

## Map-based cloning of the *ERECT PANICLE 3* gene in rice

Rihua Piao · Wenzhu Jiang · Tae-Ho Ham · Min-Seon Choi · Yongli Qiao · Sang-Ho Chu · Jung-Hyun Park · Mi-Ok Woo · Zhengxun Jin · Gynheung An · Joohyun Lee · Hee-Jong Koh

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**Abstract** Panicle architecture in rice can have a strong influence on yield. Using *N*-methyl-*N*-nitrosourea mutagenesis, we isolated an erect panicle mutant, Hep, from Hwasunchalbyeo, a glutinous *japonica* rice cultivar. Genetic analysis revealed that the erect panicle phenotype was controlled by a single recessive mutation designated *erect panicle 3* (*ep3*). Genetic mapping revealed that the *ep3* mutation was located on the short arm of chromosome 2 in a 0.1 cM region delimited by the STS markers STS5803-5 and STS5803-7. The *ep3* locus corresponded to 46.8 kb region and contained six candidate genes. Comparison of the DNA sequences of the candidate genes from wild-type and erect panicle plants revealed a single base-pair change in the second exon of LOC\_Os02g15950, which is predicted to result in a nonsense mutation. LOC\_Os02g15950 encodes a putative F-box protein containing 515 amino

acids and is expressed throughout the plant during all growth stages. A line carrying a T-DNA insertion in LOC\_Os02g15950 was obtained and shown to have the same phenotype as the *ep3* mutant, thus confirming the identification of LOC\_Os02g15950 as the *ERECT PANICLE 3* (*EP3*) gene. The *ep3* mutation causes a significant increase in the number of small vascular bundles as well as the thickness of parenchyma in the peduncle, which results in the erect panicle phenotype.

### Introduction

Among various crop breeding efforts for increasing yield, improving plant architecture has been a successful strategy. The most famous achievement in the breeding history was the introduction of the semi-dwarf gene into cultivated wheat in the late 1950s, resulting in the “Green Revolution” (Otsuka and Kalirajan 2006). In cereal crops, the architecture of panicles (compound racemes), is an important trait related to yield. Therefore, extensive studies have been carried out to improve various aspects of panicle architecture such as panicle length, number of primary branches per panicle, grain length, grain width, seed set ratio, diameter of panicle neck, and spike density (Chen et al. 2006; Qiao et al. 2008; Redona and Mackill 1998).

Donald (1968) and Yin (1961) suggested that the erect panicle in wheat is advantageous in reducing shade canopy areas to increase photosynthesis efficiency, whereas the curved panicle phenotype leads to larger shade canopy areas. In addition to increasing utilization of solar energy, erect panicles improve physiological conditions in cultivated fields including canopy illumination, temperature, humidity, and aeration of CO<sub>2</sub>, which results in increasing population growth rate and yield (Gao et al. 1999). Given

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R. Piao · W. Jiang · T.-H. Ham · M.-S. Choi · Y. Qiao · S.-H. Chu · J.-H. Park · M.-O. Woo · J. Lee · H.-J. Koh (✉)  
Department of Plant Science,  
Plant Genomics and Breeding Institute,  
and Research Institute of Agriculture and Life Sciences,  
Seoul National University, Seoul 151-921, Republic of Korea  
e-mail: heejkoh@snu.ac.kr

Z. Jin  
Agricultural College, Northeast Agricultural University,  
150030 Harbin, Heilongjiang, China

G. An  
National Research Laboratory of Plant Functional Genomics,  
Division of Molecular and Life Science,  
Pohang University of Science and Technology (POSTECH),  
Pohang 790-784, Republic of Korea

these advantages, commercial rice cultivars with the erect panicle trait were developed in the 1980s. Shennong 265 was one of the most successful erect panicle rice cultivars grown in northern China and was developed from the erect panicle Italian cultivar Ballila (Zhang et al. 2002).

Despite its usefulness, few genetic studies of the erect panicle trait in rice have been conducted and knowledge of the genes at the molecular level is limited. Both dominant and recessive genes have been reported to be responsible for the erect panicle trait. For example, Zhu and Gu (1979) developed the erect panicle cultivar, Xiaoli57, from the *japonica* cultivar Nongken57 by mutagenesis and reported that the erect panicle was controlled by a single recessive gene. Three independent studies have described dominant genes mapping to chromosome 9. Kong et al. (2007) reported a dominant gene on chromosome 9 located between the SSR markers RM5833-11 and RM5833-23, Yan et al. (2007) reported a major QTL, *qPE9-1*, between the STS marker H90 and the SSR marker RM5652, and Chen et al. (2008) reported a dominant gene linked to the SSR markers RM566, RM24423, and RM7048. Zhou et al. (2008) reported an erect panicle in introgression lines developed from *Oryza glaberrima*, an African cultivated rice, and mapped the gene controlling this trait between RM5879 and RM3332 on chromosome 4. Recently Huang et al. (2009) reported the cloning of a gene underlying a major QTL for grain yield on rice chromosome 9. This gene, *DENSE AND ERECT PANICLE1 (DEP1)*, mapped to the same location as *qPE9-1* and encodes a phosphatidylethanolamine binding protein-like domain protein (Huang et al. 2009).

We recently isolated an erect panicle mutant using chemical mutagenesis. The objectives of our study were to characterize the mutant phenotype and to clone the gene underlying this trait using a map-based cloning strategy.

## Materials and methods

### Plant materials

The erect panicle mutant line was isolated by chemical mutagenesis of the glutinous *japonica* rice cultivar Hwasunchalbyeo using *N*-methyl-*N*-nitrosourea and generation-advanced at the greenhouse and/or experimental field. The seeds of the Hwasunchalbyeo erect panicle mutant (designated Hep) were taken from  $M_8$  generation for this study. Hep was crossed with Milyang 23 (*indica*), Hwacheongbyeo (*japonica*), and Hwasunchalbyeo (*japonica*). An  $F_2$  population and segregating  $F_3$  lines from the cross between Hep and Milyang 23 and an  $F_2$  population from the cross between Hep and Hwacheongbyeo were used for genetic

analysis. Small  $F_2$  population from the cross between Hep and wild-type Hwasunchalbyeo were used for dCAPS analysis. The  $F_2$  population and one  $F_3$  family from the cross between Hep and Milyang 23 were used for fine mapping and gene cloning. To confirm the heterozygosity of selected  $F_2$  plants,  $F_3$  progenies were observed for phenotypic segregation. All plant materials for gene cloning were grown at the experimental farm of Seoul National University, Suwon, Korea in 2006 and 2007. The line containing a T-DNA insertion in LOC\_Os02g15950 was identified using the Rice Functional Genomic Express Database (<http://signal.salk.edu/cgi-bin/RiceGE>) and seeds (entry number: 1C-03432.L) were obtained from the POSTECH RISD (<http://www.postech.ac.kr/life/pfg/risd/>). The T-DNA insertion mutant lines were grown under standard greenhouse conditions.

### Observation of peduncles

Paraffin-embedded peduncle tissue sections were prepared following the methods described by Ji et al. (2006) with slight modifications. Panicles were harvested at ripening stage. The peduncle were cut 1 cm in length from the panicle node and fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) and stored at 4°C for 1 day. The fixed peduncles were dehydrated by soaking for 2 h each in an ethanol solution series 70, 85, and 95% followed by incubation in 95% ethanol overnight. Finally, the peduncles were soaked in 100% ethanol for 2 h. Peduncle samples were cleared by soaking for 2 h in the clearing solution series consisting of 75% ethanol/25% xylene, 50% ethanol/50% xylene, 25% ethanol/75% xylene, followed by soaking in 100% xylene solution overnight. For paraffin infiltration, the cleaned peduncle samples were soaked for 2 h in the solution series of 75% xylene/25% paraffin, 50% xylene/50% paraffin, 25% xylene/75% paraffin, and 100% paraffin followed by 100% paraffin at 55°C overnight. The infiltrated sample was embedded in a paraffin block and then cut into 12- $\mu$ m sections using a microtome (MICROM Lab, Walldorf, Germany) and mounted on a Superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and dried at 42°C for 1 day. The sections were deparaffinized with 100% xylene for 10 min followed by hydration by soaking for 2 min each in 50% ethanol/50% xylene, 100% ethanol, and sterile water. Peduncle samples were stained in 1% safranin/30% ethanol for 30 s followed by washing with sterile water two times. Samples were soaked for 2 min each in 30% ethanol, 50% ethanol, 70% ethanol, 85% ethanol, and 95% ethanol. Finally the sections were cleared by soaking twice in 100% xylene for 10 min and mounted in Canada balsam. The cross sections of peduncle were observed by optical microscopy at 100 $\times$  magnification.

## Fine mapping of the gene for erect panicle

A total of 987 F<sub>2</sub> plants from the cross between Hep and Milyang 23 were used for fine mapping. Genomic DNA samples were extracted from rice leaves using the CTAB method (Causse et al. 1994). Eight mutant and eight wild-type F<sub>2</sub> plants were selected and an equal amount of DNA from each of the eight plants were pooled into a single sample for bulked-segregant analysis (BSA) (Michelmore et al. 1991). After BSA, fine mapping was conducted with the neighboring STS markers, which were developed by designing primers based on the differences in DNA sequences between the *indica* and *japonica* rice subspecies (Chin et al. 2007) (<http://www.ncbi.nlm.nih.gov/> for *indica* and <http://www.rgp.dna.affrc.go.jp/> for *japonica*). Primer sequences are listed in Table 1. Linkage analysis was conducted using MAPMAKER version 3.0 software (Lander et al. 1987). Map distances were estimated using the Kosambi function (Kosambi 1944).

PCR was performed in a reaction volume of 20 µl containing 40 ng of template DNA, 0.2 µM of each primer, 200 µM of each dNTP, 10 mM Tris–Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin and 0.5 U of Taq DNA polymerase. Amplifications were carried out in a PTC100 96U Thermocycler (MJ Research, Reno, NV, USA) in the following sequence: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, and 5 min at 72°C for a final extension. PCR products were separated in 3.0% agarose/0.5× TBE gels and visualized by ethidium bromide staining.

## Sequence analysis of the candidate genes

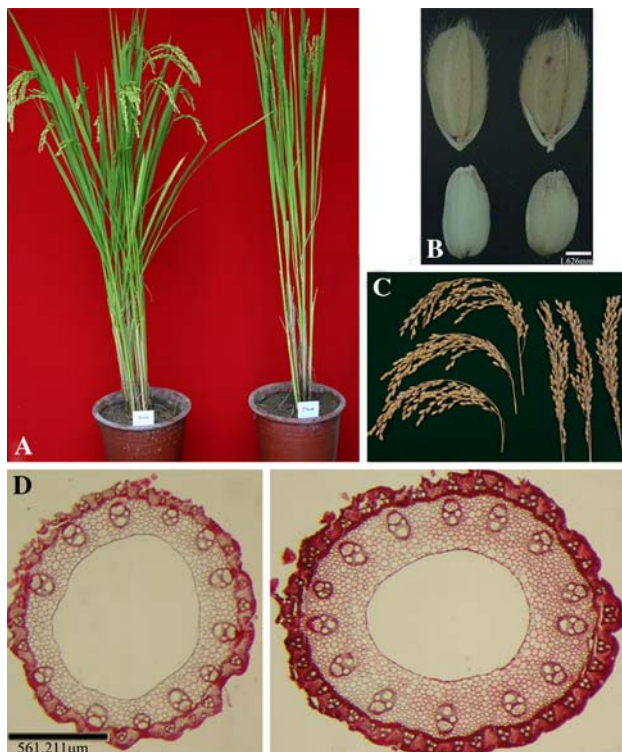
The full-length genomic DNA sequences of candidate genes were determined by dividing the genes into several overlapping segments. Specific PCR primers for each segment were used to amplify genomic DNA from wild-type Hwasunchalbyeo and Hep. PCR products were purified using a PCR purification kit (Bioneer, Deajeon, Korea) for TA cloning. Purified PCR product was introduced into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into the *E. coli* strain DH5α. Sequencing of genomic inserts was performed using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). T7 and SP6 primers were used. Sequence alignment was performed with the BLAST network services in National Center for Biotechnology Information (NCBI) and the European Bioinformatics Institute (<http://www.ebi.ac.uk>).

## dCAPS maker analysis

The sequencing results were aligned with the original parent. For dCAPS analysis, PCR amplification with the primer set dcaps-F (5'-TCGTGTGCAACCCAATAAC-TAAGGTCT-3') and dcaps-R (5'-GTGAAGGGAGACACCCATGTA-3') were used. Primers were designed using the dCAPS finder 2.0 program (<http://helix.wustl.edu/dcaps/dcaps.html>). Each product (5 µl) was digested with *Xba*I in a total volume of 15 µl at 37°C for 3 h. After digestion, 5 µl of each digest was visualized on a 3% agarose/0.5× TBE gel.

**Table 1** The PCR-based molecular makers used in fine mapping of the *EP3* gene

STS marker	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size amplified in <i>japonica</i> (bp)	Originated clone
S2039	CGCAAACAACATTCCAAAAA	CAGCGGCATTCTCTAAAA	248	AP004875
S6844-1	GTGGAGACAAGAGCGGTAGC	CAAACCTTGCTGACGGTGCT	207	AP006844
S6844-2	GTTGGATTCCACTCCTTTCG	TCAGTGTACAGGCATCACA	160	AP006844
S4801	GAAGCACAAGCTGAGGAAGG	TTCCTGTCCATTC AATTCTCG	237	AP004801
S5803-1	CCTGCACAGCTTTACATTGG	TCCAAGGGTGATTATGTACG	247	AP005803
S5803-5	CCATGACAATCGATGCTGAA	TGGTTGTGAAGGTGATTGCT	243	AP005803
S5803-6	AAGTACGTGTGGTGCCCTA	GGGATACTATGTTGGATGGA	181	AP005803
S5803-7	GAATGGATGGATGGATCGAG	GTAGGGTCCGGCGAGATATG	171	AP005803
S5803-8	AGGCAACCATGCAAATTAGG	GTGGGCCAACTCAATTCAAG	191	AP005803
S5777	TGCAGAACCGTGACTCTCTG	GAGGAGGGTGATCAAGGTGA	213	AP005777
S5631	TCTATGCTGCTTGTGCCATC	ACATGTCAACATGGAAGTGTGAG	150	AP005631
S2043	GTGCGGATCCGAATCAACT	TACTGCAGCGGGGAGTATTT	273	AP004683
S4771	AAGAGGGCGCAGATATAGGG	CACCTTTTCATTTGATAGTTGGA	220	AP004771
S4872	AAACCGATGTGCAAGGATTC	TCTTTGCCTCATTCGAAGAAA	177	AP004872
S5414	CAGGTCAACGTCACCACATC	GCTCTGTATGGCCTGGAATG	241	AP005414
S2052	GCAGTCGGTTCTCAATTGGT	GATTTTCCAGCCCATCTCA	192	AP005743



**Fig. 1** Phenotype of the Hep. **a** Plants at 3 weeks after anthesis. **b** Shape of paddy rice (*top*) and brown rice (*bottom*) grains. **c** Mature panicles. **d** Cross section of peduncles. In each case, the Hep and wild-type Hwasunchalbyeo are shown on the *right* and *left*, respectively

## RT-PCR

Total RNA was isolated from different tissues of wild-type plants at the heading stage using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). An aliquot of total RNA was reverse-transcribed using an oligo (dT) primer and a M-MLV reverse transcriptase kit (Promega, Madison, WI, USA). For the amplification of full-length cDNA of the target gene, we designed a pair of primers based on the cDNA sequence of LOC\_Os02g15950 from the MSU Rice Genome Annotation Project Database (<http://rice.plantbiology.msu.edu/index.shtml>), erect-C-F (5'-AGGGACCAAATGCCCCCTCC-3') which contains the 5'UTR sequence with start

code and erect-C-R (5'-GGGAAGTTTAGACGCCCTTT-3') which was derived from 3'UTR sequences. The amplification reaction was carried out using the following conditions: 5 min at 95°C, 35 cycles of 30 s at 94°C, 39 s at 58°C and 1 min 30 s at 72°C, with a final extension of 15 min at 72°C. Amplification products were recovered and sequenced. To analyze the RNA expression pattern, RT-PCR amplification primer sets 15950E1-E2-F (5'-CTGGCCGGAATCGGATTGAAG-3') and dcaps-R (5'-GTGAAGGGAGACACCCATGTA-3') were used.

## Genotyping of T-DNA insertion mutant

For genotyping T-DNA insertion mutant, we designed three primers based on the sequence information of T-DNA insertion position available at POSTECH RISD (<http://www.postech.ac.kr/life/pfg/risd/>). PCR with primer combinations F1 (5'-CATGGGTGTCTCCCTTCACT-3')/R1 (5'-GGTGAATGGCATCGTTTGAA-3') and F2 (5'-CTAGGAAGCAATGTCCAGCC-3')/R1 was conducted to test the co-segregation between the flanking sequence and the mutation phenotype. F1 and R1 primers were designed using the rice genome sequences containing the T-DNA inserted region, and F2 primer was made using the left border sequence of the T-DNA (Fig. 6a).

## Results

### Morphological and agronomic characterization of the erect panicle mutant

The most prominent feature associated with the Hep mutant is the upright nature of its panicles even when the grains are fully matured (Fig. 1a–c). In wild-type plants, panicles begin to deflect or bend 3 weeks after anthesis as grain weight increases. The leaves of Hep also appear to be more erect, and the overall shape of the plant appears narrower or more compact than that of wild-type plants. For general agronomic traits (Table 2), the culm length, heading date, and grain weight of the mutant were similar to wild type. However, the number of panicles (10 tillers vs. 14), panicle

**Table 2** Agronomic traits of the Hep and wild-type cultivar Hwasunchalbyeo

Traits	HD	CL (cm)	PL (cm)	PN (No.)	SN (No.)	SF (%)	Wt (g)	GL (mm)	GW (mm)	GS	NPB (No.)	LPB (cm)
Wild type	Aug. 20	72.6	17.0	13.6	122	96.1	25.7	5.69	3.04	1.88	10.9	5.66
Hep	Aug. 19	71.3	15.3	9.7	80.5	92.3	25.7	5.61	3.27	1.71	12.4	4.97
Difference	NS	NS	**	**	**	**	NS	NS	**	**	**	**

HD heading date, CL culm length, PL panicle length, PN number of panicle per hill, SN number of spikelets per panicle, SF spikelet fertility, Wt 1,000-grain weight, NPB number of primary rachis branch per panicle, LPB length of primary rachis branch per panicle, GL grain length, GW grain width, GS grain shape (length/shape), NS not significant

Asterisks indicate the significant difference between the Hep and wild-type plants as determined by Student's *t* test. \* 0.01 < *P* < 0.05; \*\* *P* < 0.01

length (15 vs. 17 cm), number of spikelet per panicle (80 vs. 122), and spikelet fertility (92.3 vs. 96.1%) was reduced in the mutant. Grains of the mutant were wider and rounder than the wild type (Table 2; Fig. 1b). For stem traits (Table 3), the thickness and diameter of the peduncle (first internode from the top) were significantly greater than the wild type, but the difference between the mutant and wild-type internodes gradually diminished below the second internode. In addition, the number of vascular bundles of the peduncle was significantly higher in the mutant, but was similar to wild type from the second internode (Fig. 1d). Although the leaves of the mutant appeared to be more upright compared to the wild-type plant, no differences between the mutant and wild type were observed in leaf cross sections (data not shown).

### Genetic analysis of the erect panicle trait

Using Hep (*japonica*) as the female parent, crosses were made to two wild-type plants, Milyang 23 (*indica*) and Hwacheongbyeon (*japonica*). All F<sub>1</sub> individuals from these crosses had wild-type panicle architecture, indicating that the erect panicle phenotype was recessive. In the two F<sub>2</sub> populations, segregation of erect panicles was observed and no intermediate phenotype was detected (Table 4). In the Hep/Hwacheongbyeon F<sub>2</sub> population, 239 wild-type and 63 erect panicle plants were observed, which fits the 3:1 ratio expected for single gene inheritance. However, in the F<sub>2</sub> population derived from Hep/Milyang 23, the observed phenotypes (811 wild type:176 erect panicle type) did not fit the 3:1 segregation ratio. This result was likely due to the segregation distortion as this F<sub>2</sub> population was derived

from an *indicaljaponica* cross, where partial sterility is an important factor controlling distorted segregation (Lin et al. 1992). F<sub>3</sub> progeny testing of a Hep/Milyang 23 F<sub>2</sub> plant exhibiting wild-type panicles (heterozygous) revealed 245 wild-type and 91 erect panicle type plants (3:1;  $\chi^2 = 0.39$ ) confirming that a single recessive mutation controls the erect panicle phenotype.

### Fine mapping of the erect panicle mutation

Initial mapping of the erect panicle mutation was performed using bulked-segregant analysis of F<sub>2</sub> plants from the Hep/Milyang23 cross and 125 STS markers distributed over the 12 chromosomes (Michelmore et al. 1991). Three markers (S2039, S2043, and S2052) on the short arm of chromosome 2 appeared to be linked to the mutant phenotype and were used to genotype 987 F<sub>2</sub> individuals. Linkage analysis revealed that the mutation maps between S2039 and S2052 with genetic distances of 8.5 and 2.5 cM, respectively (Fig. 2a). This causal gene differs from the loci previously reported for the erect panicle trait in rice, *DEP1* on chromosome 9 (Huang et al. 2009; Kong et al. 2007; Yan et al. 2007) and *EP2(t)* on chromosome 4 (Zhou et al. 2008), and thus it was designated as *ERECT PANICLE 3* (*EP3*).

To refine the position of the *EP3* gene, 14 additional STS markers located between S2039 and S2052 were designed based on the sequences available at the rice genomic databases (<http://www.ncbi.nlm.nih.gov/> for *indica* and <http://www.rgp.dna.affrc.go.jp/> for *japonica*) (Table 1; Fig. 2b). Subsequent linkage analysis with the 16 markers and 987 F<sub>2</sub> individuals revealed that the *EP3* gene was

**Table 3** Comparison of internode characters between the Hep and wild-type plants

Traits	Thickness of internode (mm)				Diameter of internode (mm)				NSVB (No.)		NLVB (No.)	
	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	1st	2nd
Wild type	0.29	0.46	0.61	0.72	1.57	3.44	4.30	4.82	21.8	32.5	11.2	32.5
Hep	0.56	0.57	0.70	0.86	1.83	3.80	4.63	5.29	34.5	33.5	12.1	33.5
Difference	**	**	*	NS	**	*	NS	NS	**	NS	*	NS

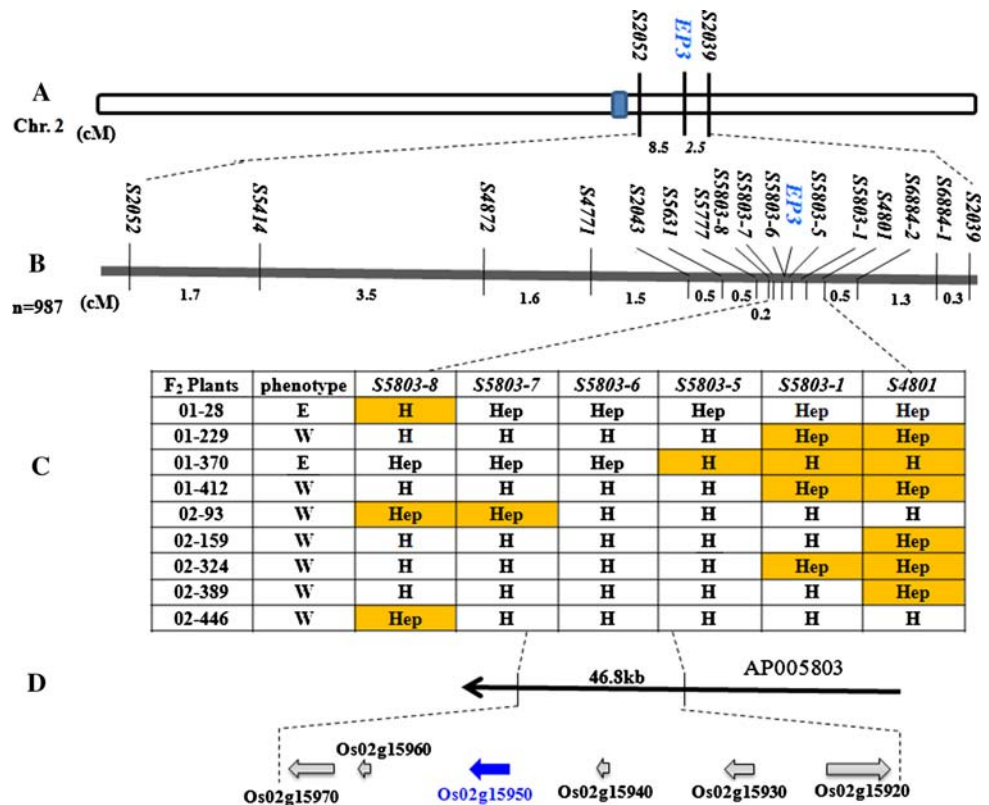
NSVB number of small vascular bundles, NLVB number of large vascular bundles, Thickness of internode [(outside diameter – inside diameter)/2], Diameter of internode (outside diameter), NS not significant

Asterisks indicate the significance of differences between the Hep and wild-type plants as determined by Student's *t* test. \* 0.01 < *P* < 0.05; \*\* *P* < 0.01

**Table 4** Genetic segregation of the erect panicle in rice

Cross combination	F <sub>1</sub> plants			F <sub>2</sub> plants			F <sub>3</sub> plants			$\chi^2$ (3:1)
	WT	ep	Total	WT	ep	Total	WT	ep	Total	
Hep/Milyang23	37	0	37	811	176	987	–	–	–	27.04**
Hep/Hwacheongbyeon	4	0	4	231	63	294	–	–	–	2.00
Hep/Milyang23	–	–	–	–	–	–	254	91	345	0.39

**Fig. 2** Genetic and physical maps of the *EP3* gene. **a** Linkage map of chromosome 2 constructed using the F<sub>2</sub> population from Hep/Milyang 23. **b** High-resolution genetic map of the *EP3* gene in the F<sub>2</sub> population. **c** Graphical genotype of key recombinants at loci nearby the *EP3* gene. **d** Relative location and orientation of candidate genes residing in the 46.8 kb locus in the BAC clone AP005803. Phenotype: *E* Hep plant, *W* wild-type plant but segregating in offspring Genotype: *Hep* Hep type, *H* heterozygote



flanked by the STS markers S5803-5 and S5803-7 and the physical distance between these two markers was 46.8 kb on a Nipponbare BAC clone AP005803. Based on available sequence annotation databases (<http://www.tigr.org> and <http://www.gramene.org>), there are six predicted genes in this region: LOC\_Os02g15920, LOC\_Os02g15930, LOC\_Os02g15940, LOC\_Os02g15950, LOC\_Os02g15960, and LOC\_Os02g15970 (Fig. 2d).

#### Identification and characterization of the *EP3* gene

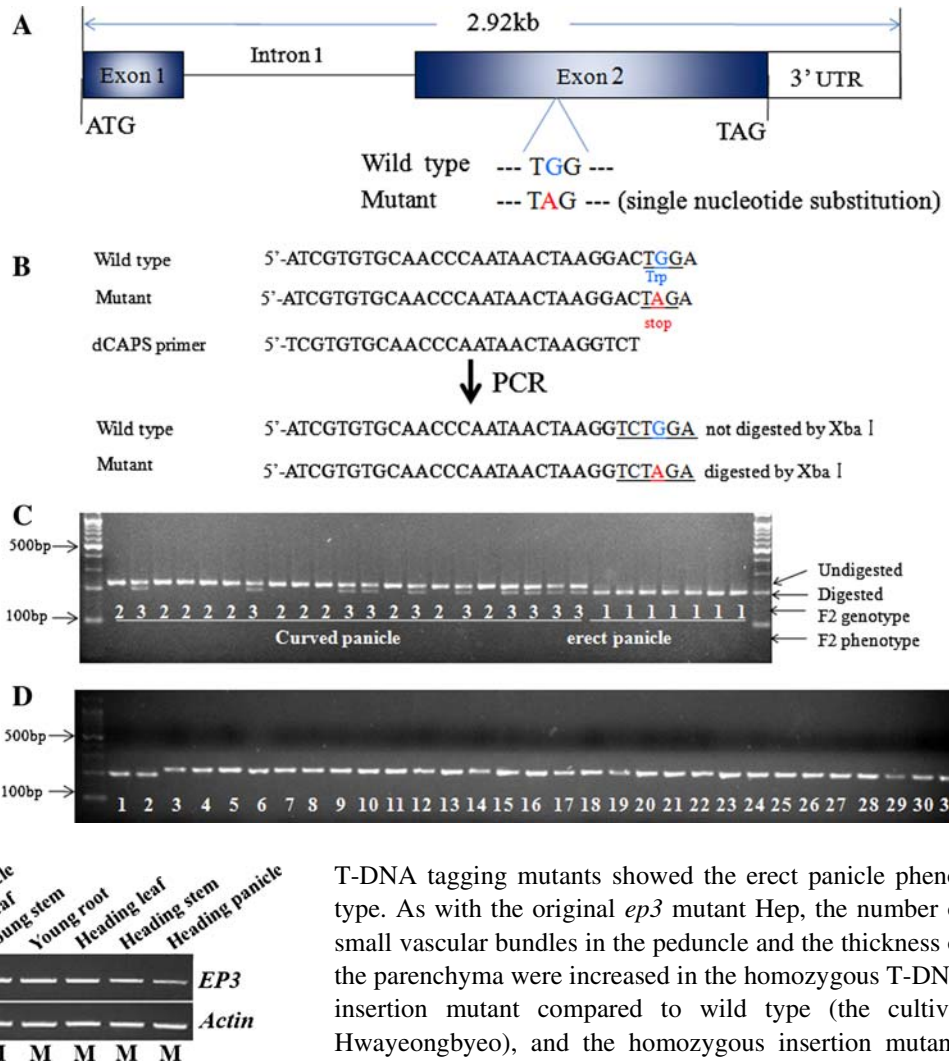
To identify the *EP3* gene, we compared the genomic sequences of all six candidate genes between Hep and wild-type Hwasunchalbyeo. Of the six candidate genes, only LOC\_Os02g15950 exhibited a sequence difference between the wild type and Hep. This gene is 2,921 bp in length and consists of two exons and one intron. A single base-pair change was detected in position 466 located in the second exon (G/C → A/T), which is predicted to result in a change from tryptophan to a stop codon (Fig. 3a). To verify the SNP, we designed the dCAPS markers, dcaps-xbaI, and screened the F<sub>2</sub> mapping population. We found complete co-segregation of the dCAPS genotypes with the matching phenotypes (Fig. 3b, c). Also, SNP genotyping of 19 *japonica* and 10 *indica* rice cultivars with wild-type panicle curvature revealed that all these cultivars have the G/C base pair (Fig. 3d).

To detect the full cDNA sequence, we designed PCR primers based on the MSU Rice Genome Annotation Project Database (<http://rice.plantbiology.msu.edu/index.shtml>). Amplifying mRNA with the primers resulted in the detection of a 1,548 bp cDNA which was identical in sequence to the Nipponbare cDNA. Semi-quantitative RT-PCR of *EP3* in wild-type and Hep tissues revealed that the gene was expressed in all tested growth stages and plant parts including young stage (35-day-old seedlings), heading stage, leaf, stem, root, and panicle (Fig. 4).

#### *EP3* gene encodes a putative F-box protein

A BLAST search of LOC\_Os02g15950 revealed that it encodes a putative protein of 515 amino acids containing an F-box protein domain located in the N-terminal region and a kelch-2 motif located in the C-terminal region. The F-box domain has approximately 50 amino acid residues and binds substrates in the ubiquitin (Ub) 26S proteasome pathway (Kipreos and Pagano 2000; Ni et al. 2004). The LOC\_Os02g15950 protein has more than 50% identity to proteins encoded by another rice gene (Os01g47050), two unknown *Zea mays* genes (zeaACG37382 and zea-ACG41053), one *Sorghum bicolor* gene (Sb04g009700), and one *Arabidopsis* gene (Os01g47050) (Fig. 5). The *Arabidopsis* homologue (AT3G61950) encodes the UNUSUAL FLORAL ORGAN (UFO)-like protein, which

**Fig. 3** *EP3* gene structure and mutation position. **a** The structure of the *EP3* gene (LOC\_Os02g15950) and the position of the mutation. **b** The mutation within the *EP3* gene in the Hep. **c** Co-segregation analysis in F<sub>2</sub> plants of the Hep×Hwasunchalbyeo cross using dCAPS marker. The dCAPS marker using a mismatch primer selectively generates a restriction site (*Xba*I) in the Hep. **d** Genotype of the dCAPS marker among the Hep and 29 rice cultivars having curved panicles. Lane 1 and 2 are Hep plants, and lanes 3–31 are rice cultivars



**Fig. 4** *EP3* gene expression. Semi-quantitative RT-PCR of the *EP3* gene in organs, including leaf, stem, root at young stage and leaf, stem, panicle at heading stage (W wild type, M Hep)

affects sepal morphology. Loss of function of the AT3G61950 gene induced fused sepals at the lower part of margins and affected the leaf width and silique (Gonzalez-Carranza et al. 2007).

A T-DNA insertion mutant in LOC\_Os02g15950 exhibits erect panicles

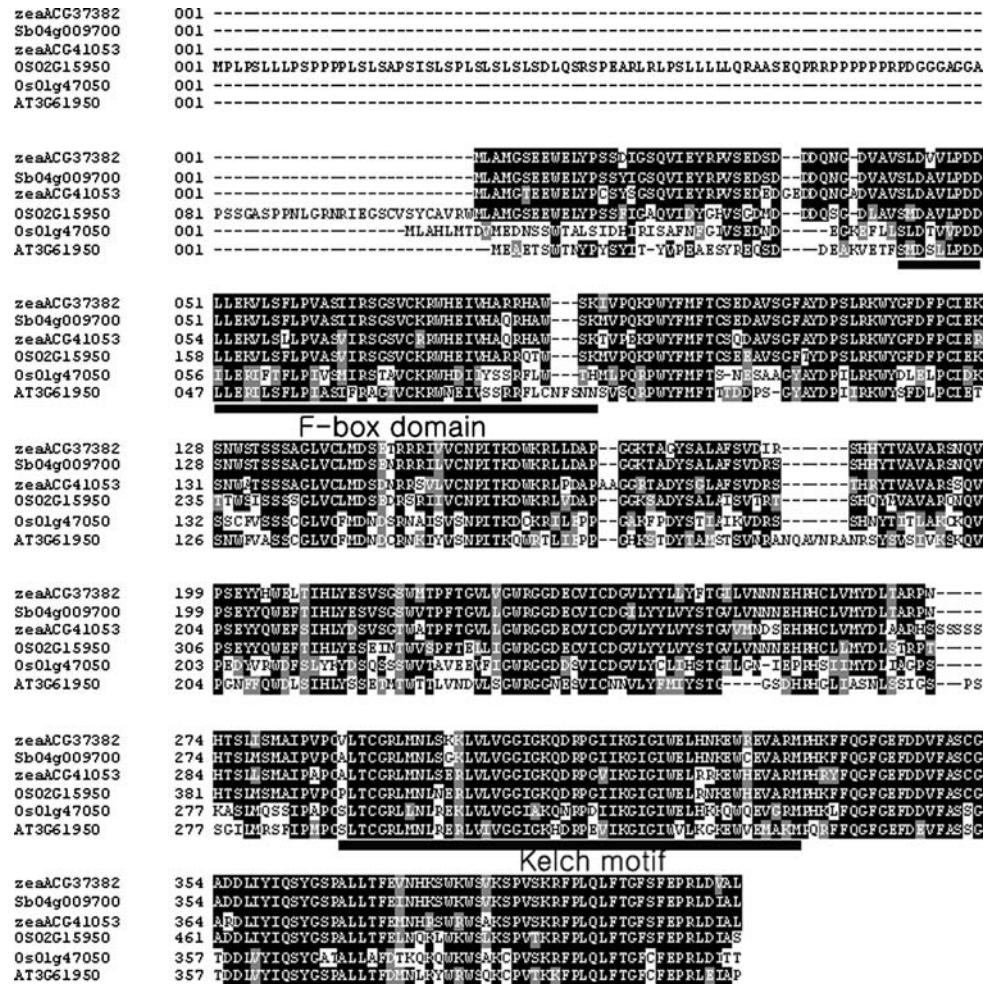
To confirm that the loss of function of LOC\_Os02g15950 caused the erect panicle phenotype, we obtained one T-DNA tagging line (1C-03432.L) from POSTECH RISD (An et al. 2003). This line has a T-DNA inserted into the second exon of LOC\_Os02g15950 (Fig. 6a), which was confirmed by PCR analysis. A total of 11 T<sub>2</sub> seeds were planted in the greenhouse in the summer of 2008. Three homozygous and five heterozygous T-DNA tagging mutants were identified (Fig. 6b). Only the homozygous

T-DNA tagging mutants showed the erect panicle phenotype. As with the original *ep3* mutant Hep, the number of small vascular bundles in the peduncle and the thickness of the parenchyma were increased in the homozygous T-DNA insertion mutant compared to wild type (the cultivar Hwayeongbyeo), and the homozygous insertion mutants exhibited a rounder grain shape (Fig. 6c–e). However, the number of large vascular bundles of T-DNA mutants was not significantly different from the wild-type cultivar.

## Discussion

Panicle type is strongly correlated with grain yield. Therefore, many studies on panicle types (e.g., erect, spreading, compact) and various aspects of panicle architecture (e.g., panicle length, number of grains on primary branch, and aberrant panicle organization) have been conducted. Several groups have recently reported the genetic characterization of the erect panicle trait using different rice germplasm (Huang et al. 2009; Kong et al. 2007; Yan et al. 2007; Chen et al. 2008; Zhou et al. 2008). Several of these studies reported the identification of genes or QTLs that mapped to very similar locations on chromosome 9, suggesting that these loci represent the same gene. Recently, Huang et al. (2009) reported the cloning of *DENSE AND ERECT PANICLE 1 (DEP1)*, a gene underlying a rice grain yield QTL on

**Fig. 5** Alignment of EP3 with F-box proteins from two *Zea mays* (zeaACG37382, accession No. zeaACG37382 and zea-ACG41053, accession No. ACG41053), *Sorghum bicolor* (gene Sb04g009700), *Arabidopsis* (AT3G61950, accession No. NP\_191718), and rice (Os01g47050, accession No. NP\_001043769). Residues that are more than 50% identical are shaded in black, conservative substitutions at an amino acid position are shaded in gray. The F-box domain and kelch motif are underlined



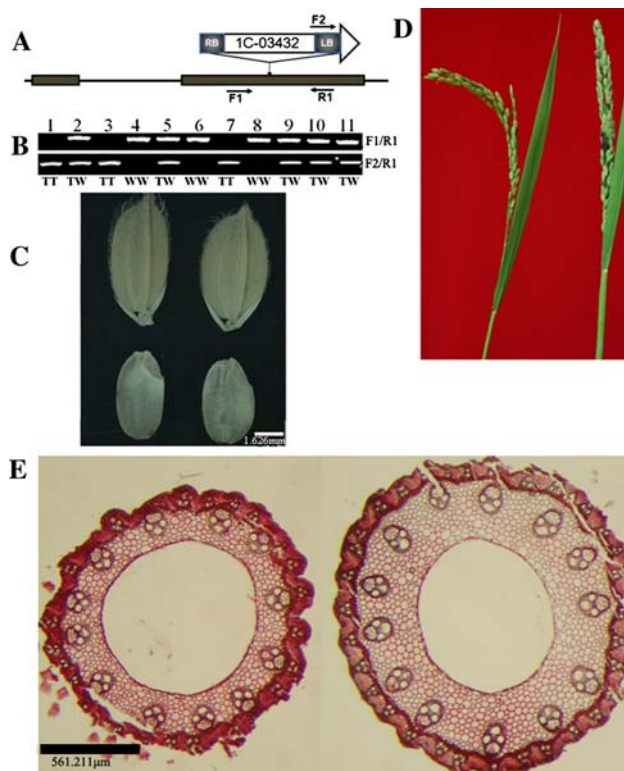
chromosome 9. A gain-of-function mutation in *DEP1* simultaneously resulted in a dense panicle, higher grain numbers per panicle, and an erect panicle. Interestingly, the location of *DEP1* coincides with that of *qPE9-1*, a QTL for panicle erectness reported by Yan et al. (2007). Another gene has been mapped to chromosome 4 (Zhou et al. 2008), thus at least two genes affect this trait.

In this study, we characterized an erect panicle mutant generated by chemical mutagenesis. Examination of the panicles revealed that the number of small vascular bundles and the thickness of parenchyma in the peduncles of Hep were greater than wild type. The number of large vascular bundles in the peduncle might not be directly affected by the mutation because it was increased in Hep but not in the T-DNA insertion mutant. In addition to the panicle architecture, Hep plants exhibited differences in the number and length of secondary branches, tiller number, and grain shape. These phenotypes co-segregated with the erect panicle trait indicating that the mutation is pleiotropic. Genetic analysis of Hep confirmed that the mutant phenotypes were due to a single recessive mutation, which we fine-mapped to a small region of rice chromosome 2. Sequence analysis

of the candidate genes at the locus resulted in the identification of a nonsense mutation in the gene, LOC\_Os02g15950. The wild-type *EP3* allele is predicted to encode a protein of 515 amino acids whereas, the Hep allele encodes a truncated protein in which 267 amino acids in the C-terminal are absent, which results in the deletion of the kelch motif\_2 (Fig. 5). Confirmation of the role of this gene in panicle erectness and other developmental/morphological traits was obtained by analysis of a T-DNA insertion mutant. As the third different gene involved in the erect panicle type to be identified, we have designated LOC\_Os02g15950 as *ERECT PANICLE 3 (EP3)*.

The *EP3* gene encodes a putative F-box protein. Members of the F-box super-family of proteins in plants have an F-box domain of about 50 amino acids in their N-terminus and bind substrates in the ubiquitin (Ub) 26S proteasome pathway. F-box proteins generally contain one or several highly variable repeat domains (e.g., kelch, leucine-rich, tetratricopeptide, or WD40), which play a key role in protein interactions and signaling pathways (Jain et al. 2007; Jarillo et al. 2001). In *Arabidopsis*, F-box proteins have been reported to be involved in regulating floral organ





**Fig. 6** Analysis of the *EP3* locus in the T-DNA line. **a** Schematic diagram of the T-DNA insertion. Two exons (*fixed boxes*) and one intron (a line between the two fixed boxes) of the *EP3* gene (LOC\_Os02g15950) are shown. *Arrows* indicate primers: *F1* forward primer in the flanking genomic sequence located on the left side of T-DNA inserted site, *F2* forward primers in the left border of T-DNA, *R1* reverse primer in the flanking genomic sequence located on the right side of T-DNA insertion site. **b** Genotype of the  $T_2$  plants evaluated by the PCR amplification with primer combinations of *F1/R1* and *F2/R1*. *TT* homozygous mutant, *WW* homozygous wild type, *TW* heterozygous. **c** Type of panicle in the  $T_2$  plants, which were homozygous for the T-DNA insertion (*right*) and wild-type Hwayoungbyeo (*left*) at 3 weeks after anthesis. **d** Seed appearance of the homozygous T-DNA mutant (*right*) and wild-type Hwayoungbyeo (*left*). **e** Anatomy of the peduncle structure in  $T_2$  plants, which were T-DNA homozygous plant (*right*) and wild-type Hwayoungbyeo plant (*left*)

development, flowering time, circadian clock, and hormone signaling (Dharmasiri et al. 2005; Hepworth et al. 2006; Schultz et al. 2001). Among the five F-box proteins reported in rice (Cao et al. 2008; Gomi et al. 2004; Ikeda et al. 2005, 2007; Itoh et al. 2003; Long et al. 2008), *ABER-RANT PANICLE ORGANIZATION 1* (*APO1*), an ortholog of the *Arabidopsis* *UFO* gene, plays a key role in the temporal regulation of meristem fate in the panicle. Hormones play an important role during pattern formation of the vascular system (Scarpella et al. 2003) and it has been shown that gibberellin treatment increases both large and small vascular bundles of the peduncle (Shimizu and Takeoka 1965). One hypothesis concerning the function of *EP3* is that it is involved in hormone signaling required for the normal development of peduncles as well as other phenotypes

observed in the Hep plants. Also, the fact that the *EP3* RNA was expressed in all growth stages and in several organs tested supports the hypothesis that *EP3* is involved in the development of several organs in rice plants.

Although the erect panicle characteristic of Hep is similar to *dep1* plants (Huang et al. 2009), grain yield related traits such as numbers of panicles and spikelets are reduced in Hep compared to wild-type plants, whereas number of spikelets per panicle in *dep1* plants (Huang et al. 2009) was significantly increased resulting in higher grain yield. For this reason, Hep may not be directly incorporated into breeding programs for higher grain yield. However, since there is no report on panicle erectness regulated by F-box protein, the Hep mutant provides a good starting point for understanding the developmental process of peduncle and panicle formation in relation to panicle architecture and grain shape in rice. Functional studies of *EP3* including the regulation of gene expression, protein interaction and developmental mechanisms for erect panicle related phenotypes are in progress.

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