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ORIGINAL ARTICLE

Molecular characterization and functional analysis of "fruit-weight2.2-like" gene family in rice

Jun Xu · Wentao Xiong · Baobao Cao · Tianze Yan · Tao Luo · Tingting Fan · Meizhong Luo

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Abstract Tomato fruit-weight 2.2 (*FW2.2*) was reported to control up to 30 % fruit weight. Recent studies demonstrated that *FW2.2*-like (*FWL*) genes also play important roles in plant growth and development. For instance, a maize homolog of *FW2.2*, named cell number regulator 1 (*CNR1*), negatively regulates plant and organ size. However, *FWL* genes in rice have not been characterized yet. In this study, eight *FWL* genes were identified in rice genome and designated as *OsFWL1-8*. The chromosome location, gene structure, protein motif, and phylogenetic relationship of *OsFWL* genes were analyzed. RT-PCR result and microarray data revealed that *OsFWL* genes exhibited diverse expression patterns and the detailed expression patterns of *OsFWL5*, *6*, and *7* negatively correlated with leaf growth activity. Rice protoplast transient transformation experiment showed that most OsFWL proteins locate at cell membrane but OsFWL8 is present in the nucleus. In addition, the functions of *OsFWL* genes were investigated by analyzing two T-DNA insertion lines for *OsFWL3* and *5*. Compared with wild type, the grain weight of *osfwl3* mutant and the plant height of *osfwl5* mutant were increased by 5.3 and 12.5 %, respectively. We also found that the increase in grain length of *osfwl3* mutant was due chiefly to incremental cell number, not cell size and the expression of *OsFWL3* negatively correlated with glume growth activity. These results provide a comprehensive foundation for further study of *OsFWL* functions in rice.

Keywords CNR1 · FW2.2 · Growth activity · OsFWL · Rice

Abbreviations

aa	Amino acid(s)
CNR1	Cell Number Regulator 1
FW2.2	Fruit-weight 2.2
FWL	FW2.2-like
OsFWL	*Oryza sativa* FW2.2-like
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

Tomato fruit-weight 2.2 (*fw2.2*) is a major quantitative trait locus which is known to contribute up to 30 % of the fruit weight variation and is a key to the evolution of fruit size (Alpert et al. 1995). A gene previously named *ORFX*, which is responsible for the *fw2.2* effect, was identified by map-based cloning and was renamed *FW2.2*. The gene *FW2.2*, which is a negative regulator of cell proliferation, regulates the tomato fruit size through controlling carpel cell number, relying on the changes in the expression level and expression timing rather than coding sequence (Cong et al. 2002; Frary et al. 2000; Liu et al. 2003). It was also reported to cause other phenotypes on fruit number and photosynthate distribution (Nesbitt and Tanksley 2001). The results of yeast two-hybrid and in vitro binding assays showed that FW2.2 directly interacts with β subunit of casein kinase II (Cong and Tanksley 2006). Casein

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J. Xu · W. Xiong · B. Cao · T. Yan · T. Luo · T. Fan · M. Luo (✉)
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
e-mail: mzluo@mail.hzau.edu.cn

Present Address:
T. Luo
Center for Neuropsychiatric Diseases, Institute of Life Science, Nanchang University, Nanchang 330031, China

kinase II (CKII) was validated to take part in cell cycle-related signaling transduction pathway in plants (Espunya et al. 1999; Moreno-Romero et al. 2008). These results indicate that *FW2.2* may participate in cell cycle regulation via CKII-mediated pathways. However, the hypothesis still lacks direct evidence so far.

Previous studies provided strong evidence that *FW2.2* has many homologs in plant, animal and fungus (Frary et al. 2000; Guo et al. 2010; Libault et al. 2010). It represents an ancient eukaryotic family of cysteine-rich proteins containing a PLAC8 domain, originally identified in mammalian placental protein with unknown function. The FW2.2-like (FWL) proteins have a highly conserved core motif: one or two transmembrane motifs locating between two cysteine/proline-rich domains (Libault and Stacey 2010). Numerous findings imply that *FWL* genes play important roles in plant development. In *Arabidopsis*, a *FWL* gene named *AtPCR1* was reported to be involved in cadmium resistance (Song et al. 2004) and two *FWL* genes (*MCA1* and *MCA2*) were found to mediate Ca^{2+} uptake (Nakagawa et al. 2007; Nakano et al. 2011; Yamanaka et al. 2010). A soybean homolog of *FW2.2* named *GmFWL1* was found to affect the nodule organogenesis when the plant was infected by the nitrogen-fixing symbiotic bacterium, *Bradyrhizobium japonicum* (Libault et al. 2010). The functions of two *FW2.2* homologs in maize were reported. Cell number regulator 1 (*CNR1*) was revealed to control plant and organ size by altering cell number. The expression of *CNR2* was found to be negatively interrelated with plant growth activity and hybrid seedling vigor (Guo et al. 2010). A *FWL* gene in avocado named *Pafw2.2-like* showed much higher expression in small fruit species than in normal ones at whole fruit growth stage (Dahan et al. 2010). With more *FWL* genes identified in other plant species, more evidences validated the hypothesis that *FWL* genes act as a general regulator of plant cell number and organ size (Guo and Simmons 2011).

Rice grain weight is a significant quantitative trait which is determined by length, width, thickness, and filling of rice (Xing and Zhang 2010). Now a series of grain weight-related genes were identified in rice, such as *GS3*, *GW2*, and *GS5*. *GS3*, a vital gene for rice grain length and weight, encodes a membrane protein composed of four different functional domains and acts as a negative regulator for grain size (Fan et al. 2006; Mao et al. 2010; Takano-Kai et al. 2009). *GW2* encodes an atypical RING-type E3 ubiquitin ligase and negatively modulates cell division through the ubiquitin–proteasome pathway (Song et al. 2007). *GS5* encodes a putative serine carboxypeptidase and acts as a positive regulator of grain width and grain weight (Li et al. 2011).

Although the *FWL* genes were reported to be general regulators of plant fruit and organ size (Guo and Simmons 2011), the molecular characteristics of *FWL* gene family in rice is still unclear. To provide a global glimpse of *FW2.2*/*CNR1* homologs in rice, we investigated their gene family members, chromosomal locations, gene structures, protein motifs, phylogenetic relationships, expression patterns, and subcellular localizations in this study. Moreover, the phenotypic test of *osfwl3* and *osfwl5* mutants indicated that *OsFWLs* may regulate seed size or plant height in rice.

Materials and methods

Identification and analysis of *OsFWL* genes

The amino acid (aa) sequence of FW2.2 was used as a query to blast The Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/) with BLASTP. Then, domain search was executed against Rice Genome Annotation Project database (RGAP, http://rice.plantbiology.msu.edu/domain_search.shtml) (Kawahara et al. 2013). The full length cDNA sequences of *OsFWL* were searched in Knowledge-Based Oryza Molecular Biological Encyclopedia database (KOME, http://cdna01.dna.affrc.go.jp/cDNA) (Kikuchi et al. 2003). Co-expression analysis was executed on Rice Oligonucleotide Array Database (ROAD, http://www.ricearray.org/index.shtml) (Cao et al. 2012).

The analysis of chromosomal localization, gene structure, protein motif, and phylogenetic relationship

The chromosomal localization data of *OsFWLs* were obtained from RGAP and mapped by MapChart 2.2 software. Gene structures of *OsFWLs* were analyzed by comparing the genomic sequences with their corresponding full length cDNA sequences. Then, the exon–intron organizations were mapped by Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) (Guo et al. 2007). Multiple sequence alignment was performed by ClustalW (Larkin et al. 2007). "DAS" Transmembrane Prediction Server (http://www.sbc.su.se/~miklos/DAS/maindas.html) (Cserzo et al. 1997) was used for membrane topology analysis. The phylogenetic tree was constructed using MEGA4.0 (Tamura et al. 2007) by the neighbor-joining method with 1,000 replicates bootstrap analysis.

Expression pattern analysis of *OsFWL* gene family

Total RNA of various tissues from Zhonghua 11 (*Oryza. sativa* L. ssp. *japonica*) was extracted using Trizol reagent in accordance with manufacturer's instructions (Invitrogen). First-strand cDNA was reverse transcribed from 1 μg RNA using PrimeScript RT reagent Kit (TaKaRa). The GAPDH gene was used to normalize the

cDNA concentration. RT-PCR was performed on ABI 9700 PCR instrument in 20 μl reaction volume including 1 μl cDNA sample, 0.2 mM dNTPs, 0.25 μM gene-specific primers, 1× PCR buffer and 1 U rTaq polymerase (Takara). The reaction profiles were the following: 94 °C for 4 min, 94 °C for 30 s, 55–65 °C for 30 s, 72 °C for 1 min (28–40 cycles) and 72 °C for 7 min. All of the primers are listed in Supplemental Table S1. The expression profiles were obtained from Collection of Rice Expression Profiles (CREP, http://crep.ncpgr.cn/crep-cgi/home.pl) (Wang et al. 2010) and Rice Expression Profile Database (RiceXPro, http://ricexpro.dna.affrc.go.jp/) (Sato et al. 2013).

Subcellular localization analysis

Subcellular localization was predicted with Plant-mPLoc software (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (Chou and Shen 2010) and further confirmed through rice protoplast transient transformation. The protoplast preparation and plasmid transformation were performed as described (Bart et al. 2006). Each target protein was fused with green fluorescent protein (GFP). A reported rice copper transporter COPT1 (Yuan et al. 2010) was fused with red fluorescent protein (RFP) and used as an indicator for cell membrane localization. Another reported rice transcription factor Ghd7 (Xue et al. 2008) was fused with cyan fluorescent protein (CFP) and used as a reference for nuclear localization. The fusion proteins of OsFWLs were introduced into rice protoplasts with COPT1 or Ghd7. Then, the protoplasts were maintained at 28 °C for 18–24 h in dark. At last, the images were captured with a confocal laser scanning microscope (Leica, Germany).

Analysis of T-DNA insertion mutants of *OsFWLs*

The insertion mutant lines were searched against Rice Functional Genomic Express Database (RiceGE, http://signal.salk.edu/cgi-bin/RiceGE). The mutant lines we received were collected from Rice Mutant Database (RMD) (Zhang et al. 2006) and Pohang University of Science and Technology (POSTECH) (Jeong et al. 2006). For genotyping analysis, the gene-specific PCR primers (P1 and P2) flanking the T-DNA insertion site and a vector border primer (P3) were designed. The primers for *OsFWL3* and *OsFWL5* are listed in Supplemental Table S1. The details about the two sets of PCR were as described (Dai et al. 2009). Grain length, grain width, and 1,000-grain weight were measured three times for each plant. The yield traits including panicle length, panicle number per plant, primary branch number per panicle, secondary branch number per panicle, and plant height were contrasted between wild type and mutant following the described method (Thomson et al. 2003). The cell length of glume inner epidermis was analyzed according to a previously reported method (Li et al. 2012).

Histochemical analysis of glucuronidase (GUS) activity

The promoter of *OsFWL3* (approximately 2 kb upstream of the translation start site) was fused to GUS gene and the construct was transformed into rice Zhonghua 11.

Expression of GUS was assayed following previous description (Li et al. 2012).

Results

Identification of *FW2.2*-like gene family in rice

To identify homologs of *FW2.2* in rice, the aa sequence of *FW2.2* was used as a query to search against RAP-DB with BLASTP. Twelve putative proteins were identified. Then using each putative protein sequence as a query we searched PLAC8 domain against RGAP database. Eventually, we identified 8 putative *FW2.2*-like genes in rice genome and named them from *OsFWL1* to *OsFWL8* (Table 1). The full length cDNAs of *OsFWL1, 2, 3, 5,* and *7* were found in KOME database. The functions of *OsFWL* genes were unknown except for *OsFWL5,* which was reported as *OsPcr1* (Plant cadmium resistance) and validated to increase cadmium resistance when expressed in yeast (Song et al. 2004).

Chromosomal locations, gene structures, protein motifs, and phylogenetic relationships of *OsFWL* genes

The eight *OsFWL* genes are distributed on three rice chromosomes: *OsFWL1*, *2*, and *3* are located on chromosome 2, where *OsFWL2* and *3* are located in adjacent gene locus; *OsFWL4*, *6*, *7*, and *8* form a gene cluster on chromosome 3; *OsFWL5* is located on chromosome 10 (Fig. 1a). For further study of *OsFWLs*, the genomic and coding sequences of all the genes were cloned and sequenced. Then, we identified the intron and exon structures of *OsFWL* genes (Fig. 1b). The coding sequences of *OsFWL* genes are all short and similar, from 411 to 561 bp. Based on the number of introns, *OsFWL* genes can be classified into three groups: Group 1 (*OsFWL4*, *6*, *7*, and *8,* have 1 intron); Group 2 (*OsFWL2* and *3* contain 2 introns); Group 3 (*OsFWL1* and *5* harbor 3 introns).

The aa sequences of deduced OsFWL proteins range from 136 to 186 aa. The contents of cysteine residues in these proteins range from 10 to 20, which are higher than those in general proteins. Topology prediction of membrane

Table 1 The summary information of *OsFWL* gene family

TIGR locus	Gene name	cDNA[a]	ProbeSet[b]	Length[c]	Cys	Localization[d]
LOC_Os02g52550	OsFWL1	AK241465	Os.47727.1.S1_s_at	181	15	Membrane
LOC_Os02g36940	OsFWL2	AK059931	Os.17177.1.S1_at	162	15	Membrane
LOC_Os02g36950	OsFWL3	AK111738	Os.8146.1.S1_at	151	13	Membrane
LOC_Os03g61440	OsFWL4	NA	OsAffx.25790.1.S1_at	136	20	Membrane
LOC_Os10g02300	OsFWL5	AK108480	Os.46839.1.S1_at	186	18	Membrane
LOC_Os03g61470	OsFWL6	NA	OsAffx.22650.2.S1_x_at	150	19	Membrane
LOC_Os03g61500	OsFWL7	AK071173	Os.34496.1.S1_at	141	16	Membrane
LOC_Os03g61480	OsFWL8	NA	OsAffx.22650.1.S1_x_at	166	10	Nucleus

[a] cDNA accession number in KOME database

[b] Probeset ID of *OsFWL* genes in CREP database

[c] Deduced aa lengths of *OsFWL* genes

[d] Subcellular localization predicted by Plant-mPLoc software

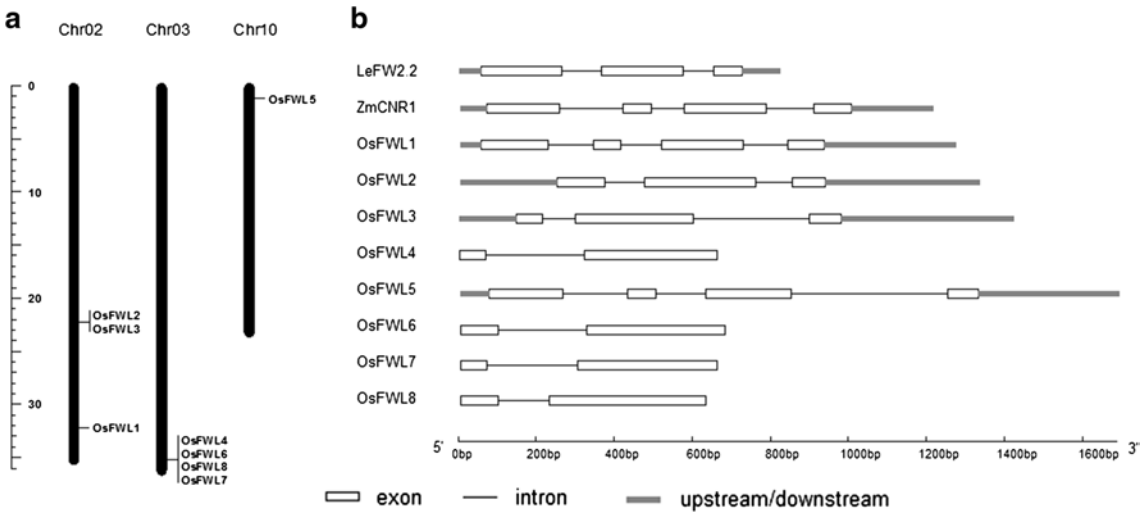


Fig. 1 Chromosomal locations of *OsFWL* gene family and gene structures of *OsFWLs*, *CNR1,* and *FW2.2*. **a** Chromosomal locations of *OsFWL* gene family. **b** Gene structures of *OsFWLs, CNR1,* and *FW2.2*

protein using "DAS" software showed that the OsFWL proteins contained a predicted transmembrane (TM) domain (Fig. 2a). For example, OsFWL1 harbors two potential TM segments, aa 58–70 and aa 86–101, which may function as a TM domain (Fig. 2b). Most OsFWLs (OsFWL2, 4, 5, 6, 7, and 8) include the CCXXXXCPC motif in this predicted TM domain (Fig. 2a). This motif was found to be the cadmium resistance-conferring motif of Pcr family (Song et al. 2004).

According to the phylogenetic tree (Fig. 2c) of OsFWL, CNR1, and FW2.2 proteins constructed using the Neighbor-Joining method, with 1,000 replicates bootstrap, OsFWL proteins can be divided broadly into two groups (OsFWL1, 2, 3, 4, and 5 are in Group I; OsFWL6, 7, and 8 constitute Group II).

Expression patterns of *OsFWL* genes

To analyze the expression patterns of *OsFWL* genes, RT-PCR of seven tissue materials, i.e. calli, seedling, leaf blade at the tillering stage, four tissues (stem, root, flag leaf, panicle) at the heading stage was performed. The result showed that the expression patterns of *OsFWL* genes are various in different organs (Fig. 3a). The *OsFWL1* is expressed in root, stem, panicle, and seedling. The *OsFWL2* is mostly expressed in root, flag leaf, leaf blade, and calli. The *OsFWL3* has the strongest expression in panicle and low expressions in calli and seedling. The *OsFWL4* has a low expression in all seven materials. The *OsFWL5* is mainly expressed in root, seedling, flag leaf, leaf blade, and panicle. The *OsFWL6* is expressed in root, leaf blade, and flag

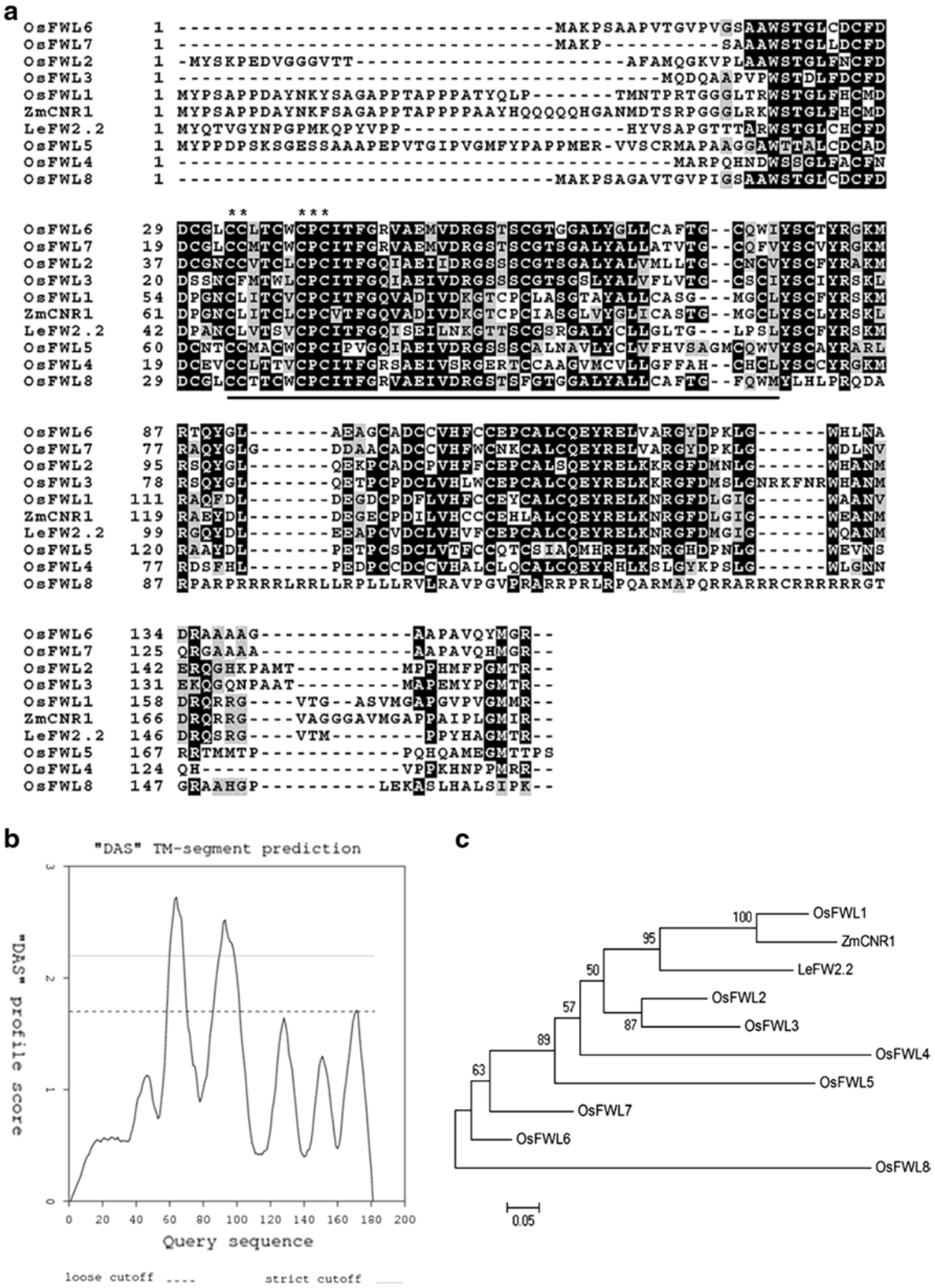
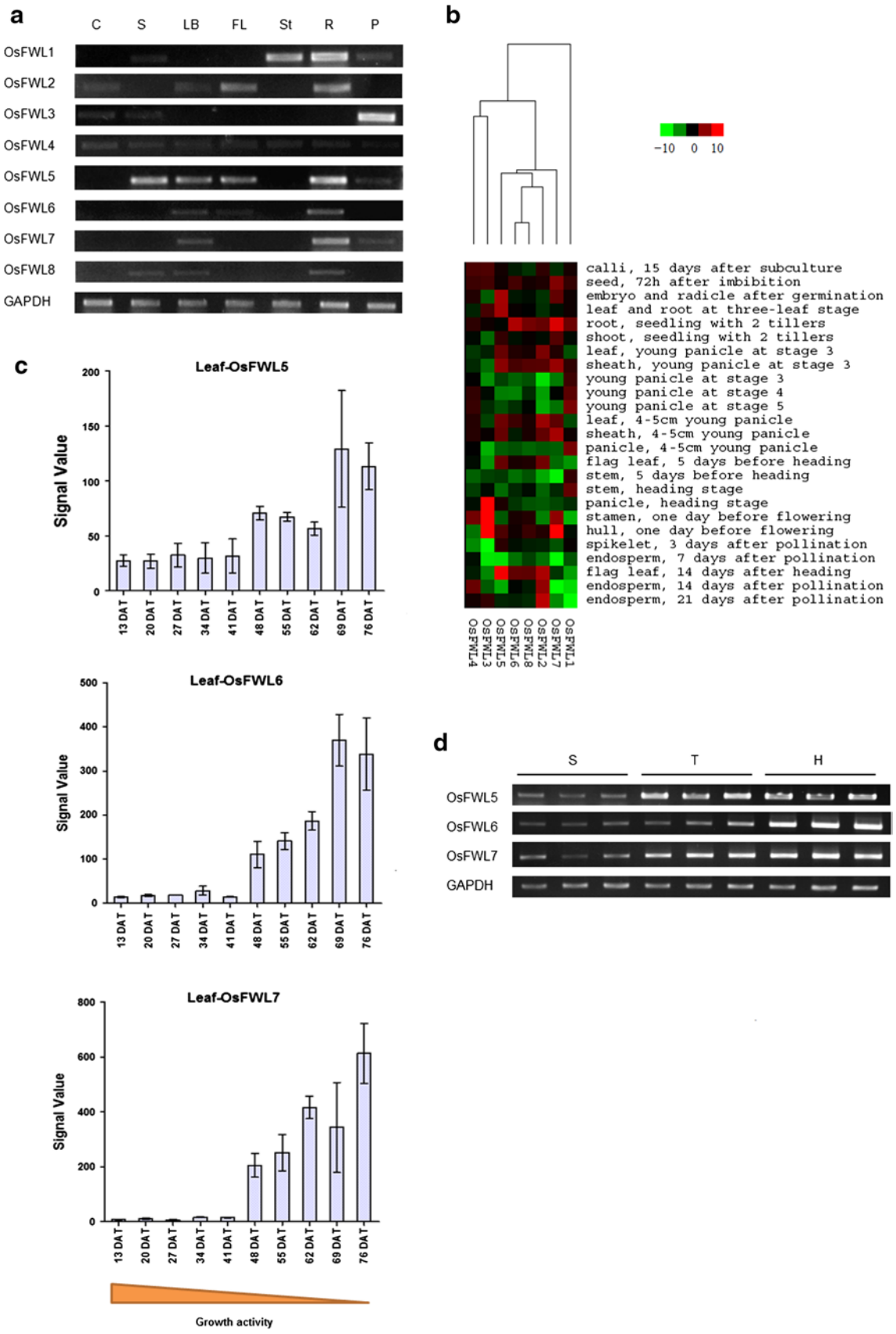


Fig. 2 Multiple sequence alignment and phylogenetic relationship of OsFWLs, CNR1, and FW2.2. **a** Multiple sequence alignment of OsFWLs, CNR1, and FW2.2. Identical and similar amino acids were showed by *dark* and *gray* shadow, respectively. The predicted transmembrane domain was indicated by *underline*. The conserved motif (CXXXXXCPC) was mentioned by *asterisks*. **b** Hydrophobic profile of OsFWL1. Hydrophobicity was determined by "DAS"—Transmembrane Prediction Server. **c** Phylogenetic relationships of OsFWLs, CNR1, and FW2.2. Phylogenetic tree was created by MEGA 4.0 using the neighbor-joining method. The numbers at the clades are percentages of bootstrap using 1,000 replicates. Only values >50 % are given


a
C
S
LB
FL
St
R
P
OsFWL1
OsFWL2
OsFWL3
OsFWL4
OsFWL5
OsFWL6
OsFWL7
OsFWL8
GAPDH
b
−10 0 10
calli, 15 days after subculture
seed, 72h after imbibition
embryo and radicle after germination
leaf and root at three-leaf stage
root, seedling with 2 tillers
shoot, seedling with 2 tillers
leaf, young panicle at stage 3
sheath, young panicle at stage 3
young panicle at stage 3
young panicle at stage 4
young panicle at stage 5
leaf, 4-5cm young panicle
sheath, 4-5cm young panicle
panicle, 4-5cm young panicle
flag leaf, 5 days before heading
stem, 5 days before heading
stem, heading stage
panicle, heading stage
stamen, one day before flowering
hull, one day before flowering
spikelet, 3 days after pollination
endosperm, 7 days after pollination
flag leaf, 14 days after heading
endosperm, 14 days after pollination
endosperm, 21 days after pollination
OsFWL4
OsFWL3
OsFWL5
OsFWL6
OsFWL8
OsFWL2
OsFWL7
OsFWL1
c
Leaf-OsFWL5
Signal Value
13 DAT
20 DAT
27 DAT
34 DAT
41 DAT
48 DAT
55 DAT
62 DAT
69 DAT
76 DAT
Leaf-OsFWL6
Leaf-OsFWL7
Growth activity
d
S
T
H
OsFWL5
OsFWL6
OsFWL7
GAPDH

◂ **Fig. 3** The expression patterns of *OsFWL* genes. **a** RT-PCR analysis of *OsFWL* genes in seven tissues: calli (C); seedling (S); leaf blade (LB) at the tillering stage; stem (St), root (R), flag leaf (FL), and panicle (P) at the heading stage. GAPDH gene was used as an internal control. The results of RT-PCR were similar by three biological replicates. **b** Hierarchical cluster display of expression profile for 8 *OsFWL* genes with corresponding probes in rice variety Minghui 63. Twenty-five samples used for expression analysis are mentioned at the *top* of each *column*. *Color* key shows log2 expression values: *green* indicates low expression, *black* represents medium expression, *red* represents high expression. **c** The detailed expression patterns of specific *OsFWL* genes in relation to leaf growth activity in RiceXPro database. The *x* axis is the day after transplant (DAT). From *left* to *right*, the growth activity is decreasing and maturation is increasing. The *y* axis is the probe signal value which is the results of three biological replicates and is shown with SEM. **d** RT-PCR analysis of the three *OsFWL* genes in leaf at seedling (S), tillering (T), and heading (H) stage. GAPDH gene was also used as the internal control

leaf. The *OsFWL7* has a relatively high expression in root but faint expression in leaf blade and panicle. The *OsFWL8* is expressed in root, seedling, and leaf blade.

To reveal the expression patterns of *OsFWL* genes in the whole life cycle, we have also searched CREP and RiceXPro expression profile databases. All 8 *OsFWL* genes can be found in CREP and 7 genes in RiceXPro. We extracted the signal values of the probes corresponding to the 8 *OsFWL* genes in CREP database. The information of the 8 *OsFWL* genes in 25 samples was normalized and subjected to Hierarchical cluster analysis (Fig. 3b). The expression patterns of *OsFWL2*, *5*, *6*, *7*, and *8* are relatively similar. They are mostly detected in seed after imbibition, root at 2-tillers stage, and leaf, flag leaf, sheath at the whole life cycle. The *OsFWL1* holds a high expression in root, stem and young panicle. The *OsFWL4* has an absolutely low expression at the whole life cycle. The expression pattern of *OsFWL3* is special. It possesses a low expression level in most tissues/organs except for panicle at heading stage. As *OsFWL* genes were expected to be negative regulators in rice cell number, we analyzed whether their expression patterns related to tissue growth activity. According to microarray data in RiceXPro database, the expressions of *OsFWL5*, *6*, and *7* negatively correlated with leaf growth activity (Fig. 3c). RT-PCR results of the three *OsFWL* genes in leaf at different development stage confirmed the microarray data (Fig. 3d).

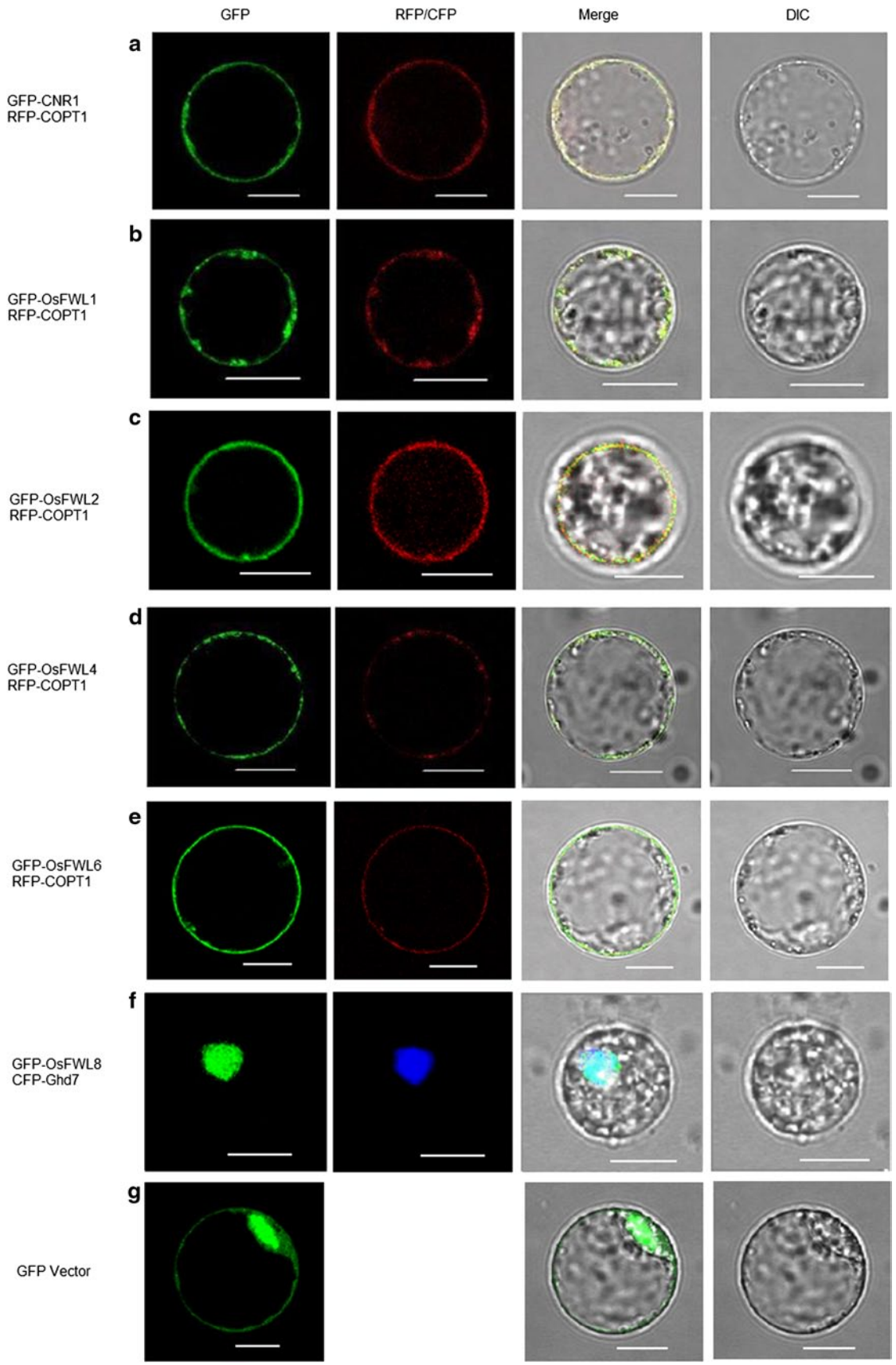
Subcellular localization of OsFWL proteins

To understand the protein properties of OsFWLs, we investigated their subcellular localization. Firstly, Plant-mPLoc software was used to predict their subcellular localization. The results suggested that seven OsFWLs (OsFWL1-7) are cell membrane proteins. This result is consistent with that of FW2.2 which was localized on the plasma membrane (Cong and Tanksley 2006). Only OsFWL8 is predicted in the nucleus. To provide experimental evidences for the above analysis, we carried out a transient gene expression assay in rice protoplast. Since the subcellular localization of CNR1 was only predicted by computer analysis but not confirmed by experiment, we included CNR1 in our study. CNR1 and OsFWL1, 2, 4, 6, and 8 were fused to GFP. The GFP empty vector was used as a negative control. The reported cell membrane protein COPT1 and nuclear protein Ghd7 were tagged with RFP and CFP, respectively to be used as positive controls. The OsFWLs and CNR1 were used to co-transform rice protoplasts with COPT1 or Ghd7. The result showed that the fusion proteins of OsFWL1, 2, 4, 6 and CNR1 were co-located with COPT1 on the cell membrane (Fig. 4a–e), while OsFWL8 was co-located with Ghd7 in the nucleus (Fig. 4f). OsFWL8 is the lowest homolog of OsFWL protein family and its subcellular localization is possibly different from other OsFWL proteins. GFP alone was distributed in cell membrane, cytoplasm, and nucleus (Fig. 4g).

Functional analysis of *OsFWL3* and *OsFWL5*

For studying the biological function of *OsFWL* genes, T-DNA or Tos17 insertion mutants were searched against RiceGE. In total, we found 8 putative T-DNA mutant lines with insertions in 5 *OsFWL* genes (Supplemental Table S2). Through two sets of PCR for genotyping, only the insertion sites of two mutant lines for *OsFWL3* (03Z11HZ85) and *5* (PFG_3A-50652) were confirmed. The other 6 lines we obtained were not confirmed due to <10 seeds or no specific flanking sequences.

The T-DNA mutant line 03Z11HZ85 contains an insertion in the 5′-UTR region of *OsFWL3* (Fig. 5a). PCR genotyping resulted in expected band pattern (Fig. 5b). RT-PCR result showed that *OsFWL3* transcript was absent in *osfwl3* homozygous mutant (Fig. 5c). We tested the yield-related traits of 14 homozygous plants and 12 wild-type plants, and found that panicle length, panicles, primary branches, second branches, and plant height of mutants were not significantly different from that of control (data not shown). Neither the grain width nor thickness was affected. However, the 10-seed grain length of mutant was about 7.70 ± 0.06 cm, which was approximately 5.8 % longer than that of wild type (7.28 ± 0.04 cm; Fig. 5d, f). So the 1,000-grain weight of mutant (25.56 ± 0.28 g) was increased by 5.3 % compared with wild type (24.27 ± 0.35 g; Fig. 5e). The size of the rice glume is an important determinant of rice grain size (Hong et al. 1996). To investigate whether cell number or cell size was affected in *osfwl3* mutant, the length of glume inner epidermal cells was measured by scanning electron microscopy (Fig. 5g). The result showed that cell length of *osfwl3* is not significantly longer than those of wild type (Fig. 5h). The data indicated that the


GFP
RFP/CFP
Merge
DIC
a
GFP-CNR1
RFP-COPT1
b
GFP-OsFWL1
RFP-COPT1
c
GFP-OsFWL2
RFP-COPT1
d
GFP-OsFWL4
RFP-COPT1
e
GFP-OsFWL6
RFP-COPT1
f
GFP-OsFWL8
CFP-Ghd7
g
GFP Vector

◂ **Fig. 4** The subcellular localizations of CNR1 and OsFWL1, 2, 4, 6, 8 in rice protoplast cells. **a** GFP-CNR1 and RFP-COPT1 were co-localized on the rice protoplast cell membrane. **b** GFP-OsFWL1 and RFP-COPT1 were co-localized on cell membrane. **c** GFP-OsFWL2 and RFP-COPT1 were co-localized on cell membrane. **d** GFP-OsFWL4 and RFP-COPT1 were co-localized on cell membrane. **e** GFP-OsFWL6 and RFP-COPT1 were co-localized on cell membrane. **f** GFP-OsFWL8 and CFP-Ghd7 were co-localized in the nucleus. **g** GFP vector was expressed in cell membrane, cytoplasm, and nucleus

increase in grain length of *osfwl3* is due chiefly to incremental cell number, not cell size. To further understand the role of *OsFWL3* in grain length control, we examined the expression pattern of *OsFWL3* in glume development via histological analysis of GUS activity. The promoter of *OsFWL3* (approximately 2 kb upstream of the translation start site) was fused to GUS and the construct was transformed into rice Zhonghua 11. The GUS expression level of mature glume was higher than that of developing glume (Fig. 5i). The result revealed that *OsFWL3* expression negatively correlated with glume growth activity.

The T-DNA mutant line PFG_3A-50652 contains an insertion in the first intron of *OsFWL5* (Fig. 6a). Figure 6b showed the genotypes. The expression of *OsFWL5* was suppressed in homozygous mutant (Fig. 6c). The plant height of homozygous mutant (9 plants; 94.27 ± 0.96 cm) was increased by 12.5 % compared with that of wild type (11 plants; 83.78 ± 0.89 cm; Fig. 6d, e). The primary contribution to this phenotype is that the first internode of mutant was increased (Fig. 6f). The other yield traits were not distinctly different from that of control (data not shown). As mentioned above, the detailed expression of *OsFWL5* negatively correlated with leaf growth activity (Fig. 3c, d).

Discussion

Studies showed that gene families were produced by four main mechanisms: segmental duplication, tandem duplication, transpositional duplication and genome duplication (Cannon et al. 2004; Freeling 2009). It was reported that there were 18 distinct pairs of duplicated segments which cover 65.7 % genome sequences in rice (Yu et al. 2005). After large-scale genome duplications, approximately 30–65 % duplicated genes were lost because of chromosomal deletions and rearrangements (Wang et al. 2005). *OsFWL1* and *5* were found to be located in the duplicated segments on chromosome 2 and 10, respectively. But their duplicated counterparts at their corresponding duplicated regions are absent. The chromosomal deletions and rearrangements may be responsible for it. *OsFWL2* and *3* are located in tandem on chromosome 2. *OsFWL6*, *7*, and *8* are located in tandem on chromosome 3 (LOC_Os03g61490 which located between *OsFWL7* and *OsFWL8* is identified as a pseudogene). The data indicate that tandem duplication may contribute to the *OsFWL* family expansion.

The aa sequence comparison between OsFWL1 and CNR1 exhibited 82 % similarity and 73 % identity. The aa sequence comparisons between other OsFWLs and CNR1 showed <51 % similarity and 41 % identity. FW2.2 and CNR1 were reported to possess a specific CLXXXX-CPC domain (Guo et al. 2010). Only OsFWL1 owns a CLITCVCPC domain, whereas in other OsFWL proteins the leucine residue was substituted by cysteine or phenylalanine (Fig. 2a). It is reported that there are numerous collinear regions between rice and maize genomes (Salse et al. 2004). For example *ZmGS3,* a homolog of rice *GS3,* regulates maize kernel development with a similar role of *GS3* in rice. The homologs of genes surrounding the rice *GS3* region are present in the corresponding *ZmGS3* region (Li et al. 2010). We chose 16 putative maize genes in the surrounding *CNR1* region to Blast against the rice genome (RGAP) and found ten genes with high homologies in the rice region surrounding *OsFWL1* (Supplemental Fig. S1). The result showed a collinear relationship between maize *CNR1* and rice *OsFWL1* regions. The protein similarity and regional collinear relationship suggested that *OsFWL1* is an ortholog to *CNR1*. We received two T-DNA mutant lines (PFG_3D-01373 and PFG_1A-08235) for *OsFWL1* but the inserted sites were not confirmed. Thereby, we are performing the over-expressing and RNAi silencing experiments of *OsFWL1*.

The expression patterns of *OsFWL* genes (Fig. 3a–d) can offer valuable clues to the function of the corresponding gene. *OsFWL1* and *2* widely expressed in multiple organs indicated that they might have pleiotropic functions at different development stages. *OsFWL3* specifically expressed in panicle at heading stage suggested that it might be associated with pollen and grain development. *OsFWL4* had a very low expression in the whole life cycle and its transcripts were even absent in RiceXPro database. *OsFWL5*, *6*, and *7* showed similar expression patterns and may play redundant roles in rice development. The detailed expression patterns of *OsFWL5*, *6*, and *7* negatively correlated with leaf growth activity (Fig. 3c, d), but other *OsFWL* did not show such characteristic. *OsFWL8* might be involved in different biological processes in contrast to other *OsFWLs*, as it was located in the nucleus. Co-expression analysis was executed on Rice Oligonucleotide Array Database (ROAD). The results showed that OsFWL1, 2, and 5 were co-expressed with a series of zinc finger proteins. OsFWL1, 2 and 5 were also co-expressed with the ubiquitination-related proteins, such as C3HC4 type domain-containing proteins, RING-H2 finger proteins, and ubiquitin-conjugating enzymes, suggesting that the three OsFWL proteins may be involved in ubiquitination pathway (Supplemental Table S3). The zinc finger and ubiquitination-related

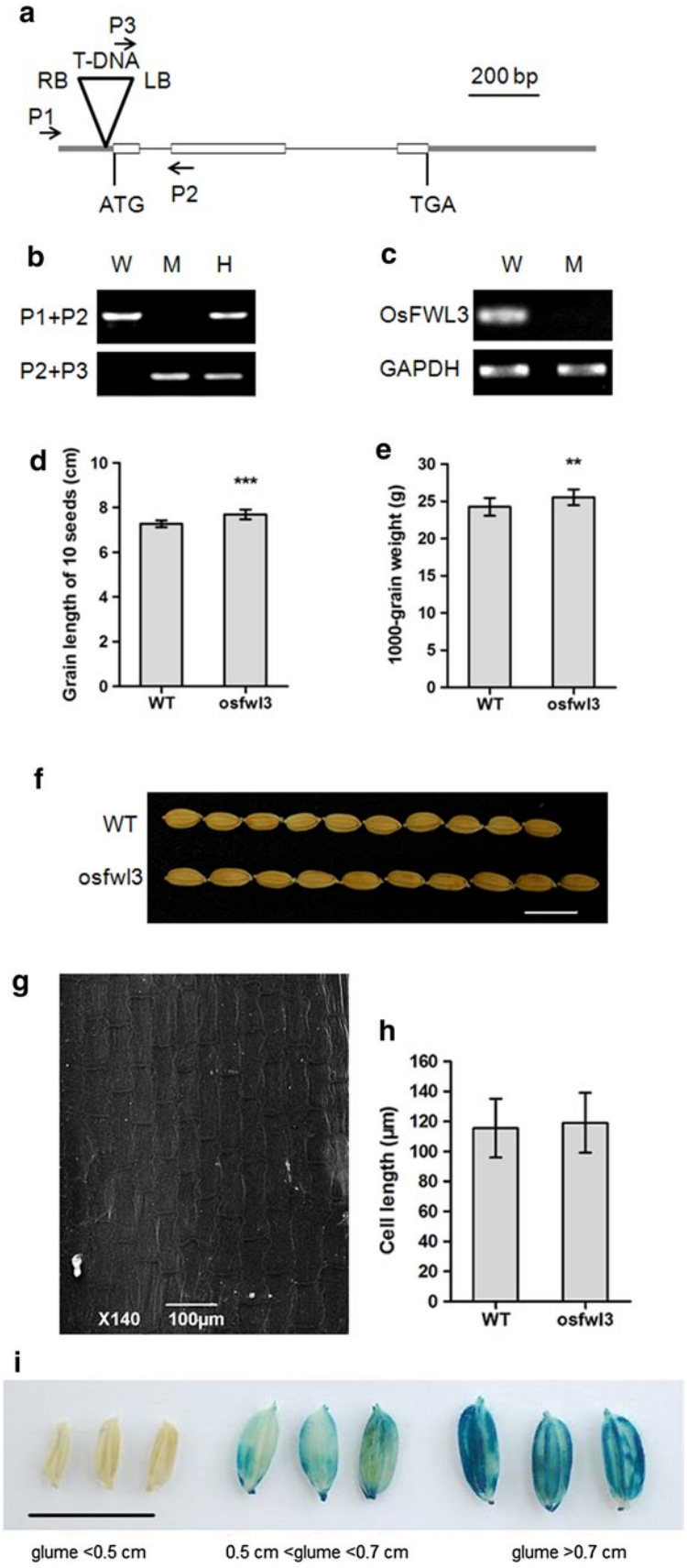


Fig. 5 The functional analysis of *OsFWL3*. **a** *OsFWL3* gene structure and T-DNA insertion site. T-DNA was inserted into the 5′-UTR region. *White boxes*, *thin lines* and *gray boxes* represent the exons, introns and UTRs, respectively. *LB* and *RB* represent the *left* and *right border* of T-DNA, respectively. *P1* and *P2* represent the gene-specific PCR primers flanking the T-DNA insertion site and *P3* represents the vector border primer. **b** PCR result for genotyping. **c** RT-PCR result of *OsFWL3* in homozygous mutant and wild type. GAPDH gene was used as the control. In part **b** and **c** *W*, *H*, and *M* indicate the wild type, heterozygous mutant, and homozygous mutant, respectively. **d** Grain length of 10 seeds in wild type and *osfwl3* mutant. **e** 1,000-grain weight in wild type and *osfwl3* mutant. In part **d** and **e**, *t* test was generated between wild types and *osfwl3* mutants. The *columns* are present as mean ± standard deviation ($n \geq 12$). Three *asterisks* indicate $P < 0.001$, two *asterisks* represent $0.001 \leq P < 0.01$. **f** Grain phenotype of wild type and *osfwl3* mutant. **g** An example image of glume inner epidermal cell in wild type by scanning electron microscope. **h** The analysis of glume inner epidermal cell length between wild type and *osfwl3* mutant. The *columns* are present as mean ± standard deviation ($n = 50$). P value = 0.37 was generated by *t* test. **i** The expression pattern of *OsFWL3* in relation to glume growth activity by GUS activity test

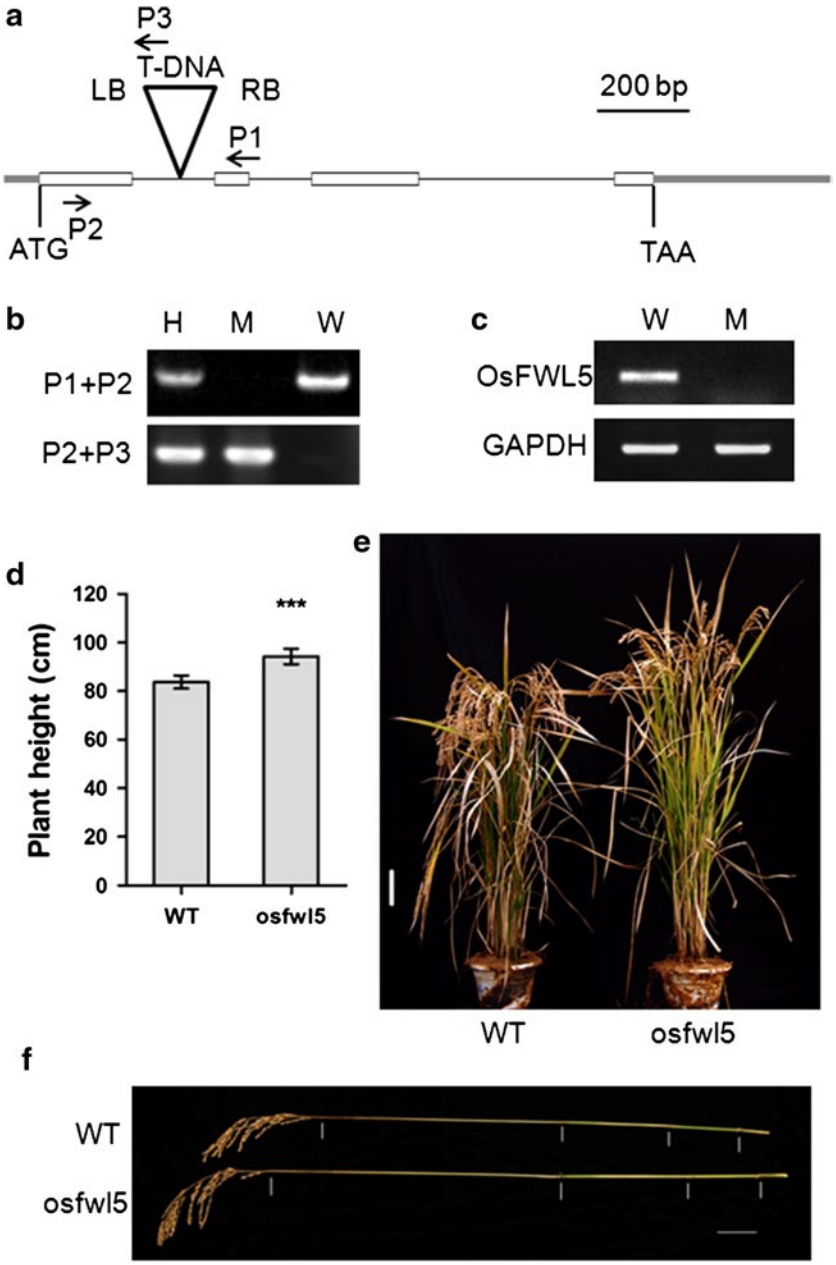


Fig. 6 The T-DNA insertion mutant analysis of *OsFWL5*. **a** *OsFWL5* gene structure and T-DNA insertion site. *White boxes*, *thin lines* and *gray boxes* represent the exons, introns and UTRs, respectively. *LB* and *RB* represent the *left* and *right border* of T-DNA, respectively. *P1* and *P2* represent the gene-specific PCR primers flanking the T-DNA insertion site and *P3* represents the vector border primer. **b** PCR result for genotyping. **c** RT-PCR result of *OsFWL5* in homozygous mutant and wild type. GAPDH gene was used as the control. In part **b** and **c** *W*, *H*, and *M* indicate the wild type, heterozygous mutant, and homozygous mutant, respectively. **d** Plant height of wild type and *osfwl5* mutant. *t* test was generated between wild types and mutants. The *columns* are present as mean ± standard deviation ($n \geq 9$). Three *asterisks* indicate $P < 0.001$. **e** Adult plants of wild type and *osfwl5* mutant. **f** Main culms of wild type and *osfwl5* mutant

proteins were not found in co-expression analysis of other OsFWLs.

We found that *OsFWL3* negatively regulated grain length (Fig. 5d–f), and the increase in grain length of *osfwl3* mutant was due chiefly to incremental cell number, not cell size (Fig. 5h). The data suggested that *OsFWL3* might have similar functional mechanisms to tomato *FW2.2* and maize *CNR1*. The expression of *OsFWL3* negatively correlated with glume growth activity (Fig. 5i). We also found that *OsFWL5* negatively regulated plant height (Fig. 6d, e) and its expression level negatively correlated with leaf growth activity (Fig. 3c, d). These results suggested that *OsFWL3* and *OsFWL5* might act as negative regulators in rice growth and development. The impacts of the two genes are not strong, possibly because functional redundancy exists among *OsFWL* genes. It was reported that both *AtPcr1* and *OsPcr1*/*OsFWL5* can increase cadmium resistance when expressed in yeast and the *Arabidopsis* plants of reduced *AtPcr1* expression are more sensitive to cadmium. (Song et al. 2004). These data suggested that compared with wild-type *ospcr1*/*osfwl5* mutant may be more sensitive to cadmium, too. CCXXXXCPC is the cadmium resistance-conferring domain of *Pcr* family. All OsFWL proteins contain this domain except for OsFWL1 and 3 (Fig. 2a). It indicated that most *OsFWL* genes may have the similar function as *AtPcr1*. In *Arabidopsis*, MCA1 and MCA2 which

mediate Ca^{2+} uptake (Nakagawa et al. 2007; Yamanaka et al. 2010) were considered as *FWL* genes, as they include a PLAC8 motif in the C-terminal half. However, the N-terminal half containing an EF hand-like region and a coiled-coil motif was found to be responsible for Ca^{2+} uptake activity (Nakano et al. 2011). Because OsFWLs do not contain the two functional regions, they may not be involved in Ca^{2+} uptake.

The *FWL* genes are ubiquitous in plant, animal and fungal genomes. A total of 136 *FWL* genes were identified based on similarity to the tomato *FW2.2* gene (Guo et al. 2010; Libault and Stacey 2010). *FWL* genes were known to play important roles in plant development. In this study, we identified *OsFWL* gene family and focused on functional analysis of *OsFWL3* and *OsFWL5*. Based on the differences in protein domain, expression pattern and T-DNA insertion mutant impact, *OsFWLs* may play diverse roles in rice growth and development.

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