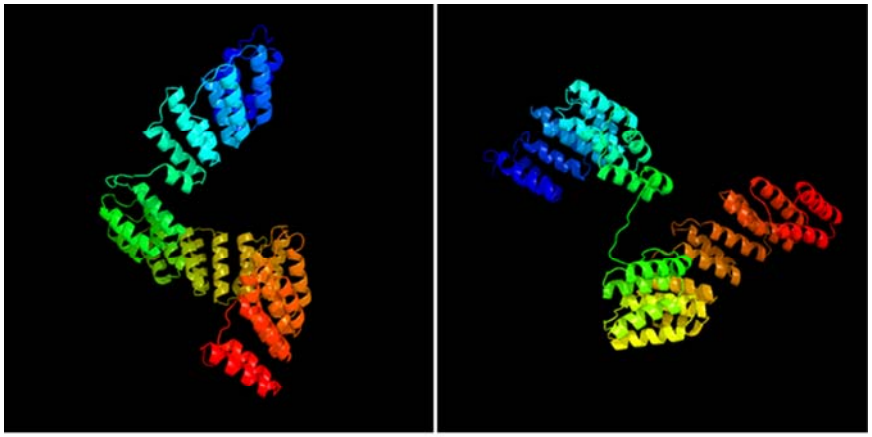
E)



SPED1 sped1-D

Figure S4 Sequence and structure of SPED 1

A) SPED 1 is a PPR protein containing 12 PPR motifs. The positions of the two changed amino acid, K70T and 414L in sped1-D are indicated by asterisks.

B) Phylogenetic analysis showed that SPED or sped has the highest sequence similarity with at1g31430 and SLO1 in Arabidopsis. Sequences used in this analysis were as follows: At1g31430, MEF9 (At1g62260), SLO1 (At2G22410), CRR4 (At2g45350), CRR2-At3g46790, AtPPR4 (At5g04810), LOJ (At2g39230), PGR3 (At4g31850), P67 (At4g16390), AtC401 (At5g21222), GRP23 (At1g10270), EMB175 (At5g03800) and HCF152 (At3g09650) from Arabidopsis; sped (NP_001057989), OSPPR1 (AAS93059), Rf1a (ABC42330), Rf1b (ABC42331), EEE58126 and OGR1 (ACL79585) from rice; PPR4 (NP_001105869), CRP1(NP_001105879) and PPR2 (ACG30078) from maize; ABL85032 from *Brachypodium sylvaticum*; Rfo (ACJ70132) from *Brassica napus*; PPR13 (XP_002436844) from sorghum; and Rf1 (AAM52341) from petunia.

C) Sequence alignment of sped 1, Slo1, MEF9, At1g31430 and CRR4 also showed that the sped 1 contains a truncated E motif.

D)Protein sequence alignment of SPED1 homologs from Arabidopsis (AtSPED1, At1g31430), Setaria italic (SiSPED1, XP_004967201.1), Sorghum bicolor (SbSPED1, XP_002438633), Zea mays (ZmSPED1, AFW76438) and Brachypodium distachyon (BdSPED1, XP_003563682).The two changed amino acid,K70T and I414L in sped1-D are both located in a conservative domain. Their positions are indicated by asterisks.

E) Prediction of 3-D structures of SPED1 and sped1-D proteins by using phyre2.

A)

B)

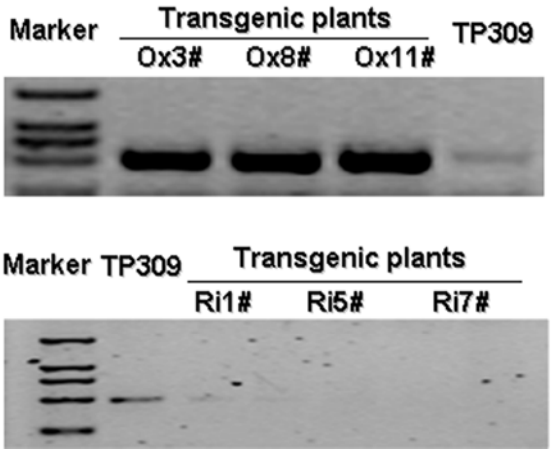


Figure S5 The phenotypes of TP309 plants containing SPED-OX or miR528-sped-2.

A) Two to three spikelets clustered in TP309 transgenic plant #8 containing *SPED-OX*; Statistical analysis of the spikelet sterility of TP309 transgenic plant containing *miR528-sped-2* showed that the number of fertile spikelets or grain yield per panicle was about reduced to 5% (The phenotype of Ri5# is shown).

B) RT-PCR analysis showed that the expression levels of *SPED1* are much higher in plants with *SPED-OX*, while they are lower in plants with *miR528-sped-2* than that in TP309

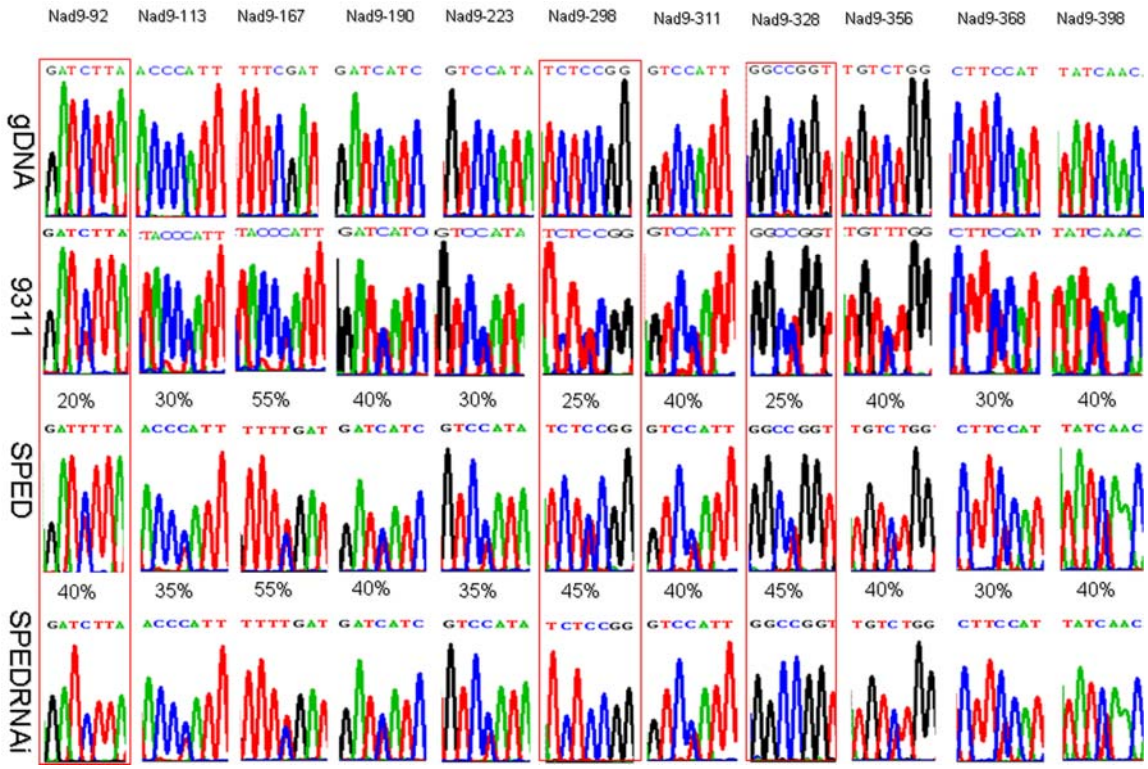


Figure S6 SPED is involved in the editing of Nad9 gene. RT-PCR products containing the 11 partial editing sites of Nad9 gene were directly sequenced. Besides the editing efficiency of C92, C298 and C328 are 50% increase in average in SPED plant, that of all the 11 sites are increased to 70-80% in sped-RNAi plant. Asterisks indicate the editing sites.

Table S1 Nine plant hormones that were examined to determine the effects of hormones on the elongation of sped1-D's pedicels and peduncles.

Phytohormone	Concentration	Panicle type
IAA	20mg/L and150mg/L	Clustered panicle
ZT	20mg/L and150mg/L	Clustered panicle
ABA	20mg/L and150mg/L	Clustered panicle
6-BA	20mg/L and150mg/L	Clustered panicle
NAA	20mg/L and150mg/L	Clustered panicle
Epibrassinolide	0．01mg／L	Clustered panicle
Cytokinin	0.001g/L	Clustered panicle
2，4-D	40ppm,80ppm, 120ppm, 160ppm	Clustered panicle
GA3	50mg/L,100mg/L,150mg/L	Clustered panicle

Table S2 The incompletely and completely dominant inheritance of sped1-D in different background.

Cross combination	Number of F1 plant (Mutant type: wild type)	Number of F2 plant (Mutant type: wild type)	Dominant or recessive
9311×sped1-D	6:0	252:88	incomplete dominant
R498×sped1-D	10:0	137:43	incomplete dominant
Balila×sped1-D	7:0	110:39	incomplete dominant
R549×sped1-D	9:0	66:21	complete dominant
R527×sped1-D	9:0	178:53	incomplete dominant
TP309×sped1-D	11:0	112:42	complete dominant
Kitaake×sped1-D	6:0	100:30	complete dominant

Table S3 Molecular markers used for map-based cloning of *sped1-D* gene.

Molecular Marker	Forward primer（F）	Reverse primer （R）
RM5957	ACTGCTGCACTGCACAAGAC	AGCTAGCTAGGCGTGAGCTG
RM3287	GGACGGATTGTAGGTAGGAC	CCTTTCTTCAATCTGCATTC
RM275	GCATTGATGTGCCAATCG	CATTGCAACATCTTCAACATCC
RM20384	TCAGATCACTGTGCTCCAACTCC	TCTATCTATCATGCCGACCTTGC
RM20297	TTGGCACGGCCATATAACAAGC	AAGTTGATGGCCTTTGGTTTGC
RM20311	ATAATTCATCCGGCCACCAACACC	CTCCGGGACAAGGTTGCTGAGG
RM20315	CGTCCTCCAGGAAACCCTGTAAGC	CAGAAACTCGCCGAAGCAGAGC
RM1340	TCCAAACTAGTGGGAACGC	CTCAACGCCATGAACCTC
RM20303	ACCTCCGCGTCGTAGAAGTAGC	CAAACCCAAACCCAAGGAGAGG
CSP1	AGGCTTCTTGGAATGGAACTGC	GGGAATATACGTGGATGTGAGAGG
CSP4	AGCGTCCTTCTCCATCAT	TTTGTCACTTGTCCCGTA
CSP7	TGTGACAGCATAGGAGTG	AGGAGTAGCTTTTGGTTC
CSP8	ACGCCTCTCCTTCACGCT	GTATTCCCCATCTCCAGTACG
CSP25	TGCCAGTGCCAGCCATCAAAC	GCCAAGCCGGTGCGACGAG
CSP30	CAATTCGCGGCCCTATGAT	GGCGGCCGAGCTGGTGGAC
CSP320B	CAATGAGGGAGGTTTATCAGC	CCAAACAGTGGCATAGCATCCTTT

Table S4 Flowering-related genes used for RT-PCR analysis.

Gene	Forward primer	Reverse primer	Annealing temperature (℃)	PCR fragment (bp)
RFL	CAGAGGGAGCATCCTTTCGTG	CGCATCTTGGGGCTTGTTGA	62	227
FCP1	GTCTCATGGGATTCTGGGT	GTACACGATTGAGGCGCGG	62	626
OMADS3	CCTGTTGCTGCTTCTGCC	CGGTTCGTCGTGTTCTCG	62	264
OsWUS	CCGCATCGAGGGCAAGAA	GCCGACTGGGAAGAGTGGAA	62	397
WOX3	TCGACCACGCTAATTCCCTTCT	CATGCTGCTCTTCCTTGAGGC	62	438
OSH1	AAGGTAAACAACAAGGCACA	GCTCAAGACACGCAGGATA	62	201
Fon4	TGTTTGGTGGTTGCATGGTGTT	TGCTTCGTCTTCGGCTCTGTC	52	146
Fon1	GGATCAGGCACCGGAACA	AGCCGTAGGAGCCAGCAAT	60	348
Fon2	GTTAGCCGAAGACGAAGC	TCCACTATGCAGGAGCAG	58	382
OsMADS6	CACCAGCAACTACAGAGCC	CCACGCAAGACCATTAGG	55	341
OsPck1	GTTACTGAAGAATAAAAAAT	GATCAAATCAAGATGCTTTC	43	253
OsMe1	CGCCGCGGAGGTCAAATTTT	ATGCTGCCCATTACAATGGG	55	265
OsRAA1	ATGTCAGGGGTTTGGGTGTTC	GGCGTCGACGACGCGGAAGGA	52	340
LAX1	TTCCTCAAGGCGCAGGTCA	ATCTCCAGCGTCGTCATCCC	55	176
SP1	CGGTAACCAAGAGGAAACAAGTG	CACCACGCACAGTAGCACCTT	56	246
APO1	CCGCCGGCCCGACCTCCATCATC	CTCCACCAGCGCCGGCGACCTCAG	62	317
AK070205	GCCACTGCGCCAACCTGCTCTC	TGTCCGGATTGCTTGCCTTGATGC	56	402
AK106784	TCGTCGTCGGCTGTCTTCCCACTG	GCGCTCGCGGCCTGCTCCATC	62	329
AK120659	ACCAACGGCCGGCCCCTCTCG	CGCCCCTTCCCCCGCATCTCG	62	404
SPEDJ	GAAGCAATTCCATGCAATGAGG	GGTCCAGTCAAACTAATGG	60	450
HYG	GACGGTGTCGTCCATCACAGTTT	ACTCACCGCGACGTCTGTCGAGAA	56	495
Actin	AGCAACTGGGATGATATGGA	CAGGGCGATGTAGGAAAGC	56	450

Table S5 Rice materials used in this study

Rice variety and material	Rice type	Inflorescence phenotype	Genotypes	Source or reference
9311	*Indica*	Normal	Sped1	Restorer line (YU *et al.* 2002)
TP309	*Japonica*	Normal	Sped1	Taipei 309 (SONG *et al.* 1995)
R948	*Indica*	Normal	Sped1	Restorer line, from Sichuan Agricultural University, China
R549	*Indica*	Normal	Sped1	Restorer line, from Sichuan Agricultural University, China
Ballila	*Japonica*	Normal	Sped1	Introduced from Itali (JI *et al.* 2012)
R527	*Indica*	Normal	Sped1	Restorer line, from Sichuan Agricultural University, China
Kitaake	*Japonica*	Normal	Sped1	Introduced from USA (CHEN *et al.* 2014)
sped1-D	*Indica*	clustered spikelets	sped1-D	The rice spontaneous mutant of 9311(This study)
Tsped1	*Japonica*	clustered spikelets	sped1-D	BC3 plant from the backcross of TP309 with sped1-D.This study (This study)
R948sped1	*Indica*	clustered spikelets	sped1-D	BC3 plant from the backcross of R948 with sped1-D.This study (This study)
R549sped1	*Indica*	clustered spikelets	sped1-D	BC3 plant from the backcross of R549 with sped1-D.This study (This study)
Ballilasped1	*Japonica*	clustered spikelets	sped1-D	BC3 plant from the backcross of Ballila with sped1-D.This study (This study)
R527sped1	*Indica*	clustered spikelets	sped1-D	BC3 plant from the backcross of R527 with sped1-D.This study (This study)
Ksped1	*Japonica*	clustered spikelets	sped1-D	BC3 plant from the backcross of Kitaake with sped1-D (This study)

Files S1-S4

Available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.163931/-/DC1

File S1 672 genes that were up-regulated in sped1-D plant.

File S2 574 genes that were down regulated in sped1-D plant.

File S3 Secondary structure and disorder prediction of SPED1.

File S4 Secondary structure and disorder prediction of sped1-D.

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