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Identification And Functional Analysis of 3-Phosphatidylinositol Kinase *FAB1/PIKfyve* Gene Family in Rice (*Oryza sativa*)

Yichun Xu, Wanqi Liang, Canhua Wang, Jing Yu
School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, 200240, China
Corresponding author email: jingleyj@163.com

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Abstract FAB1/PIKfyve is a key enzyme that catalyzes phosphatidylinositol 3-phosphate (PtdIns3P) to form phosphatidylinositol 3,5-bisphosphate (PtdIns (3,5) P_2). Its product PtdIns (3,5) P_2 plays an important role in the development of eukaryotic cells. In order to figure out the function of PtdIns (3,5) P_2 in rice reproductive development, this study combined bioinformatics and genetics methods to identify the rice *FAB1/PIKfyve* genes, analyzed the physical and chemical properties, gene structures, conserved domains, phylogenetic tree, cis-acting elements, tissue expression profiles and used CRISPR/Cas9 gene editing technology to obtain *osfab1b* mutants. Bioinformatics analysis results showed that nine *FAB1* gene family members were identified from the whole genome of *Oryza Sativa*. Gene structure analysis indicated the differences existed in the gene structure of *FAB1* family, the number of exons are 8-12. Conserve domain analysis showed that OsFAB1A and OsFAB1B contained the N-terminal FYVE domain, and the remaining members contained the Cpn60_TCP1 domain and PIPKc kinase domain. Phylogenetic analysis indicated that the functions of *FAB1* family were highly conserved in mono- and dicotyledonous plants. Prediction of cis-element in the upstream regulatory region of *FAB1* genes revealed a variety of growth-related, light-responsive, and hormone and stress-responsive cis-elements. Tissue expression profiles showed that most of *FAB1* genes were global-expressed, and the high expression of *FAB1C* sub-cluster genes in lemma and palea suggested that they might be involved in floral organ development. Finally, the *osfab1b* mutants were obtained through the CRISPR/Cas9 system, and the pollen vitality of the *osfab1b* mutants showed no significantly abnormality, implied that *FAB1* family had functional redundancy in the reproductive development of rice. The present results provide a theoretical reference for biological function studies of the phosphatidylinositol regulatory network in rice.

Keywords Rice (*Oryza sativa*); *FAB1/PIKfyve* gene family; Phosphatidylinositol 3,5-bisphosphate; CRISPR/Cas9

Phosphatidylinositols (PIs) is one of the components of glycerol phospholipids in biofilm. The hydroxyl groups at positions 3, 4 and 5 of its D-inositol ring can undergo multiple phosphorylation reactions to form a variety of derivatives, which together constitute a complex phosphatidylinositol metabolic network (Balla, 2013). Among them, phosphatidylinositol 3,5-bisphosphate (PtdIns (3,5) P_2) is the lowest content phosphatidylinositol derivative in eukaryotic cells. On the contrary, PtdIns (3,5) P_2 has key regulatory functions in many cell development processes such as intimal transport, signal transduction, cell polar growth and cytoskeleton reorganization (Michell et al., 2006; Takasuga and Sasaki, 2013). Catalyzing PtdIns3P by FAB1 (Formation of aploid and binucleate cells 1)/PIKfyve (FYVE finger-containing phosphoinositide kinase) kinase is the only way to synthesize PtdIns (3,5) P_2 (Takasuga and Sasaki, 2013). Typical FAB1/PIKfyve kinases such as yeast Fab1p, human PIKfyve and mouse P235 contain three conserved domains: N-terminal FYVE (FAB1p, YOTB, Vac1p, EEA1) domain, Cpn60_TCP1 chaperone like domain in the middle as well as C-terminal PIPKc kinase domain, and animal PIKfyve also contains a DEP domain (Michell et al., 2006; Botelho et al., 2008; Takasuga and Sasaki, 2013). Biochemical studies have shown that the FYVE domain can specifically bind PtdIns3P, and this binding is crucial for the intimal localization of FAB1 kinase (Gaullier et al., 1998), Cpn60_TCP1 domain is related to the effective folding of actin and tubulin (Botelho et al., 2008). PIPKc kinase domain is the key domain that catalyzes PtdIns3P to form PtdIns(3,5)P_2. Unlike *Fab1p/PIKfyve* being a single copy gene in yeast and human, there are

four *FAB1A-D* genes encoding FAB1 kinase in *Arabidopsis thaliana* genome, of which only FAB1A/B shows the classical Fab1p/PIKfyve homologous protein structure, FAB1C/D lacks the N-terminal FYVE domain, and FAB1A-D has no DEP domain (Mueller-Roeber and Pical, 2002; Whitley et al., 2009).

The balance of PtdIns(3,5)P_2 levels is essential for the normal development of eukaryotic cells. Studies in yeast and animals have shown that the functional defect of *Fab1p/PIKfyve* affects the morphology of vacuole, endosome and lysosome, osmotic regulation, vacuole pH regulation and other processes, and is closely related to the maintenance of intimal homeostasis (Gary et al., 1998; Ikonomov et al., 2001). Whitley et al. (2009) found that the functional deletion of the *FAB1A/B* single gene in the model plant *Arabidopsis thaliana* only caused slight leaf curl. However, the vacuoles of *fab1a/fab1b* double mutant pollen could not normally cleave and die after the first mitosis. Conditional *fab1a/fab1b* double mutants induced by estradiol showed pleiotropic developmental abnormalities, such as plant dwarfing, blocked root elongation, imbalance of root gravitropism and insensitivity to exogenous auxin treatment. At the same time, plants overexpressing *FAB1A/B* showed similar developmental defects and abnormal floral morphology, suggesting redundancy and dose effect of *FAB1* family function (Hirano et al., 2011). The delayed stomatal closure induced by abscisic acid can be observed in inhibition of PtdIns(3,5)P_2 synthesis or *fab1b/fab1c* T-DNA mutants, and the stomatal closure rate in *fab1b/fab1c* double mutants is lower than that in single mutants, which verifies the redundancy of *FAB1* family function (Bak et al., 2013). In addition, PtdIns (3,5)P_2 is involved in regulating the polar growth of pollen tubes. The transport of pollen tube membrane in *fab1b* and *fab1d* mutants is disturbed and accompanied by a decrease in the production of reactive oxygen species (Serrazina et al., 2014). The above studies showed that these developmental defects are related to vacuolar acidification affected by intimal transport. However, at present, the direct downstream acting factor of FAB1 kinase is not clear. Some studies have shown that it can affect the proton transport process on the vacuolar membrane by binding V-PPase (Vacuolar proton pyrophosphatase) (Bak et al., 2013).

Plant phosphatidylinositol metabolic regulatory network has been gradually established with the research in recent years, but its molecular mechanism and function need to be improved, especially there are few studies in monocotyledonous model rice. Therefore, in this study, 9 members of *FAB1* gene family in rice were identified by bioinformatics methods, their physical and chemical properties, gene structures, conserved domains, phylogenetic tree and tissue expression profiles were analyzed, and *osfab1b* mutants were obtained by CRISPR/Cas9 gene editing technology, which provided theoretical basis and genetic materials for studying the biological function of *FAB1* family and its product PtdIns(3,5)P_2 in rice reproductive development.

1 Results and Analysis

1.1 Identification of *FAB1* family genes in rice

There are four known genes encoding FAB1 protein in *Arabidopsis thaliana* genome, namely *FAB1A* (*At4g33240*), *FAB1B* (*At3g14270*), *FAB1C* (*At1g71010*) and *FAB1D* (*At1g342260*) (Mueller-Roeber and Pical, 2002). The full-length protein sequences of these four genes were used as the target sequences and performed Blastp comparison in NCBI and Phytozome v12.1 database to preliminarily obtained the candidate genes. Then, the CD-search tool was used to screen genes having Cpn60_TCP1 and C-terminal PIPKc kinase domain at the same time, and 9 members of *FAB1* gene family in rice were identified (Table 1). These genes are named according to their homology with *Arabidopsis thaliana FAB1A-D* gene, which is convenient for subsequent description. The nucleic acid length of these genes ranged from 5 600 to 14 836 bp, and the CDs length ranged from 4 146 to 5 496 bp. The longest protein product contained 1 831 amino acid residues and the shortest protein product contained 1 381 amino acid residues. The physical and chemical properties of the protein were analyzed by ProtParam tool. The protein with the largest molecular weight was OsFAB1A (LOC_Os03g28140), which was 203.39 kD, and the protein with the smallest molecular weight was osfab1d-3 OsFAB1D-3 (LOC_Os04g59540), which was 155.72 kD, and the isoelectric point ranged from 5.21 to 6.5.

Table 1 Information of *FAB1* family members identified in japonica rice and characteristics

Gene name	Genome accession number	Nucleic acid length	CDS length	Amino acid residues	Molecular weight (kD)	PI
OsFAB1A	LOC_Os03g28140	10728	5457	1818	203.39	5.72
OsFAB1B	LOC_Os08g34950	10755	5496	1831	202.56	5.83
OsFAB1C-1	LOC_Os06g14750	7797	4698	1565	175.28	6.02
OsFAB1C-2	LOC_Os08g33200	7433	4893	1630	182.21	6.50
OsFAB1C-3	LOC_Os09g23740	8237	4902	1633	181.68	6.33
OsFAB1D-1	LOC_Os12g13440	14386	5034	1677	187.51	5.32
OsFAB1D-2	LOC_Os09g10650	6686	4575	1524	170.25	5.21
OsFAB1D-3	LOC_Os04g59540	5600	4146	1381	155.72	5.72
OsFAB1D-4	LOC_Os08g01390	8206	4833	1610	180.13	5.56

1.2 Analysis of *FAB1* gene structure and chromosome location in rice

Except that *OsFAB1D-1* has two transcripts, all members of *FAB1* gene family in rice have only one transcript. The gene structure was analyzed (Figure 1). The results showed that *OsFAB1A* and *OsFAB1B* have highly similar exon and intron arrangement patterns, and there are certain differences in the structure of other genes. The number of exons is 8~12 and the number of introns is 7~11. In addition, *OsFAB1D-1* has an upstream and downstream untranslated region of more than 4 kb, *OsFAB1C-1* and *OsFAB1C-3* lack a 5'-untranslated region (5'UTR), and *OsFAB1A* and *OsFAB1D-2* do not have UTR.

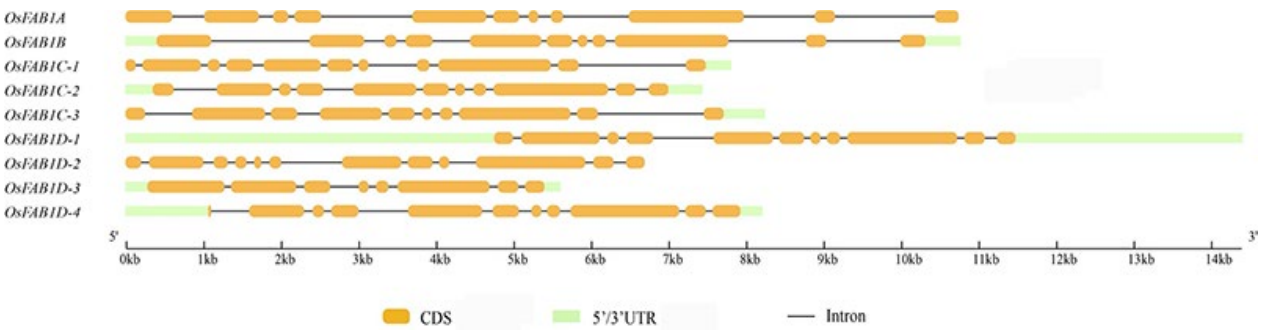

Figure 1 Gene structure of *FAB1* in rice

The chromosome location of the family genes was analyzed by MG2C software (Figure 2). It was found that these 9 genes were located on 6 chromosomes of rice. Among them, the most genes are located on chromosome 8, namely *OsFAB1B*, *OsFAB1C-2* and *OsFAB1D-4*, two genes are located on chromosome 9, and *OsFAB1A*, *OsFAB1D-3*, *OsFAB1C-1* and *OsFAB1D-1* are located on chromosomes 3, 4, 6 and 12 respectively.

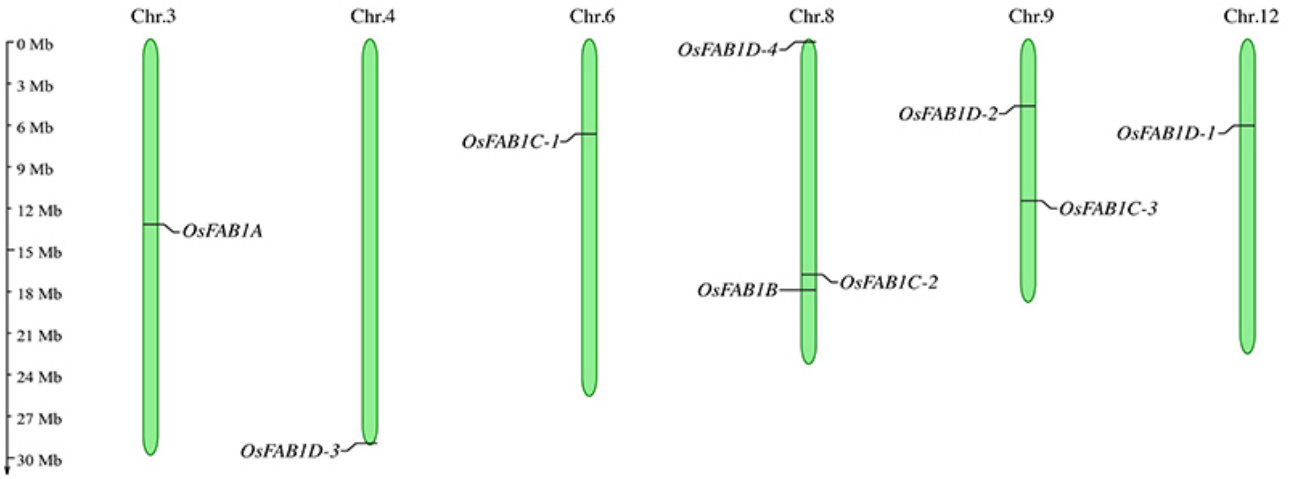

Figure 2 The chromosome location of *FAB1* genes in rice

1.3 Analysis of conserved domains of FAB1 proteins in rice

The traditional Fab1p and its homologous protein structure consist of N-terminal FYVE domain and Cpn60_TCP1 chaperone like domain in the middle and C-terminal PIPKc kinase domain. Previous studies have shown that only FAB1A and FAB1B in *Arabidopsis thaliana* have N-terminal FYVE domain, and FAB1C and FAB1D are truncated forms without FYVE domain (Mueller-Roeber and Pical, 2002; Whitley et al., 2009). The conservative domain of FAB1 protein in rice was predicted by CD-search tool. The results showed that only OsFAB1A and OsFAB1B contained N-terminal FYVE domain, and the other members only contained Cpn60_TCP1 and PIPKc kinase domains (Figure 3A), which was consistent with the results in *Arabidopsis thaliana*. It is worth noting that the truncated forms of FAB1 protein without FYVE domain in rice (7) are significantly more than that in *Arabidopsis thaliana* (2), indicating that the structure of FAB1 protein is more diverse in the evolution of monocotyledonous plants and may have new functions different from that of dicotyledonous plants. The PIPKc kinase domain consists of about 260 amino acids. The protein sequence alignment of the PIPKc kinase domain of rice and *Arabidopsis thaliana* by DNAMAN software showed that the homology reached 74.51% (Figure 3B), suggesting that its kinase function is highly conserved in rice and *Arabidopsis thaliana*.

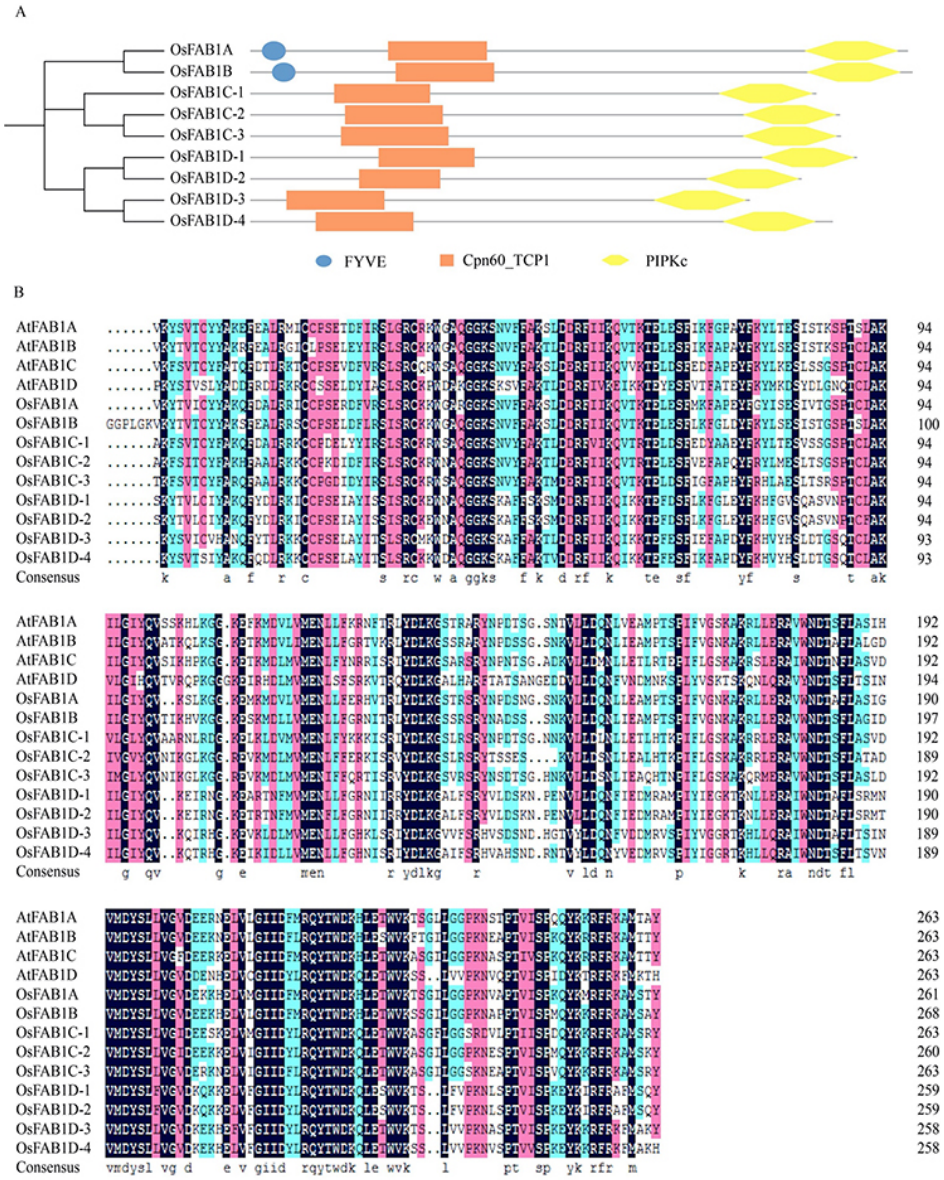

Figure 3 Analysis of conserved domains of FAB1 proteins in rice

Note: A: Conserved domain of FAB1 proteins; B: Amino acid sequence alignment of PIPKc kinase domain

1.4 Phylogenetic analysis of *FAB1* family in plants

In order to further study the phylogenetic relationship of *FAB1* family in mono- and dicotyledonous plants, the phylogenetic tree was constructed by using the FAB1 protein sequences of *Oryza sativa* (9), *Hordeum vulgare* (8), *Sorghum bicolor* (8), *Arabidopsis thaliana* (4), *Glycine max* (7) and *Populus trichocarpa* (6) (Figure 4). The results showed that the *FAB1* family could be divided into three subclasses. *FAB1A* and *FAB1B* in rice clustered into class I with *FAB1A/B* genes in other plants, three *FAB1C* genes in rice clustered into class II with *FAB1C* genes in other plants, and four *FAB1D* genes in rice clustered into class III with *FAB1D* genes in other plants. There are monocotyledonous and dicotyledonous *FAB1* members in each subclass, indicating that the functional differentiation of these three subclasses is earlier than the differentiation of mono- and dicotyledonous plants. In addition, the *FAB1* gene in mono- and dicotyledonous plants clustered into different evolutionary branches in each subclass, speculating that its function has differentiated in mono- and dicotyledonous plants.

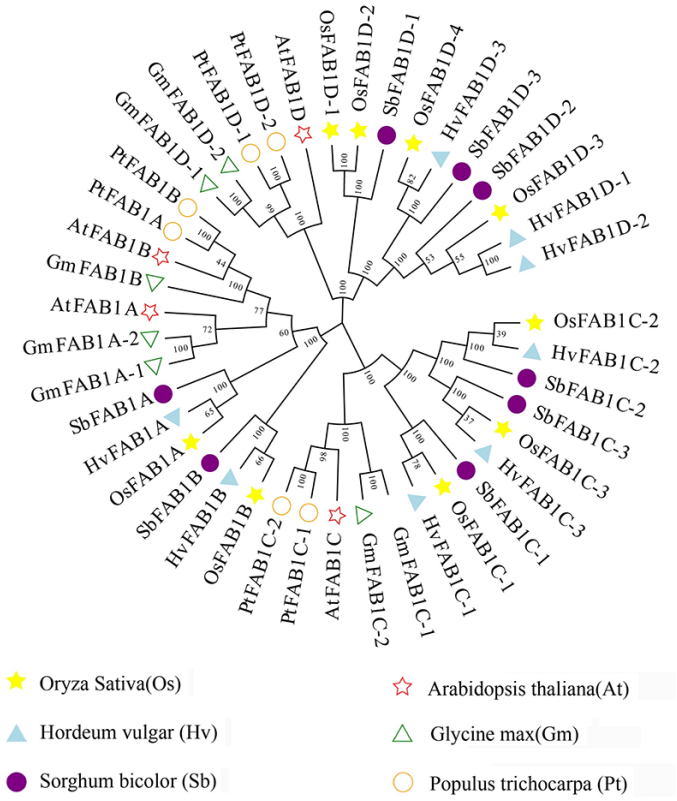

Figure 4 Phylogenetic analysis of *FAB1* family in mono- and dicotyledonous plants

1.5 Analysis of cis-elements in the promoters of *FAB1* gene family in rice

In order to study the possible mechanisms of *FAB1* gene family in rice in response to biological stress, abiotic stress and hormone signals, the cis-acting elements in the 2 kb regulatory region of the upstream of its start codon were predicted (Table 2). The results showed that the promoters of *FAB1* gene family in rice was rich in a variety of light response elements (20 kinds, including Sp1, Box4, G-box), hormone response elements and stress response elements. The hormone response elements include TGA-element auxin- responsiveness element, ABRE abscisic acid responsiveness element, P-box gibberellin-responsive element, TCA-element salicylic acid responsiveness element, TGACG/ CGTCA -motif methyl jasmonate responsiveness element; Stress response elements include LTR low-temperature responsiveness element, MYB binding site MBS involved in drought-inducibility and ARE element involved in anaerobic induction. In addition, the promoters also contain 5 tissue-specific cis-elements (CAT-box, motif I, GCN4_motif, HD-Zip1, RY-element). The above results suggested that *FAB1* gene family may participate in multiple growth processes of rice, and have functions in a variety of hormone responses and resistance to abiotic stress.

Table 2 Cis-elements in the promoters of *FAB1* gene family

Cis element	Typical sequence	Function	Gene								
			OsFAB1A	*OsFAB1B*	*OsFAB1C-1*	*OsFAB1C-2*	*OsFAB1C-3*	*OsFAB1D-1*	*OsFAB1D-2*	*OsFAB1D-3*	*OsFAB1D-4*
TGA element	AACGAC	Auxin- responsiveness	-	1	-	1	2	1	1	1	-
ABRE element	CGCACGTGTC, ACGTG, AACCCGG	Abscisic acid responsiveness	2	1	3	2	2	3	2	3	1
P box	CCTTTTG	Gibberellin-responsive	1	-	1	1	-	1	-	1	-
TCA element	CCATCTTTTT	Salicylic acid responsiveness	1	1	-	-	-	-	-	1	-
TGACG motif	TGACG	MeJA-responsiveness	-	-	3	5	4	3	3	-	3
CGTCA motif	CGTCA	MeJA-responsiveness	-	-	3	5	4	3	3	-	3
Sp1 element	GGGCGG	Light responsiveness	3	1	-	-	3	1	-	-	1
Box 4	ATTAAT	Light responsiveness	4	1	-	3	3	-	1	4	9
LTR element	CCGAAA	Low-temperature responsiveness	-	-	2	2	-	1	2	1	-
MBS site	CAACTG	Drought-inducibility	1	3	-	-	-	1	-	1	-
ARE element	AAACCA	Anaerobic induction	2	4	3	3	3	3	5	1	-

1.6 Expression analysis of *FAB1* family in rice

The temporal and spatial expression patterns of *FAB1* family genes in rice were studied to provide clues for further understanding the biological processes involved. The microarray data were used to analyze the expression pattern in tissues (Figure 5). The results showed that most *FAB1* genes in rice were expressed in all tissues. *OsFAB1A*, *OsFAB1C-2* and *OsFAB1C-3* are significantly expressed in leaves, *OsFAB1C-1* and *OsFAB1D-4* are mainly expressed in roots, and *OsFAB1D-2* is mainly expressed in stems, but the expression of *FAB1* gene in anthers is relatively low. In addition, *OsFAB1C-1* and *OsFAB1C-2* are highly expressed in palea and lemma, indicating that *FAB1C* subclass genes may have functions in the development of flower organs. *OsFAB1D-3* is significantly expressed in embryos, and other members are almost not expressed in endosperm except *OsFAB1D-3* and *OsFAB1D-4*, suggesting that *OsFAB1D* subclass genes may be involved in regulating seed development.

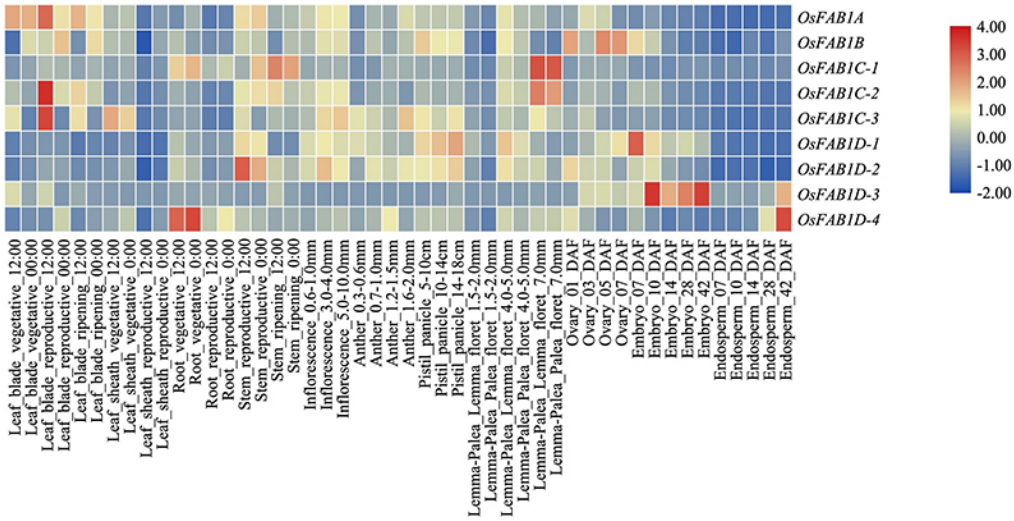

Figure 5 *FAB1* gene expression profiles in different tissues

1.7 Acquisition of *osfab1b* mutant and evaluation of pollen fertility

In order to verify whether the *FAB1* family in rice has a function in the process of reproductive development, *OsFAB1B* knockout lines under the background of Wuyunjing 7 (9522) were constructed by CRISPR/cas9 system. The selection requirements of targets are based on the description of Xie et al. (2015), and two targets were designed close to the 5' start codon and on the Cpn60_TCP1 conserved domain respectively. Genotyping of the obtained transgenic seedlings showed that *osfab1b* mutants with two mutation types were obtained (Figure 6). *osfab1b-1* and *osfab1b-2* were double allelic mutants, in which *osfab1b-1* lost a base T at +162 and the other allele lost 3 bp continuously at +162; *osfab1b-2* lost a base T at +162, the other allele lost 2 bp at +163 and a base G was lost at +168. The above mutation types both caused the translation to terminate or shift in front of target 2, so the mutation types of target 2 were not analyzed. And Cpn60_TCP1 and PIPKc domains deletion leads to the complete loss of gene function, which provides important genetic materials for the functional research of *FAB1* family in rice.

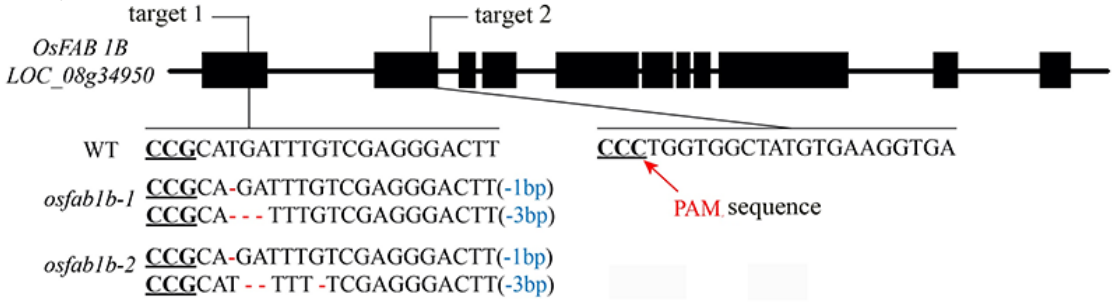

Figure 6 *osfab1b* CRISPR targets and mutation type analysis

The pollen fertility of T_0 generation CRISPR transgenic plants was evaluated. The iodine staining results showed that the pollen viability of *osfab1b-1* and *osfab1b-2* mutants had no significant difference compared with the wild type, indicating that there may be functional redundancy in *FAB1* family in rice during the pollen development process, and the knockout of a single gene could not lead to reproductive development defects (Figure 7).

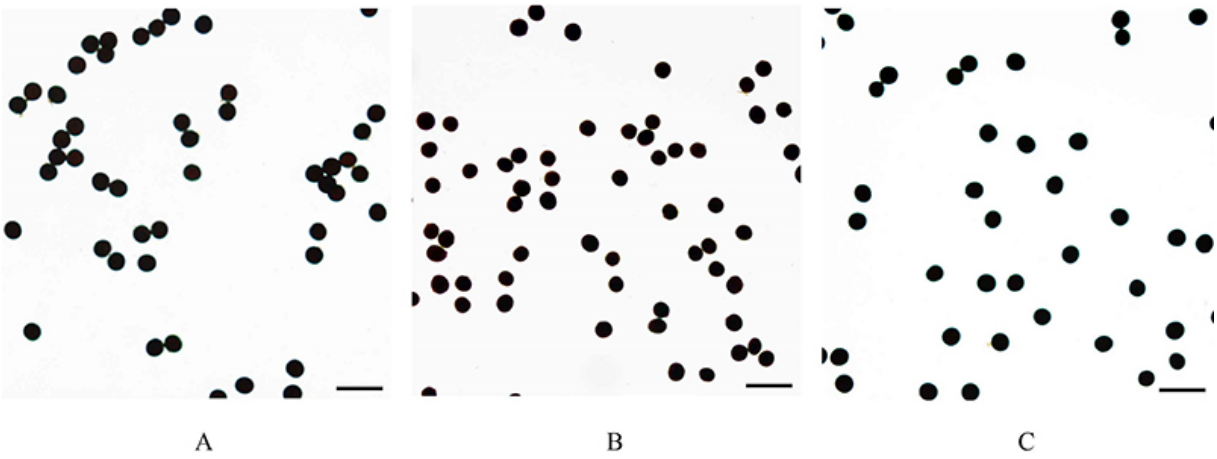

Figure 7 Pollen vitality analysis of *osfab1b*
Note: A: WT; B: *osfab1b-1;* C: *osfab1b-2*; Bars=100 μm

2 Discussion

PtdIns (3,5)P_2, as a kind of phosphatidylinositol derivatives with very small amounts, has important regulatory functions in the development of eukaryotic cells. FAB1/PIKfyve kinase jointly maintains the level of PtdIns (3,5)P_2 in cells by recruiting effector proteins to the vacuolar membrane, and participates in the regulation of diverse cell development processes (Balla, 2013). However, the function of PtdIns (3,5)P_2 and the mechanism of FAB1 kinase in monocotyledonous model rice remain to be clarified. In this study, 9 *FAB1* family members were identified from the rice genome, and their nucleic acid length, molecular weight, isoelectric point and other physical and chemical properties were different. Meanwhile, *OsFAB1A* and *OsFAB1B* have similar gene structure, and the exon intron arrangement patterns of other members are quite different. Through conservative domain analysis, it was found that except OsFAB1A and OsFAB1B, which are similar to the traditional FAB1/PIKfyve family proteins and have N-terminal FYVE domain, the other members only have Cpn60_TCP1 and PIPKc kinase domains, which is consistent with the description of FAB1 protein in *Arabidopsis thaliana* by Mueller-Roeber and Pical (2002). In addition, the number of truncated forms of FAB1 protein without FYVE domain in rice was significantly higher than that in dicotyledonous *Arabidopsis thaliana*, indicating that the forms of FAB1 protein were more diversified in the evolution of monocotyledonous plants.

In order to further study the evolutionary relationship of *FAB1* family in mono- and dicotyledonous plants, a phylogenetic tree containing *FAB1* family genes in 6 species of *Oryza sativa*, *Hordeum vulgare*, *Sorghum bicolor*, *Arabidopsis thaliana*, *Glycine max* and *Populus trichocarpa* was constructed. The results showed that the *FAB1* family genes were clustered into three subclasses, that is, *FAB1A/B*, *FAB1C* and *FAB1D*, and the members of mono- and dicotyledonous plants were distributed in each subclass, suggesting that the function of *FAB1* family was conservative and the functional differentiation was earlier than the differentiation of mono- and dicotyledonous plants. Previous studies have shown that the truncated form of FAB1 protein without FYVE domain is unique to plants (Whitley et al., 2009). Although the FYVE domain is crucial for FAB1 kinase to recognize and target its substrate PtdIns3P, studies in yeast have shown that the FYVE domain is not necessary for PtdIns (3,5)P_2 synthesis (Botelho et al., 2008), so it is speculated that this truncated form may be involved in plant development different from traditional FAB1 kinase.

There is increasing evidence that PtdIns (3,5)P_2 can be used as a signal molecule in response to hormones and stress. When yeast cells were in hypertonic shock, PtdIns (3,5) P_2 levels immediately increased by 20 times (Duex et al., 2006). Under salt stress, the volume of tobacco pollen tube cells decreased, at the same time, PtdIns (3,5) P_2 levels increased by 2 times (Zonia and Munnik, 2004). The imbalance of PtdIns (3,5) P_2 level also affects stomatal closure induced by abscisic acid, suggesting its function in drought stress (Bak et al., 2013). In this study, the upstream regulatory region of *FAB1* family genes in rice was analyzed and found to have a variety of light responsiveness elements, growth and development related elements, hormone and stress response cis-elements. It is worth noting that the promoter regions of all *FAB1* family genes in rice contain abscisic acid responsiveness element ABRE. It is speculated that there is a similar stomatal closure regulation mechanism in response to abscisic acid in rice.

Dynamic vacuole change is an important feature of rice pollen development. The use of inhibitors (YM201636) to reduce PtdIns (3,5)P_2 levels can lead to abnormal maintenance of late endosomal and vacuolar morphology (Hirano et al., 2017). In mutants related to PtdIns (3,5)P_2 metabolic pathway, such as *Arabidopsis thaliana fab1a/fab1b* and *vac14*, it was found that pollen vacuoles could not cleave normally after the first mitosis, resulting in pollen abortion. This pollen abortion is considered to be related to the defect of vacuolar membrane surface polar transport and vacuolar acidification caused by the decrease of PtdIns (3,5)P_2 level (Whitley et al., 2009; Zhang et al., 2018). In this study, *osfab1b* mutant was obtained by CRISPR/Cas9 gene editing technology. It was found that there was no obvious defect in pollen viability of *osfab1b* mutant compared with wild type. According to the research in *Arabidopsis thaliana*, *FAB1A/B* has functional redundancy in pollen development (Whitley et al., 2009), and only conditionally knock out *FAB1A* and *FAB1B* at the same time, will nutritional growth defects cause, which will not be observed in a variety of single mutants (Hirano et al., 2011). Therefore, it is speculated that there is redundancy in the function of *FAB1A/B* in monocotyledonous plants, and multiple mutants need to be constructed for follow-up research to determine whether *FAB1* family has function in rice reproductive development.

3 Materials and Methods

3.1 Plant materials

Wild rice (*Oryza sativa*) was Wuyunjing 7 (9522), which was planted in the Transgenic Experimental Base of Shanghai Jiao Tong University from May to October in 2019.

3.2 Main reagents

KOD FX high fidelity polymerase was purchased from Toyobo (Shanghai) Biotech Co., Ltd.. Taq master mix was purchased from Nanjing Vazyme Biotech Co., Ltd.. FokI and BsaI restriction endonuclease were purchased from NEB company. T7 ligase was purchased from Takara Biomedical Technology (Beijing) Co., Ltd.. *Escherichia coli* competent DH5α was self-made by our laboratory. *Agrobacterium* competent EHA105 was purchased from Tiangen Biotech (Beijing) Co., Ltd.. Vectors pRGEB32 and pGTR were provided by Xie et al. (2015). DNA sequencing service was provided by Shanghai Paisennuo Gene Technology Co., Ltd.. Primer synthesis was provided by Shanghai Generay Biotech Co., Ltd..

3.3 Identification of *FAB1* family members in rice

From *Arabidopsis thaliana* TAIR (https://www.arabidopsis.org/) database, the full-length protein sequence of FAB1A-D was obtained as the query sequence, which was performed Blastp comparison in Public database such as NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome v12.1 (https://phytozome.jgi.doe.gov/) respectively to obtain candidate genes. In Rice Genome Annotation Project (RGAP) database (http://rice. plantbiology.msu.edu/), the protein sequence of candidate genes was downloaded and the conserved domains were predicted by using CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Chen et al., 2017) to ensure that the protein sequence in the candidate genes has Cpn60_TCP1 and PIPKc kinase domains at the same time. The physical and chemical properties such as protein molecular weight (kD) and isoelectric point (pI) were predicted by ProtParam website (https://web.expasy.org/protparam/).

3.4 Bioinformatics analysis of *FAB1* family in rice

The full-length genome sequence and CDS sequence of *FAB1* gene in rice were obtained from RGAP database, and GSDS (http://gsds.cbi.pku.edu.cn/) was used to draw the schematic diagram of gene structure. The data such as the location of genes on chromosomes and the length of rice chromosomes were obtained in RGAP database, and MG2C (http://mg2c.iask.in/mg2c_v2.0/) was used to draw chromosome location map. The location of conserved domains of FAB1 protein in rice was obtained by CD-search and then iTOL (https://itol.embl.de/) was used for drawing. The multi sequence alignment of FAB1 proteins in *Arabidopsis thaliana* and rice was completed by DNAMAN software. From Phytozome v12.1 database, the FAB1 homologous protein sequences of *Sorghum bicolor*, *Hordeum vulgare*, *Glycine max* and *Populus trichocarpa* were obtained, and MEGA7.0 adjacency method (the parameter is set to Possion model, Bootstrap 1000) was used to complete the construction of the phylogenetic tree, and EvolView (https://www.evolgenius.info/evolview/) was used for editing and annotation. The 2 kb sequence of the ATG upstream of *FAB1* gene in rice was obtained in RGAP database, and Plantcare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict cis-elements.

3.5 Expression analysis of *FAB1* family in rice

The expression data of *FAB1* gene in different tissues of rice were from Rice X pro (https://ricexpro.dna.affrc.go.jp/) database, and TBtools software (Chen et al., 2020) was used to complete the drawing of heat map.

3.6 Acquisition and genotype identification of *osfab1b* mutant

The gene sequence of *OsFAB1B* was input on CRISPR-P 2.0 (http:// crsripr.hzau.cn/CRISPR2) website to find suitable targets. Two targets with low miss probability were selected close to 5' start codons ATG and near Cpn60_TCP1 conserved domain respectively. The target sequence is PAM (Protospacer adjacent motif) sequence and 20 bases of upstream (He et al., 2017). According to the method described in Xie et al. (2015), connectors were added at both ends of the target sequence. After CRISPR/Cas9 plasmid was constructed, *Agrobacterium tumefaciens* transformation and wild rice callus infection were carried out. The DNA of transgenic plants was extracted as a template, and the target DNA fragments were amplified by PCR and sequenced. The sequencing results were decoded and analyzed by online tool DSDecodeM (http://dsdecode.scgene.com/home/) and the identification of genotype was completed. Targets and genotyping primer sequences (Table 3).

Table 3 CRISPR construction and genotyping primers

Primer name	Forward primer	Reverse primer
OsFAB1B_Cri_T1	TAGGTCTCCCGACAAATCATG	CGGGTCTCAGTCGAGGGACTT
	GTTTTAGAGCTAGAA	TGCACCAGCCGGG
OsFAB1B_Cri_T2	TAGGTCTCCACATAGCCACCA	CGGGTCTCAATGTGAAGGTGA
	GTTTTAGAGCTAGAA	TGCACCAGCCGGG
OsFAB1B_GT	TGCCATCTGTGGTGATCTGA	TCGAACTGGACTTGTCGCTC

3.7 Evaluation of pollen fertility of *osfab1b* mutant

The wild and mutant anthers were placed in I_2-KI solution (0.2% iodine and 2% potassium iodide), the pollen was evenly released by crushing the anthers with medical tweezers, and pictures were taken under the microscope (Leica DM2500) for the evaluation of pollen fertility.

Authors' Contributions

XYC was the experimental design and executor of this research; XYC completed data analysis and wrote the first draft of the manuscript; LWQ, WCH and YJ were the conceivers and the persons in charge of the project, guiding the experimental design, data analysis, manuscript writing and modification. All authors read and approved the final manuscript.

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