A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Molecular genetic mechanism of *OsFKF1* in flowering time in rice

벼 *OsFKF1* 유전자의 개화기 조절에 대한 분자유전학적 연구

By

SU-HYUN HAN

AUGUST, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Molecular genetic mechanism of *OsFKF1* in flowering time in rice

UNDER THE DIRECTION OF DR. NAM-CHON PAEK
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY

SU-HYUN HAN

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

APRIL, 2015

APPROVED AS A QUALIFIED DISSERTATION OF SU-HYUN HAN

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY THE COMMITTEE MEMBERS

JUNE, 2015

CHAIRMAN _____
Hee-Jong Koh, Ph.D.

VICE-CHAIRMAN _____
Nam-Chon Paek, Ph.D.

MEMBER _____
Hak Soo Seo, Ph.D.

MEMBER _____
Byoung-Cheorl Kang, Ph.D.

MEMBER _____
Yong Weon Seo, Ph.D.

Molecular genetic mechanism of *OsFKF1* in flowering time in rice

SU-HYUN HAN

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

ABSTRACT

Flowering time (or heading date) is a critical trait that determines seasonal and regional adaptation in plants. Many important regulators in photoperiodic flowering pathway are conserved between *Arabidopsis thaliana* (*At*) and rice (*Oryza sativa; Os*), however some are diversified in their functions in the regulation of flowering time. In the facultative long-day (LD) plant *Arabidopsis thaliana*, FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), a blue-light receptor, regulates photoperiodic flowering through the transcriptional and post-translational regulation of CONSTANS (CO) under inductive LD

conditions. By contrast, the facultative short day (SD) plant rice (*Oryza sativa*) flowers early under inductive SD and late under non-inductive LD conditions, and the regulatory function of *OsFKF1*, an ortholog of *Arabidopsis FKF1*, remains elusive. In this study, we characterized the T-DNA insertion *osfkf1* knockout mutants which showed a late flowering phenotype under SD, LD, and natural LD conditions. Transcriptional analysis revealed that *OsFKF1* up-regulates expression of the floral activator *Early heading date 2* (*Ehd2*) and down-regulates expression of the floral repressor *Grain number, plant height and heading date 7* (*Ghd7*) under both SD and LD conditions. Consequently, the expression levels of potent floral inducers *Early heading date 1* (*Ehd1*), *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*), downstream of *Ehd2* and *Ghd7*, decrease in *osfkf1* mutants. Notably, we found that OsFKF1 can up-regulate *Ehd1* expression in a blue light-dependent manner, without modulating the expression of *Ehd2* and *Ghd7*. In contrast to the LD-specific floral activator *AtFKF1*, *OsFKF1* likely acts as an autonomous floral activator because it promotes flowering independent of photoperiod, probably via its distinct roles in controlling expression of rice-specific genes including *Ehd2*, *Ghd7*, and *Ehd1*. In contast, OsFKF1 also interacts with OsGI and OsCDF1 (also termed OsDof12) like *Arabidopsis* FKF1, which interacts with GIGANTEA (GI) and CYCLING DOF FACTOR 1 (CDF1). Taken together, our data indicate that *OsFKF1* is a floral inducer in rice and we have identified similar and distinct roles of FKF1 in *Arabidopsis* and rice.

Key words: *OsFKF1*; knockout mutant; flowering time; *Ehd2*; *Ghd7*; *Ehd1*; blue-light response; rice.

Student number: 2012-30303

CONTENTS

LIST OF FIGURES

LIST OF TABLE

LIST OF ABBREVIATIONS

FKF1	FLAVIN-BINDING, KELCH REPEAT, F-BOX 1
LD	Long-day
SD	Short-day
NLD	Natural long-day
CO	CONSTANS
Ehd	Early heading date
Ghd	Grain number, plant height and heading date
GI	GIGANTEA
CDF	CYCLING DOF FACTOR
FT	FLOWERING LOCUS T
Hd	Heading date
RFT1	RICE FLOWERING LOCUS T
WT	Wild-type
DTH	Days to heading
ELF3	EARLY FLOWERING 3
SCF	SKP-Cullin-Rbx-F-box
DAS	Days after sowing
UBQ5	UBIQUITIN 5
ZT	Zeitgeber Time

WAS	Weeks after sowing
BIFC	Bimolecular fluorescence complementation

INTRODUCTION

Flowering time, also referred to as heading date in rice (*Oryza sativa*), determines the seasonal and regional adaptability of crops. The floral transition is regulated by the interaction between endogenous and environmental cues. Photoperiod (day length) is one of the most important environmental signals for floral transition, and photoperiodic flowering is regulated by light input signals and the circadian clock (Searle & Coupland 2004; Imaizumi & Kay 2006). Recent progress in plant molecular biology has revealed the molecular mechanisms of photoperiodic flowering in *Arabidopsis thaliana*, a model long-day (LD) dicot plant. In *Arabiodpsis*, external light signals perceived by photoreceptors such as phytochromes and cryptochromes regulate *CONSTANS* (*CO*) and the florigen, *FLOWERING LOCUS T* (*FT*), through the circadian clock, then finally promote flowering in response to faborable LD signals (Kardailsky *et al*. 1999; Kobayashi *et al*. 1999; Imaizumi & Kay 2006; Kobayashi & Weigel 2007; Turck *et al*. 2008). The clock-associated gene *GIGANTEA* (*GI*) functions as a mediator

between *CO* and the circadian clock by positively regulating *CO* expression, and this *GI-CO-FT* pathway functions as a central mechanism for flowering induction under inductive LD conditions in *Arabidopsis* (Park *et al*. 1999; Huq *et al*. 2000; Suarez-Lopez *et al*. 2001; Mizoguchi *et al*. 2005).

Rice is a facultative short day (SD) plant that flowers early under SD conditions (<10-h light/day) and shows delayed flowering under LD conditions (>14-h light/day) (Thomas 1997; Izawa 2007; Tsugi *et al*. 2008). Emerging work has also identified complex genetic networks controlling photoperiodic flowering in rice. Interestingly, rice shares a similar genetic pathway in photoperiodic flowering with *Arabidopsis* (Hayama *et al*. 2003; Izawa 2007; Tsuji *et al*. 2011; Shrestha *et al*. 2014). Under inductive SD coniditons in rice, *OsGI*, the rice orholog of *GI*, activates *Heading date 1* (*Hd1*), the rice ortholog of *CO*; *Hd1* promotes flowering by activating *Heading date 3a* (*Hd3a*), the rice ortholog of *FT*; this *OsGI-Hd1-Hd3a* regulatory pathway is similar to *GI-CO-FT* pathway under inductive LD conditions in *Arabidopsis* (Yano *et al*. 2000; Kojima *et al*. 2002; Hayama *et al*. 2003; Tamaki *et al*. 2007).

However, recent studies revealed that rice has a unique pathway distinct from the conserved *OsGI-Hd1-Hd3a* pathway. Under non-

inductive LD conditions, *Hd1* suppresses *Hd3a* expression, which is opposite to its function in SD, whereas *CO* has no effect on flowering in *Arabidopsis* under non-inductive SD conditions (Putterill *et al*. 1995; Yano *et al*. 2000; Suarez-Lopez *et al*. 2001; Hayama *et al*. 2003). *RICE FLOWERING LOCUS T 1* (*RFT1*), another rice homolog of *FT* and the closest paralog of *Hd3a*, functions as an LD-dependent florigen, acting independently of an LD-dependent floral repressor *Hd1* (Komiya *et al*. 2008; Komiya *et al*. 2009). Furthemore, there have been identified some rice-specific photoperiodic flowering time genes that have no orthologs in *Arabidopsis* such as *Ehd1*, *Ghd7*, *Ehd2*, *Ehd3*, *Ehd4*, and *OsMADS51*. *Early heading date 1* (*Ehd1*), a B-type response regulator, mediates the *Hd1*-independent flowering pathway by positively regulating *Hd3a* and *RFT1* under both SD and LD (Doi *et al.* 2004; Komiya *et al.* 2009). The expression of *Ehd1* is activated by blue-light illumination through the morning phase set by *OsGI*-dependent circadian clocks (Itoh *et al.* 2010). *Grain number, plant height and heading date 7* (*Ghd7*)/*Hd4*, encoding a CCT (CO, CO-LIKE and TIMING OF CAB1) motif-containing protein, strongly delays flowering by downregulating *Ehd1* expression in LD, but rarely affects flowering time in SD (Xue *et al*. 2008). *Ghd7* expression is induced by phytochrome signaling and this induction represses *Ehd1*

expression the next morning under LD (Itoh *et al.* 2010). *Early heading date 2* (*Ehd2*)/*Rice Indeterminate 1* (*RID1*)/*Oryza sativa Indeterminate 1* (*OsId1*) is a rice ortholog of maize *INDETERMINATE1* and promotes *Ehd1* expression in both SD and LD (Matsubara *et al.* 2008; Park *et al.* 2008; Wu *et al.* 2008). *Early heading date 3* (*Ehd3*) downregulates *Ghd7* transcription to promote flowering in LD, and upregulates *Ehd1* expression in SD independently of *Ghd7* (Matsubara *et al.* 2011). *Early heading date 4* (*Ehd4*), a novel CCCH-type zinc finger protein, promotes flowering by positively regulating *Ehd1* expression independently of other known *Ehd1* regulators in both SD and LD (Gao *et al.* 2013). *OsMADS51*, encoding a type I MADS-box protein, positively regulates *Ehd1* expression in SD (Kim *et al.* 2007).

Several molecular genetic approaches have also identified other rice photoperiodic flowering genes that are orthologous to those of *Arabidopsis*, in addition to the *OsGI-Hd1-Hd3a* pathway. *Days to heading 8* (*DTH8*)/*Hd5*, encoding a putative HAP3 subunit of the CCAAT-box-binding transcription factor, strongly repress *Ehd1* expression to delay flowering in LD (Wei *et al.* 2010; Yan *et al.* 2011). *OsMADS50*, a rice ortholog of *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), acts upstream of *Ehd1* to promote flowering, and

another rice ortholog of *SOC1*, *OsMADS56*, attenuates *Ehd1* expression in LD (Lee *et al.* 2004; Ryu *et al.* 2009). The rice ortholog of *EARLY FLOWERING 3* (*ELF3*), *OsELF3*/*Hd17*, regulates circadian rhythm and promotes flowering by down-regulating *Ghd7* expression in SD and LD, and is involved in blue light-mediated *Ehd1* expression (Matsubara *et al.* 2012; Saito *et al.* 2012; Zhao *et al.* 2012; Yang *et al.* 2013). *OsPRR37*/*Hd2*, the gene homologous to *Arabidopsis PSEUDO-RESPONSE REGULATORS 3* (*AtPRR3*) and *Arabidopsis PSEUDO-RESPONSE REGULATORS 7* (*AtPRR7*), mainly acts as a suppressor of LD-dependent flowering through the downregulation of *Hd3a* expression (Murakami *et al*. 2003; Murakami *et al*. 2005; Murakami *et al*. 2007; Shibaya *et al.* 2011; Koo *et al.* 2013). *Early flowering 1* (*EL1*)/*Hd16*, which encodes casein kinase I, inhibits flowering by phosphorylating GHD7, resulting in the suppression of *Ehd1*, *Hd3a* and *RFT1*, mainly in LD (Dai & Xue, 2010; Hori *et al.* 2013; Kwon *et al.* 2014). *Hd6*, encoding the alpha subunit of casein kinase II, enhances the repressor function of *Hd1* in LD through the phosphorylation of an as-yet unknown protein (Ogiso *et al.* 2010).

In *Arabidopsis*, *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1* (*FKF1*) encodes a blue light receptor involved in LD-dependent

promotion of flowering (Nelson *et al*. 2000; Imaizumi *et al*. 2003). FKF1 has three domains: a LOV (Light, Oxygen, or Voltage, a subfamily of PAS domain) domain for the perception of blue light, an F-box for the formation of SKP-Cullin-Rbx-F-box (SCF) E3 ubiquitin ligase complex, and six Kelch-repeat domains for the recognition of substrate proteins (Nelson *et al*. 2000; Andrade *et al*. 2001; Yasuhara *et al*. 2004; Imaizumi et al. 2003; Imaizumi *et al*. 2005). *FKF1* expression is rhythmic and peaks around dusk; also, FKF1 protein is activated by blue light (Nelson *et al*. 2000; Imaizumi *et al*. 2003). In LD, FKF1 interacts with GI, and the FKF1-GI complex activates *CO* transcription by degradation of CYCLING DOF FACTORs (CDFs), which repress *CO* transcription, in a blue light-dependent manner (Imaizumi et al. 2005; Sawa et al. 2007; Fornara et al. 2009). A recent report showed that FKF1 also stabilizes CO protein in the LD afternoon by interacting with CO though its LOV domain, independent of interaction with GI (Song *et al*. 2012). Thus, FKF1 confers robust induction of *FT* mRNA for flowering though multiple feed-forward mechanisms including transcriptional and post-translational regulation of *CO* (Song *et al*. 2012). The rice genome encodes three *FKF1* homologs, *OsFKF1* (LOC_Os11g34460), *OsZTL1* (LOC_Os02g05700), and *OsZTL2*/*OsLKP2* (LOC_Os06g47890)

(Murakami *et al*. 2007). Similar to the expression of *Arabidopsis ZTL* and *LKP2*, the expression of *OsZTL1* and *OsZTL2*/*LKP2* does not fluctuate in day and night (Murakami *et al*. 2007). By contrast, *OsFKF1* expression oscillates in a circadian manner, similar to *FKF1* (Nelson *et al*. 2000; Imaizumi *et al*. 2003; Murakami *et al*. 2007). Although *OsFKF1* was suggested as the orgholog of *Arabidopsis FKF1* based on their sequence similarity and expression profiles, the molecular genetic and biochemical function of *OsFKF1* in rice floral induction has not been reported yet.

In this study, to examine *OsFKF1* function in rice floral induction, we characterized the phenotype of a T-DNA insertion knockout mutant of *OsFKF1* (hereafter termed *osfkf1*). The *osfkf1* mutants exhibited late flowering and altered expression of rice flowering time genes, down-regulating *Ehd2* and up-regulating *Ghd7*, resulting in decreased expression of *Ehd1*, *Hd3a* and *RFT1* in both SD and LD conditions. In addition, blue light-mediated activation of *Ehd1* was considerably impaired in *osfkf1* mutants. Interestingly, the pairwise interaction between the components of OsFKF1-OsGI-OsCDF1 module was observed, but the regulatory mechanism of this module appears to be different from that of the FKF1-GI-CDFs module in *Arabidopsis*. We

discuss an evolutionary view of the regulatory pathway of *OsFKF1* and its differences from that of *Arabidopsis FKF1* in floral induction.

MATERIAL AND METHODS

Plant materials and growth conditions

The T-DNA insertion knockout mutant of *OsFKF1* (LOC_Os11g34460; PFG_1B-03633.R; *osfkf1*) in rice was isolated in the Korean *japonica* cultivar 'Dongjin' (hereafter termed wild type; WT) and was obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/RiceGE) (Jeon *et al.* 2000; Jeong *et al.* 2002). Rice plants were grown in the paddy field of the Seoul National University Experiment Farm under natural long day (NLD) conditions (37° N latitude, Suwon, Korea). Seeds were sown on the seed beds in the greenhouse on April 27 and transplanted to the paddy field on June 1. The day lengths during rice culture in the field were 13.7 h in late-April, 14.2 h in May, 14.7 h in June, 14.4 h in July, and 13.5 h in August. Rice cultivation followed normal agricultural practices for Korean rice varieties. Rice was also grown in growth chambers under SD (10-h light, 30℃/14-h dark, 24℃) or LD (14.5-h light, 30℃ /9.5-h dark, 24℃) conditions with 60% relative humidity. The light source was light-

emitting diodes producing mixed red, blue and white light, and photon flux density was around 500 μmol m^{-2} s^{-1}. Flowering time (or heading date) was recorded from sowing to emergence of the first panicle in the main culm.

Indentification the T-DNA insertion *osfkf1* mutant

To identify the homozygous *osfkf1* mutant, we extracted genomic DNAs from the *osfkf1*-segregating population using a cetyl trimethyl ammonium bromide (CTAB) method (Murray & Thompson 1980) and performed PCR analysis. PCR was conducted with a T-DNA plasmid pGA2715 right border primer (pGA2715-RB) in combination with an *OsFKF1* left genomic primer (*OsFKF1*-LP) and right genomic primer (*OsFKF1*-RP) (Table 1). PCR was performed with 32 cycles of 95℃ for 30s, 60℃ for 30s, and 72℃ for 1 min.

Vector construction and plant transformation

The full-length cDNA clone of *OsFKF1* (GenBank Accession No. AK100677; http://cdna01.dna.affrc.go.jp/cDNA/) was amplified by RT-

PCR using the gene-specific primers *OsFKF1*-F and *OsFKF1*-R (Table 1) and then subcloned into the pCR8/GW/TOPO vector (Invitrogen, USA). After verifying the sequences, the *OsFKF1* cDNA was inserted into pMDC32 gateway binary vector containing the 35S promoter (Curtis & Grossniklaus 2003) through LR recombination (Lambda integrase/excisionase, Elpis-biotech, Daejeon, Korea). The resultant plasmid was transformed into the *Agrobacterium tumefaciens* strain GV3101 and then introduced into rice calli of *osfkf1* mutant mature embryos using *Agrobacterium*-mediated transformation (Jeon *et al.* 1999; Lee *et al*. 1999). The transgenic rice plants were selected on 2N6 media containing hygromycin (50 mg L^{-1}) and confirmed by genomic PCR using the specific primers 35S promoter-F and *OsFKF1* OX-R (Table 1).

RNA extraction, reverse transcription (RT) and quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from leaves using the MG Total RNA Extraction Kit (Macrogen, Seoul, Korea) according to the manufacturer's instructions. First-strand cDNAs for RT were synthesized from 3 μg total RNA using oligo$(dT)_{15}$ primer and M-MLV

reverse transcriptase (Promega), and diluted with water to 100 µl. Relative expression levels of *OsFKF1* and flowering-related genes were measured by RT-qPCR using gene-specific primers, and *Ubiquitin5* (*UBQ5*) was used for an internal control (Table 1) (Jain *et al*. 2006). GoTaq qPCR Master Mix (Promega) was used in a 20 µl total reaction volume. Expression levels of each gene were measured by the relative quantification method using a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) and qPCR conditions were: 95℃ for 2 min, and then 45 cycles of 95℃ for 10 s and 60℃ for 1 min.

Subcellular localization of OsFKF1

YFP was fused in front of the *OsFKF1* cDNA in the pEarleyGate 104 (pEG104) vector through LR recombination (Lambda integrase/ excisionase, Elpis-biotech, Daejeon, Korea), resulting in the *35S:YFP-OsFKF1* plasmid. The fusion constructs, as well as the control (empty pEG104 vector; *35S:YFP*), were transformed into onion (*Allium cepa*) epidermal layers using a DNA Particle Delivery System (Biolistic PDS-1000/He, BioRad). The transformed onion epidermal layers were incubated at 22℃ on Murashige and Skoog plates under the light for 20

h. Then, the cells were examined with a confocal laser scanning microscope (Carl Zeiss LSM710, Oberkochen, Germany).

Analysis of light-induced expression of *Ehd1*, *Ehd2*, and *Ghd7*

Rice plants including WT, *osfkf1*, and *oself3-1* (Zhao *et al*. 2012) were grown under day-neutral (12-h light/12-h dark) conditions for 5 weeks and then transferred at dawn (zeitgeber time [ZT] = 0) to blue light or red light (60 μmol m^{-2} s^{-1}) conditions or constant darkness. Samples were harvested every 1 h after the beginning of exposure, and RNA extraction, RT-PCR and RT-qPCR were performed as described above.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed according to the Yeast Protocols Handbook (Clontech, Palo Alto, CA, USA). To generate Gal4 DNA-BD (binding domain) and Gal4 AD (activation domain) constructs, the full-length cDNAs of *OsFKF1*, *OsGI*, *OsCDF1* (LOC_Os03g07360, also termed *OsDof12*), *OsCDF2* (LOC_Os07g13260), *OsCDF3* (LOC_Os07g4857 0), and three partial cDNAs of *OsGI* (OsGI-N, amino

acid residues [aa] 1-398; OsGI-M, aa 349-754; OsGI-C, aa 703-1160) were amplified by RT-PCR using gene-specific primers (Table 1) and cloned into both pGBKT7 and pGADT7 vectors. The pairwise of BD and AD vectors were co-transformed into the AH109 yeast strain. Chlorophenol red-β-D-galactopyranoside (CPRG; Roche) was used to measure the β–galactosidase activity.

Bimolecular fluorescence complementation (BiFC) assays

The full-length cDNAs of *OsFKF1*, *OsGI*, and *OsCDF1* were amplified using gene-specific primers (Table 1), and then subcloned into the pCR8/GW/TOPO vector (Invitrogen, USA). Through an LR recombination using the Lambda integrase/excisionase (Elpis-Biotech), the cDNAs were inserted into the BiFC Gateway vectors (Citovsky *et al.* 2006) pSAT5-DEST-cEYFP(175-end)-C1(B) (pE3130), pSAT5(A)-DEST-cEYFP(175-end)-N1 (pE3132), pSAT4(A)-DEST-nEYFP(1-174)-N1 (pE3134), and pSAT4-DEST-nEYFP(1-174)-C1 (pE3136), to generate constructs fused with the YFP N- or C-terminal fragments. Each pair of recombinant plasmids encoding nYFP and cYFP fusions was mixed 1:1 (w/w) and co-bombarded into onion (*Allium cepa*) epidermal

cell layers using a DNA Particle Delivery System (Biolistic PDS-1000/He, BioRad). The transformed onion epidermal cells were incubated at 22℃ on phytoagar plates containing Murashige and Skoog medium in the dark for 20 h. Then, the onion cells were examined by confocal laser scanning microscopy (Carl Zeiss LSM710, Germany).

Table 1. Primer sequences used in this study.

A. Primers for verification of T-DNA insertion	
Primer name	Sequence (5’ → 3’)
pGA2715-RB	TTGGGGTTTCTACAGGACGTAAC
OsFKF1-LP	GACTCAAACTTGTTTCAGCCG
OsFKF1-RP	ATGCTGGTTTTTCCTTGGTG
OsFKF1 semi-qPCR F	GCTACGCTCCTGTGATGCCTAC
OsFKF1 semi-qPCR R	CCACCGAATATGATTATCCGTC
B. Primers for plant transformation	
Primer name	Sequence (5’ → 3’)
OsFKF1-F	ATGTTTGATGCGGGGGATCGC
OsFKF1-R	TCACTCATCTTCGTCGG
35S promoter-F	CTATCCTTCGCAAGACCCTTC
*OsFKF1*OX-R	GCTCATCTCAAGCCTCACAG
C. Primers for quantitative real time PCR	
Primer name	Sequence (5’ → 3’)
UBQ5 qPCR-F	ACCACTTCGACCGCCACTACT
UBQ5 qPCR-R	ACGCCTAAGCCTGCTGGTT
OsFKF1 qPCR-F	ATGGCACAGTTCATGTACCCTGGA
OsFKF1 qPCR-R	TCCTTGGTGAGGTCAAGCAGGAAT
Hd3a qPCR-F	CTTCAACACCAAGGACTTCGC
Hd3a qPCR-R	TAGTGAGCATGCAGCAGATCG
RFT1 qPCR-F	TGACCTAGATTCAAAGTCTAATCCTT
RFT1 qPCR-R	TGCCGGCCATGTCAAATTAATAAC
Hd1 qPCR-F	TCAGCAACAGCATATCTTTCTCATCA
Hd1 qPCR-R	TCTGGAATTTGGCATATCTATCACC
Ehd1 qPCR-F	GGATGCAAGGAAATCATGGA
Ehd1 qPCR-R	AATCCCATCGGAAATCTTGG
Ehd2 qPCR-F	CGACGACAATAGCTCGATCGC
Ehd2 qPCR-R	GTGCATGGTCACGGAGCCTT
Ghd7 qPCR-F	AGGTGCTACGAGAAGCAAATCC
Ghd7 qPCR-R	GGGCCTCATCTCGGCATAG
OsELF3 qPCR-F	ACCACTTCGACCGCCACTACT
OsELF3 qPCR-R	ACGCCTAAGCCTGCTGGTT
OsGI qPCR-F	ATCGTTCTGCAGGCCGAGA
OsGI qPCR-R	TCACCAATGCTTCTGGGCTAT
Ehd3 qPCR-F	GGACCACCTCGTCACCTACAA

Ehd3 qPCR-R	CGCCGTTGGCCATGAG
Ehd4 qPCR-F	CAGCCAGCGGAATCATCAC
Ehd4 qPCR-R	CCAAATCCATCAGACCTACTCCT
DTH8 qPCR-F	CAGCGCCGGGTATGTCGTCT
DTH8 qPCR-R	GTCGTCGCCGTTGATGGTCTT
OsMADS51 qPCR-F	GAAATCAAAGAAGATGTTGGCAAA
OsMADS51 qPCR-R	CTTCCTCCTGCCCCCTAGAG
OsMADS50 qPCR-F	CAGGCCAGGAATAAGCTGGAT
OsMADS50 qPCR-R	TTAGGATGGTTTGGTGTCATTGC
OsMADS56 qPCR-F	GAGAAGAGCCTCCACAAGATAAG
OsMADS56 qPCR-R	TAAGCAAAGTCCGCTCCTTC
D. Primers for yeast two-hybrid vector construction	
Primer name	Sequence (5’ → 3’)
OsFKF1 EcoRI-F	GAATTCATGTTTGATGCGGGGGATCGCGG
OsFKF1 PstI-R	CTGCAGTCACTCATCTTCGTCGG
OsGI EcoRI-F	GAATTCATGTCAGCTTCAAATGAG
OsGI BamHI-R	GGATCCTCAGCAAGTGAGTG
OsGI-N EcoRI-F	GAATTCATGTCAGCTTCAAATGAG
OsGI-N BamHI-R	GGATCCTCGGAAAAGTAAAGCAGC
OsGI-M EcoRI-F	GAATTCGTTGAACTCCTTAGAGCAGC
OsGI-M BamHI-R	GGATCCCGCAGACAGTGCATTTAAG
OsGI-C EcoRI-F	GAATTCCTTGGCATCTTGGAAGCTC
OsGI-C BamHI-R	GGATCCTCAGCAAGTGAGTG
OsCDF1 EcoRI-F	GAATTCATGGGGGAGTGCAAGGTGGGAG
OsCDF1 BamHI-R	GGATCCTCAAGATCCCTCTTGGAAGGTC
OsCDF2 NdeI-F	CATATGATGACCTCGGCCAGCTTGCTG
OsCDF2 EcoRI-R	GAATTCTCACAGTATATTTCCAGACGTTG
OsCDF3 EcoRI-F	GAATTCATGGACGACCTCGCCGCCGCCTC
OsCDF3 BamHI-R	GGATCCTCACGTTGTCTCCTGGAAAG
E. Primers for BiFC vector construction	
Primer name	Sequence (5’ → 3’)
OsGI-F	ATGTCAGCTTCAAATGAGAAG
OsGI-R	TCAGCAAGTGAGTGGGCAGC
OsCDF1-F	ATGGGGGAGTGCAAGGTGGGAG
OsCDF1-R	TCAAGATCCCTCTTGGAAGGTC

RESULTS

Phenotypic characteristics of the *osfkf1* knockout mutant

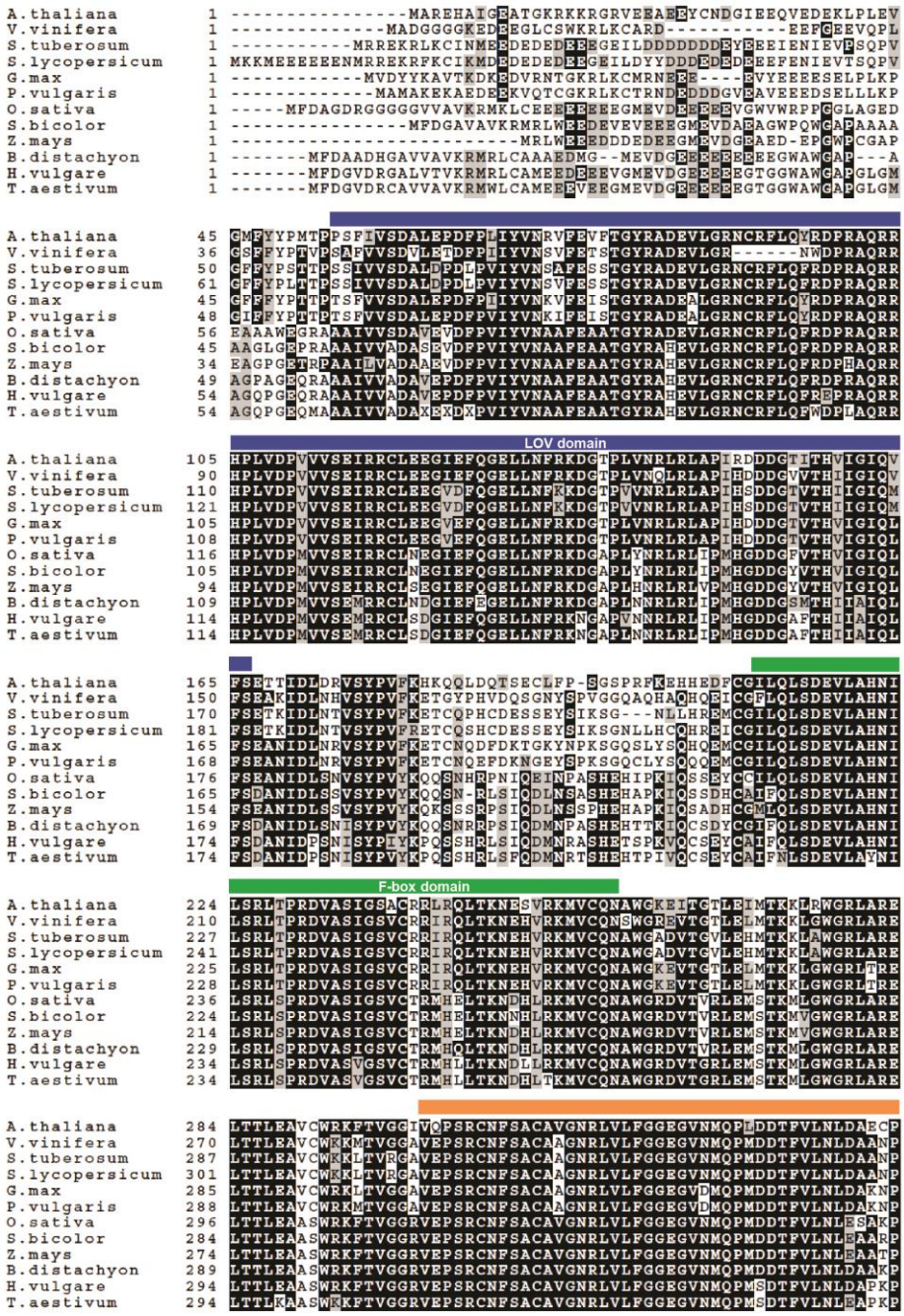
Oryza sativa FKF1 (*OsFKF1*; LOC_Os11g34460) is the rice ortholog of *Arabidopsis thaliana FKF1* (*AtFKF1*; At1g68050) (Murakami *et al.* 2007). Protein sequence alignment revealed that the LOV, F-box, and Kelch-repeat domains of FKF1 are highly conserved among plant species, and OsFKF1 shares 72% amino acid identity with AtFKF1 (**Figure 1**). Since *AtFKF1* plays an important role in photoperiodic flowering and both *AtFKF1* and *OsFKF1* show circadian expression (Nelson *et al.* 2000; Imaizumi *et al.* 2005; Murakami *et al.* 2007; Sawa *et al.* 2007; Song *et al.* 2012), we considered that *OsFKF1* likely also acts in the photoperiodic flowering pathway, similar to *AtFKF1*. To investigate the function of *OsFKF1*, we measured the heading date of an *osfkf1* knockout mutant line, which harbors a T-DNA fragment in the second exon of *OsFKF1* (**Figure 2A**), resulting in no detectable accumulation of *OsFKF1* transcripts (**Figure 2B**). Under NLD conditions (37° N latitude, Suwon, Korea), *osfkf1* (132 days to heading

[DTH]) flowered around 17 days later than its parental *japonica* cultivar 'Dongjin' (115 DTH; hereafter referred to as WT) (**Figure 2C** and **2D**). In the growth chambers, under SD conditions (10-h light/14-h dark), *osfkf1* (90 DTH) flowered around 22 days later than WT (68 DTH) (**Figure 2D**). Under LD conditions (14.5-h light/9.5-h dark), *osfkf1* (138 DTH) flowered around 28 days later than WT (110 DTH), indicating that mutation of *OsFKF1* delays flowering regardless of photoperiod conditions.

For the complementation test, we transformed *osfkf1* mutants with the full-length cDNA of *OsFKF1* driven by the 35S promoter (see Materials and Methods). Five independent T_0 transgenic lines were obtained and the flowering time of four T_0 plants was measured in the paddy field. We found that they flowered similar to WT, indicating that *OsFKF1* expression complements the late flowering phenotype of *osfkf1* mutants under NLD (**Figure 3A** and **3B**). The RT-qPCR analysis confirmed the 35S-mediated overexpression of *OsFKF1* in the four T_0 transgenic lines (**Figure 3C**). Taking these results together, we concluded that *OsFKF1* acts as an autonomous floral inducer because its mutation causes rice plants to flower late, independent of photoperiod.

A reduction in growth rate or a prolonged plastochron (leaf initiation

and appearance rate) may cause late flowering in plants (Itoh *et al.* 1998). To test whether *osfkf1* affects growth rate, we examined leaf emergence rates of WT and *osfkf1*; we found that WT flowered when 12 and 24 leaves emerged in SD and LD, respectively (**Figure 4A** and **4B**). Until heading, WT and *osfkf1* mutants showed nearly indistinguishable leaf emergence rates in both SD and LD, indicating that the *osfkf1* mutation does not affect the growth rate and plastochron in rice. Thus, the late flowering of *osfkf1* mutants is caused by delayed transition from the vegetative to the reproductive stage, not by alteration of growth rate or plastochron.


LOV domain
F-box domain

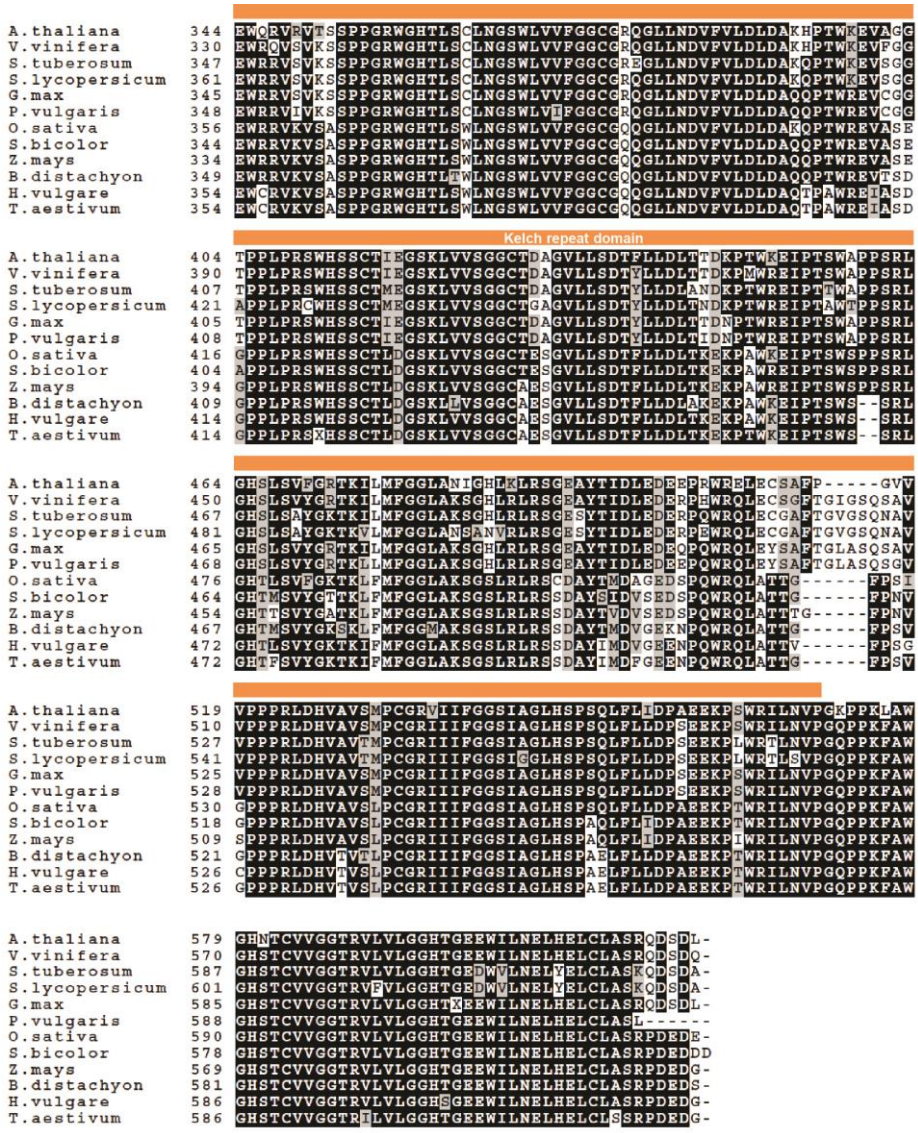


Figure 1. Protein sequence alignments of FKF1 homologs in higher plants.

Clustal W alignment of homologs of FKF1 (http://ch.embnet.org/software/ClustalW.html) was performed. All protein sequences were

obtained using the NCBI-BLASTP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Colored bars represent conserved domains of FKF1 homologs (LOV, F-box and Kelch repeat domains). A. thaliana (*Arabidopsis thaliana*, NP_564919); V. vinifera (*Vitis vinifera*, XP_002281284); S. tuberosum (*Solanum tuberosum*, XP_006364042); S. lycopersicum (*Solanum lycopersicum*, XP_004228739); G. max (*Glycine max*, NP_001235886); P. vulgaris (*Phaseolus vulgaris*, XP_007160435); O. sativa (*Oryza sativa*, NP_001068068); S. bicolor (*Sorghum bicolor*, XP_002450912); Z. mays (*Zea mays*, DAA42101); B. distachyon (*Brachypodium distachyon*, XP_003577479); H. vulgare (*Hordeum vulgare*, BAJ95049); T. aestivum (*Triticum aestivum*, ABL 11478).

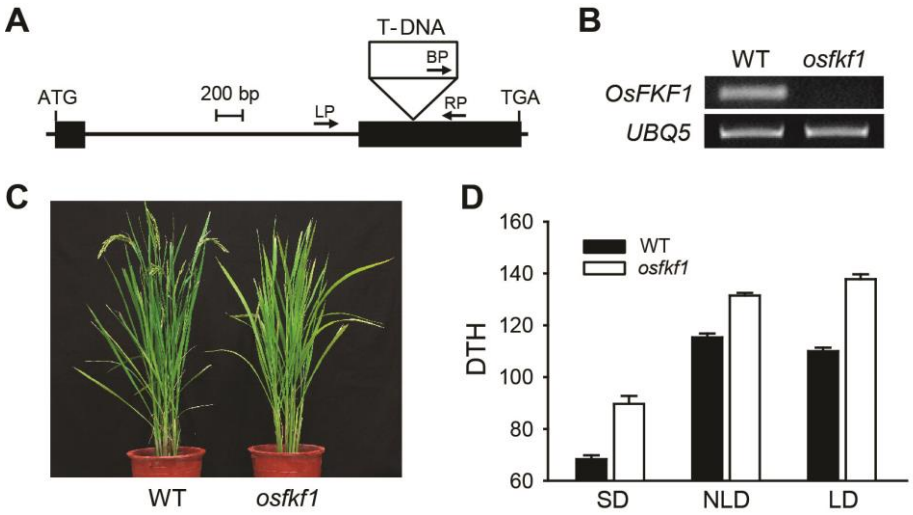


Figure 2. Late flowering phenotypes of *osfkf1* knockout mutants under different photoperiodic conditions.

(**A**) The *osfkf1* mutant has a T-DNA insertion in the 2nd exon of *OsFKF1* and was obtained in the *japonica* rice cv. 'Dongjin' (hereafter termed WT). Filled boxes and solid lines indicate exons and introns, respectively. Arrows indicate LP (left primer), BP (border primer), and RP (right primer), which were used for identification of the T-DNA insertion site in *OsFKF1*. (**B**) Expression of *OsFKF1* in WT and *osfkf1* mutants by RT-PCR. Leaf samples were collected from 90 days after sowing (DAS) plants grown under natural long-day (NLD) conditions at 10 h after dawn (zeitgeber time [ZT] = 10). *OsFKF1* transcripts in WT were not detected in *osfkf1* mutants. *Ubiquitin 5* (*UBQ5*) mRNA level was measured as a loading control. (**C**) Flowering phenotypes of WT (left) and the *osfkf1* mutant (right) grown under NLD in the paddy field. Photo was taken 1 week after heading of WT (122 DAS). (**D**) Days to heading (DTH) of

WT and the *osfkf1* mutant in SD (10-h light/day), NLD (~14-h/ day), and LD (14.5-h light/day) conditions. Means and standard deviations were obtained from 15 (in SD and LD) and 40 plants (in NLD) of each genotype.

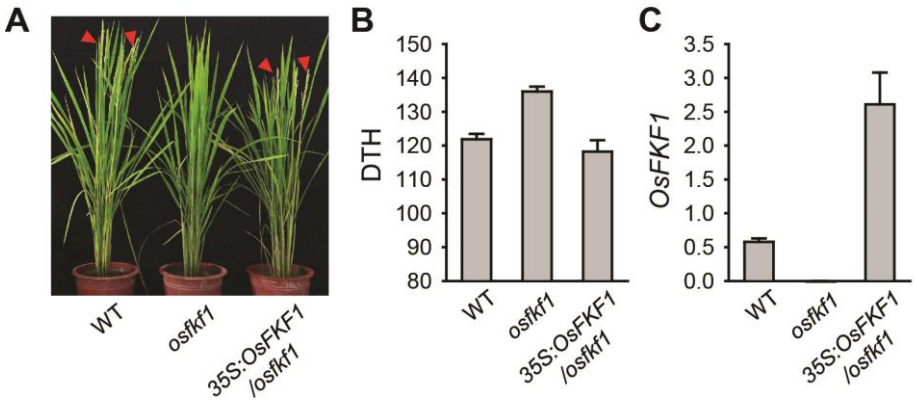


Figure 3. Flowering phenotypes of *OsFKF1* complemented plants.

(**A**) For the complementation test, heading dates of four *35S*:*OsFKF1*/ *osfkf1* T_0 plants were measured in NLD. Photo was taken at 1 week after heading in WT (122 DAS). (**B**) Days to heading (DTH) of WT, *osfkf1*, and *35S:OsFKF1/osfkf1* plants grown in NLD. Values are shown as means of 10 plants for WT and *osfkf1*, and 4 individual complemented T_0 plants. (**C**) Levels of *OsFKF1* mRNA in WT, *osfkf1*, and *35S:OsFKF1/osfkf1* plants grown under NLD, measured by RT-qPCR. Leaf samples were collected from 90 DAS plants at ZT10. Values are shown as means of 3 plants for WT and *osfkf1*, and 4 individual complemented T_0 plants. *Ubiquitin 5* (*UBQ5*) was used for an internal control and error bars indicate standard deviations.

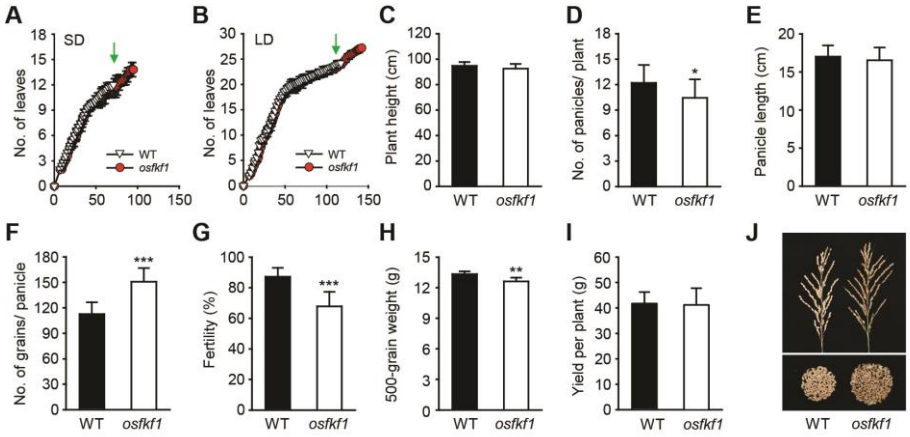


Figure 4. Analysis of agronomic traits in *osfkf1* mutants.

(**A**, **B**) Comparison of leaf emergence rates in WT and *osfkf1* in SD (**A**) and LD (**B**) during development. Values are shown as means (n = 5). Error bars indicate standard deviations. Leaf emergence rate was calculated according to the method of Itoh *et al.* (1998). The average heading date of WT is shown by an arrow in SD or LD. (**C**-**I**) Comparison of plant height (**C**), number of panicles per plant (**D**), panicle length (**E**), number of grains per panicle (**F**), grain fertility (**G**), 500-grain weight (**H**) and yield per plant (**I**) in WT (left) and *osfkf1* (right). Values are shown as means (n = 20). Error bars indicate standard deviations. Differences between means were compared using the two-tailed Student's *t*-tests (**P*<0.05, ***P*<0.01, ****P*<0.001). (**J**) Panicles (upper) and grains (lower) from the main panicle of WT (left) and the *osfkf1* mutant (right) in NLD.

Agronomic traits of *osfkf1* mutants

Flowering time in cereal crop plants is closely associated with grain yield at harvest. To evaluate the agronomic traits of *osfkf1* mutants in rice, we examined morphological traits, such as plant height, number of panicles per plant, panicle length, number of grains per panicle, grain fertility, yield per plant, and 500-grain weight of *osfkf1* mutants grown in NLD (**Figure 4C**-**4J**). Among the parameters, the number of panicles per plant, grain fertility and 500-grain weight were significantly lower in *osfkf1* mutants than in WT (**Figure 4D**, **4G**, and **4H**). By contrast, the number of grains per panicle was much higher in *osfkf1* mutants (**Figure 4F**, **4J**). Although the number of grains per panicle was considerably higher in *osfkf1*, the mutants showed no significant increase in grain yield per plant compared with WT, possibly due to a decrease in other yield-related traits, such as the number of panicles per plant, grain fertility and 500-grain weight. The significant difference in grain fertility and 500-grain weight between WT and *osfkf1* mutants can likely be attributed to different heading dates, as the late-heading *osfkf1* mutants are subjected to unfavorably low temperatures during fertilization and grain filling (19.4-28.6℃ for WT and 15.9-22.7℃ for *osfkf1* in Suwon, Korea at

37°N latitude in 2012). Thus, in low latitudes that have longer growth periods for rice culture, the *osfkf1* mutation may contribute to increased grain yield by increasing the numbers of grains per plant. The hypothesis should be further examined under different cultivation conditions.

Spatial and temporal accumulation of *OsFKF1* transcripts

Since many of the genes regulating flowering time are expressed at high levels in the leaf tissue (Xue *et al.* 2008; Gao *et al.* 2013), we examined the mRNA levels of *OsFKF1* in various organs of 30-day-old WT plants grown in LD. RT-qPCR analysis revealed that *OsFKF1* mRNAs are most abundant in the emerging young leaf (L1) and the least abundant in the root and shoot base (bottom part of stem) (**Figure 5A**, **5B**). Expanded leaves (L2-L4) showed lower *OsFKF1* mRNA levels than young leaves. The *OsFKF1* transcripts also accumulated to moderate levels in the leaf sheath during vegetative growth, as well as in pistil and young panicles at the reproductive stage. These results suggest that *OsFKF1* transcripts are most abundant in leaves, similar to *AtFKF1* transcripts, which are also most abundant in the rosette leaves (Nelson *et al*. 2000).

To examine the subcellular localization of OsFKF1 protein, we constructed an OsFKF1-YFP fusion and used particle bombardment to deliver the *35S*:*YFP-OsFKF1* fusion vector into onion (*Allium cepa*) epidermal cells. YFP-OsFKF1 proteins accumulated in both the nucleus and cytosol (**Figure 5C**). In *Arabidopsis*, FKF1 is thought to function in the nucleus by interacting with GI and CDFs, but also localizes in

cytoplasmic speckles when co-expressed with LKP2 or ZTL (Takase *et al*. 2011). Thus, our observations suggest that OsFKF1 and AtFKF1 have similar subcellular localizations.

Next, we investigated the diurnal expression of *OsFKF1* in SD and LD using RT-qPCR analysis (**Figure 5D**, **5E**). Leaf samples were collected from the leaf blades at 30 and 70 days after sowing (DAS) grown in SD and LD, respectively, which are approximately 40 days before heading in WT. *OsFKF1* showed diurnal expression with a peak just before dusk in WT under SD (ZT9) and LD (ZT12), consistent with those observed in *Arabidopsis*. Together, these results indicate that *OsFKF1* has many similarities to *AtFKF1* at the molecular level.

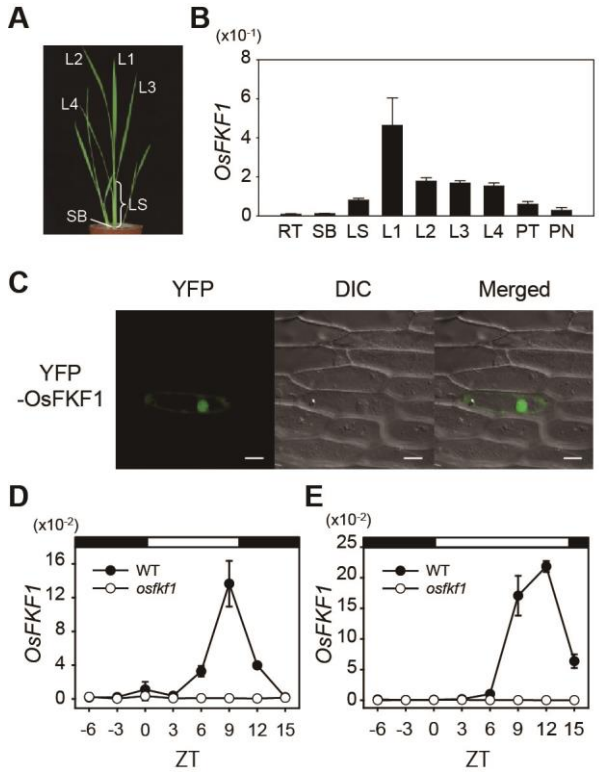


Figure 5. Spatial and temporal expression of *OsFKF1*.

(**A**) WT plant at 30 DAS under LD. (**B**) RT-qPCR results for *OsFKF1* mRNA expression in various tissues. Samples were collected at ZT10 from 30 DAS (RT, SB, LS, L1-L4) and 115 DAS (PT, PN) WT plants grown under LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. RT, root; SB, shoot base; LB, leaf blade; LS, leaf sheath, PT, pistil; PN, panicle. (**C**) Subcellular localization of YFP-OsFKF1 fusion protein in onion epidermal cells analyzed by confocal laser scanning microscopy. The fluorescent (YFP), bright-field and

merged images are shown (from left to right). Scale bar = 20 μm. (**D-E**) Diurnal changes in levels of *OsFKF1* in SD (**D**) and LD (**E**). Filled (●) and open (○) circles represent the WT and *osfkf1*, respectively. Leaf samples were collected every 3 hours from 30 DAS plants in SD and from 70 DAS plants in LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. ZT, zeitgeber time (hours after dawn).

Mutation of *OsFKF1* alters the expression of flowering time genes

To establish the regulatory role of *OsFKF1* in the floral induction pathway, we used RT-qPCR to measure the transcript levels of flowering time related genes in WT and *osfkf1* mutants (**Figure 6**). For this, we grew plants under SD and LD in the growth chambers and harvested the top two leaf blades (for example, L1 and L2 in **Figure 5A**) at 30 and 70 DAS (40 days before heading in WT), respectively. In rice, transcriptional activation of the florigen genes *Hd3a* and *RFT1* in leaves promotes the floral transition (Kojima *et al.* 2002; Tamaki *et al.* 2007; Komiya *et al.* 2008). Our RT-qPCR analysis revealed that *osfkf1* mutants had significantly lower levels of *Hd3a* and *RFT1* transcripts compared with WT, under both SD and LD conditions (**Figure 6A-6D**). In particular, the difference in *Hd3a* expression levels between WT and *osfkf1* was higher in SD than in LD, whereas the difference in *RFT1* expression levels was more pronounced in LD. These results are consistent with the role of *Hd3a* as a floral activator under SD and *RFT1* as a floral activator under LD (Komiya *et al*. 2008; Komiya *et al*. 2009).

We next measured the transcript levels of *Hd1* and *Ehd1*, which are

upstream regulators of *Hd3a* and *RFT1* (Izawa *et al.* 2002; Hayama *et al.* 2003; Itoh *et al.* 2010). Plants grown under both photoperiods showed similar patterns of *Hd1* expression in WT and *osfkf1*, but the levels of *Hd1* transcript during the evening period were slightly lower in *osfkf1* compared with WT (**Figure 6E**, **6F**). The *Ehd1* transcript levels were lower in *osfkf1* mutants, and much lower under LD (**Figure 6G**, **H**). These results indicate that *OsFKF1* upregulates expression of *Hd3a* and *RFT1* by modulating the levels of *Ehd1* transcripts.

To further reveal components of the *OsFKF1-Ehd1* pathway, we measured the transcript levels of other upstream regulators of *Ehd1*. Expression of *Ghd7*, a negative regulator of *Ehd1* in LD (Xue *et al.* 2008), was higher in *osfkf1* mutants than in WT, in SD and LD (**Figure 6I**, **6J**). The peak levels of *Ehd2*, a positive regulator of *Ehd1* (Matsubara *et al.* 2008; Park *et al.* 2008; Wu *et al.* 2008), were lower in *osfkf1* mutants under both SD and LD (**Figure 6K**, **6L**). However, other *Ehd1* regulators, including *Ehd3*, *Ehd4*, *DTH8*, *OsMADS51*, *OsMADS50*, and *OsMADS56*, showed no significant difference between WT and *osfkf1* (**Figure 7**). Moreover, *osfkf1* mutants showed no change in the transcript levels of *OsGI*, a positive regulator of *Ghd7* (Itoh *et al*. 2010), and *OsELF3*, a negative regulator of *Ghd7* (Matsubara *et al.* 2012; Saito *et*

al. 2012; Zhao *et al.* 2012; Yang *et al.* 2013) (**Figure 8**), suggesting that the *osfkf1* mutation does not affect expression of these genes in the genetic cascades regulating flowering time in rice. Therefore, our results demonstrate that *OsFKF1* positively regulates *Ehd2* transcription and negatively regulates *Ghd7* transcription, leading to up-regulation of the expression of *Ehd1*, *Hd3a*, and *RFT1* to promote flowering independent of photoperiod conditions.

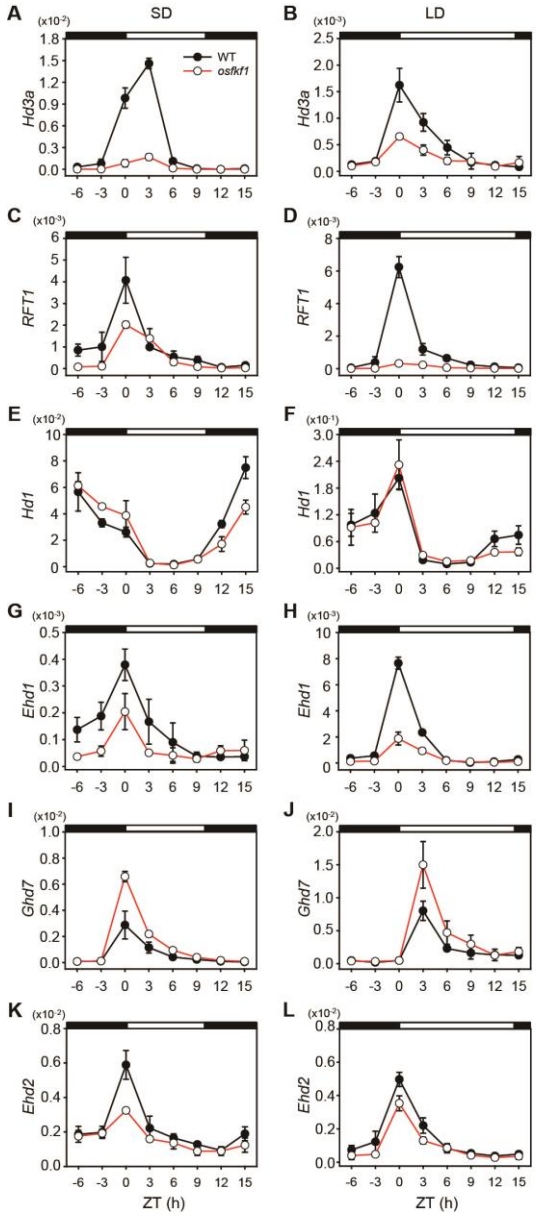


Figure 6. Diurnal expression of flowering-time genes in *osfkf1* and WT under SD and LD.

Comparison of transcript levels of *Hd3a*, *RFT1*, *Hd1*, *Ehd1*, *Ghd7*, and *Ehd2* between WT and *osfkf1* mutants in SD and LD. Filled (●) and open (○) circles represent WT and *osfkf1*, respectively. Leaf samples were collected every 3 hours from 30 DAS plants grown under SD and from 70 DAS plants grown under LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. ZT, zeitgeber time (hours after dawn). DAS, days after sowing.

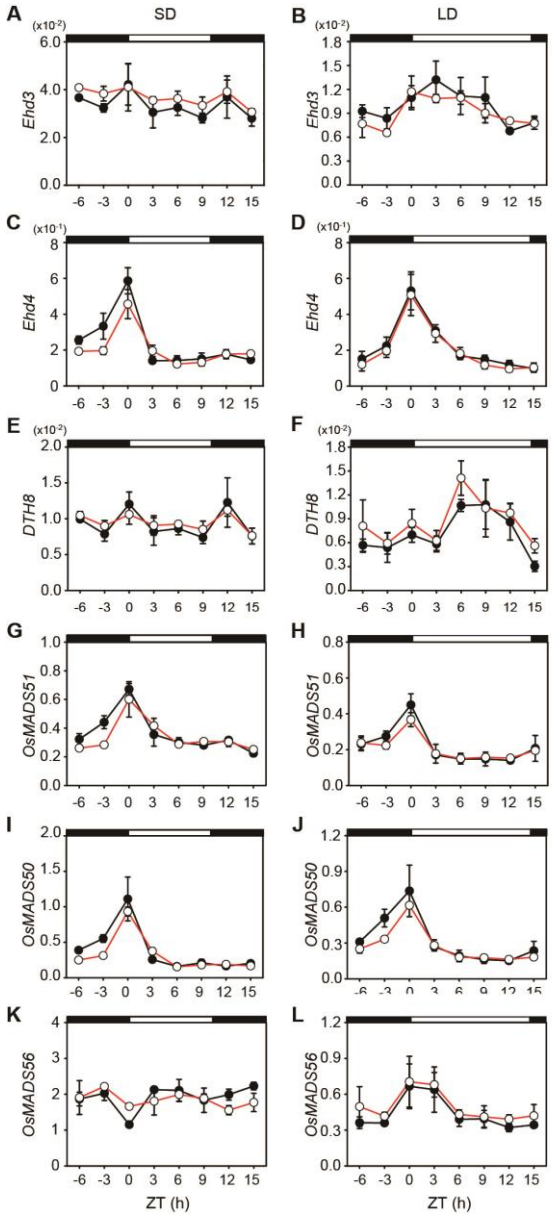


Figure 7. Comparison of transcript levels of *Ehd3*, *Ehd4*, *DTH8*, *OsMADS51*, *OsMADS50*, and *OsMADS56* between WT and *osfkf1* in

SD and LD.

Filled (●) and open (○) circles represent WT and *osfkf1*, respectively. Leaf samples were collected every 3 hours from 30-day-old plants in SD and from 70-day-old plants in LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. ZT, zeitgeber time (hours after dawn).

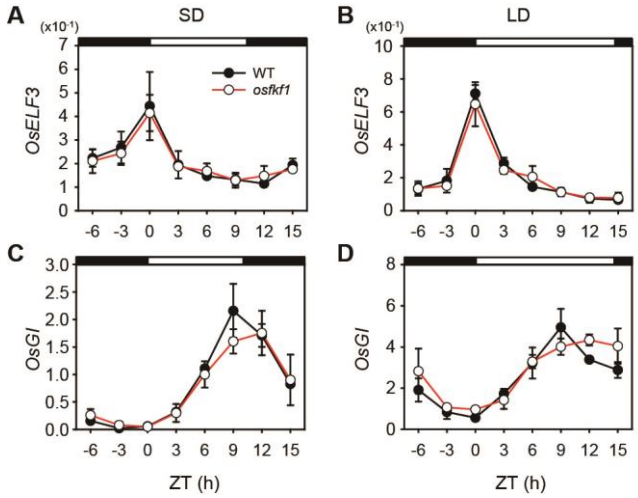


Figure 8. Comparison of transcript levels of *OsELF3* and *OsGI* between WT and *osfkf1* in SD and LD.

Filled (●) and open (○) circles represent WT and *osfkf1*, respectively. Leaf samples were collected every 3 hours from 30-day-old plants in SD and from 70-day-old plants in LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. ZT, zeitgeber time (hours after dawn).

Changes in transcript levels of the flowering time genes downstream of *OsFKF1* during development in *osfkf1* mutants

To examine the putative downstream genes of *OsFKF1*, we examined the transcript levels of flowering time genes during development in SD and LD, until heading of WT plants. We harvested fully developed leaves at ZT2 and measured transcript levels of *Ghd7*, *Ehd2*, *Ehd1*, *Hd3a*, and *RFT1*. In both WT and *osfkf1*, the levels of *Ghd7* transcripts increased and reached a peak at 5-6 weeks after sowing (WAS), and decreased thereafter in SD and LD (**Figure 9A**, **9B**). Also, *Ghd7* mRNA levels in *osfkf1* were higher than those in WT until 11 WAS, under both SD and LD (**Figure 9A**, **9B**). In SD, the mRNA levels of *Ehd2* in *osfkf1* were lower than those in WT until 7 WAS, but were similar to each other thereafter. By contrast, in LD, *Ehd2* expression remained at lower levels in *osfkf1* throughout development (**Figure 9C**, **9D**). The transcript levels of *Ehd1* gradually increased in WT and *osfkf1* mutants, but remained lower in *osfkf1* until heading (**Figure 9E**, **9F**). The mRNA levels of *Hd3a* in WT rapidly decreased at 6 WAS, increased after 7 WAS in SD, and markedly increased after 13 WAS in LD. In *osfkf1* mutants, the mRNA levels of *Hd3a* remained lower than in WT until heading (**Figure 9G**,

9H). The transcript levels of *RFT1* increased remarkably around the heading date in WT under both photoperiods, while *RFT1* transcript levels in *osfkf1* mutants remained much lower (**Figure 9I**, **9J**). Notably, the expression levels of *Hd3a* and *RFT1*, two rice florigens, remained at lower levels in *osfkf1* than in WT in both SD and LD, consistent with the late flowering phenotype of *osfkf1* (**Figure 9G-9J**). *Hd1* is the rice homolog of *Arabidopsis CO* and *FKF1* regulates *CO* transcription (Imaizumi *et al*. 2005; Sawa *et al*. 2007; Fornara *et al*. 2009). However, we observed no significant difference in the transcript levels of *Hd1* between WT and *osfkf1* mutants under SD or LD (**Figure 10**). Taken together, these results further support the notion that *OsFKF1* down-regulates *Ghd7* and up-regulates *Ehd2* at the transcriptional level in both photoperiods, and these transcriptional changes eventually result in up-regulation of the downstream genes *Ehd1, Hd3a*, and *RFT1*, to promote flowering.

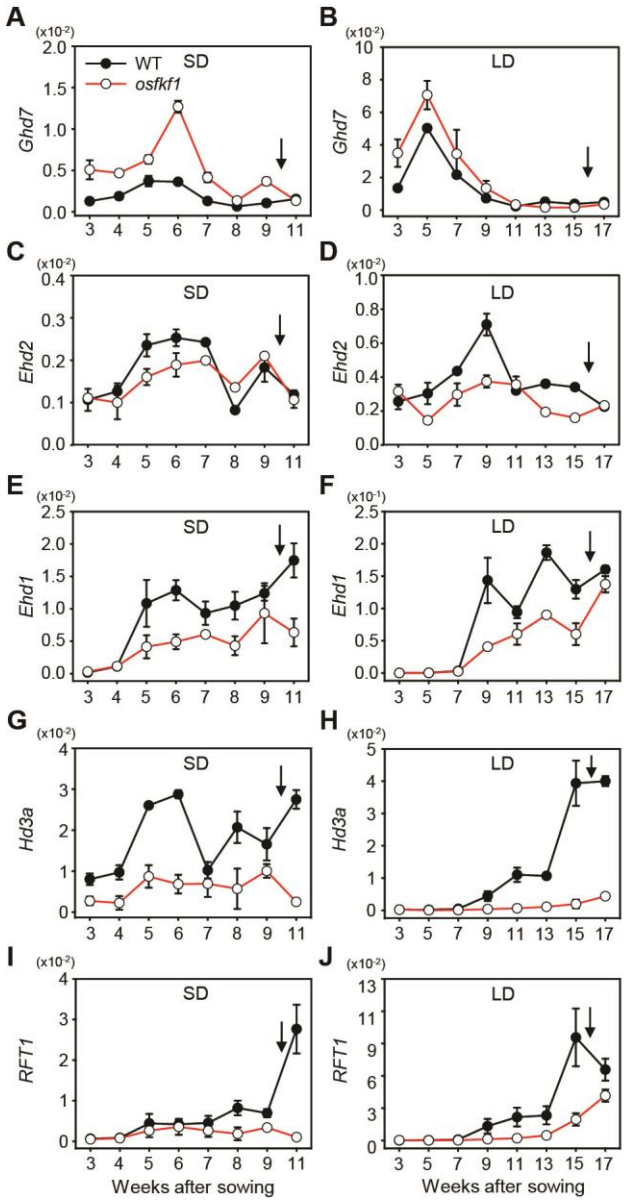


Figure 9. Altered expression of flowering time genes in *osfkf1* mutants under SD and LD during development.

Comparison of transcript levels of *Ghd7*, *Ehd2*, *Ehd1*, *Hd3a* and *RFT1* between *osfkf1* and WT in SD and LD. Filled (●) and open (○) circles represent WT and *osfkf1*, respectively. Leaf samples were collected 2 hours after dawn (ZT2). *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. The average time of heading in WT is shown by arrows.

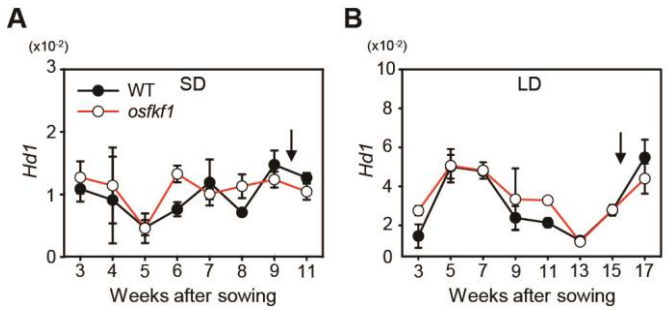


Figure 10. Expression analysis of *Hd1* in *osfkf1* mutants under SD and LD during development.

Filled (●) and open (○) circles represent WT and *osfkf1*, respectively. Leaf samples were collected 2 hours after dawn (ZT2). *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. The average time of heading in WT is shown by arrows.

OsFKF1 up-regulates *Ehd1* expression independent of *Ehd2* and *Ghd7* expression under blue light

In *Arabidopsis*, the blue-light receptor FKF1 contains a LOV domain that is involved in blue-light perception (Nelson *et al*. 2000; Imaizumi *et al*. 2003). CDFs repress *CO* transcription and FKF1 and GI physically interact with and destabilize CDFs in a blue light-dependent manner (Sawa *et al*, 2007; Fornara *et al*. 2009). Since *OsFKF1* regulates *Ehd1* expression (**Figure 6G**, **6H**; **Figure 9E**, **9F**), and AtFKF1 and OsFKF1 contain highly conserved protein domains (**Figure 1**), we examined whether OsFKF1 activates *Ehd1* expression in response to blue light. Plants were entrained in day-neutral (12-h light/12-h dark) conditions for 5 weeks and then transferred to blue light or dark the following dawn. *OsELF3* is also essential for *Ehd1* activation in response to blue light in the morning (Zhao *et al.* 2012); therefore, we used the *oself3-1* T-DNA insertion knockout mutants (Zhao *et al.* 2012) as a positive control for the blue-light response experiment (**Figure 11**). The levels of *Ehd1* activation in response to blue light were represented as relative abundance, which is calculated by dividing the values for *Ehd1* mRNA levels in the blue light-treated plants by those in the dark-treated plants.

In WT, the *Ehd1* mRNA levels gradually increased during blue light treatment and the relative abundance of *Ehd1* reached a peak at 3 hours after blue light irradiation (**Figure 11**). However, the effect of blue light irradiation on *Ehd1* transcript levels was much weaker in *osfkf1* and *oself3-1* mutants than in WT. Moreover, *osfkf1* and *oself3-1* mutants showed similar activation of *Ehd1* transcription, indicating that *OsFKF1* affects blue light-mediated induction of *Ehd1* for floral induction, similar to *OsELF3*.

Since we found that *OsFKF1* upregulates *Ehd1* and *FT*-like genes by modulating the expression of *Ghd7* and *Ehd2* (**Figure 6I-6L**), we also examined the relative abundance of *Ghd7* and *Ehd2* under blue light conditions. The relative abundances of *Ghd7* and *Ehd2* transcripts did not significantly differ between WT and *osfkf1* (**Figure 12**), indicating that *Ghd7* and *Ehd2* are not involved in the blue light-dependent *OsFKF1-Ehd1* floral induction pathway.

Itoh et al. (2010) also reported that red light pulses induce *Ghd7* expression in WT. Thus, we irradiated WT and *osfkf1* mutants with red light to examine whether *OsFKF1* is involved in red light-mediated activation of *Ghd7*. We observed no difference in the relative abundance of *Ghd7* between WT and *osfkf1*, although the relative abundance of

Ghd7 greatly increased one hour after treatment (**Figure 13A**). Moreover, we measured the transcript levels of *Ehd1* and *Ehd2* during red light treatment. The relative abundance of *Ehd1* and *Ehd2* transcript did not significantly differ between WT and *osfkf1* under red light (**Figure 13B**, **13C**). Thus, our results suggest that *OsFKF1* activates *Ehd1* transcription in a blue light-dependent manner, without modulating expression of *Ghd7* and *Ehd2*.

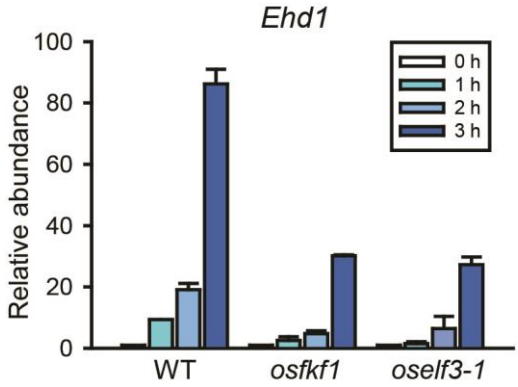


Figure 11. The *osfkf1* mutation impairs the blue light-mediated activation of *Ehd1*.

Rice seedlings were initially grown under day-neutral (12-h light/12-h dark) conditions for 5 weeks and then transferred to blue light (60 μmol m^{-2} s^{-1}) or darkness at dawn, and harvested at the indicated time points. The relative abundance of *Ehd1* mRNAs was calculated by dividing *Ehd1* mRNA levels in the blue light-treated plants by those in the dark-treated plants. Note that relative abundance of *Ehd1* was much lower in *osfkf1* compared with WT. *oself3-1* knockout mutants were used as a positive control for the blue-light response in this experiment (Zhao *et al*. 2012). *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations.

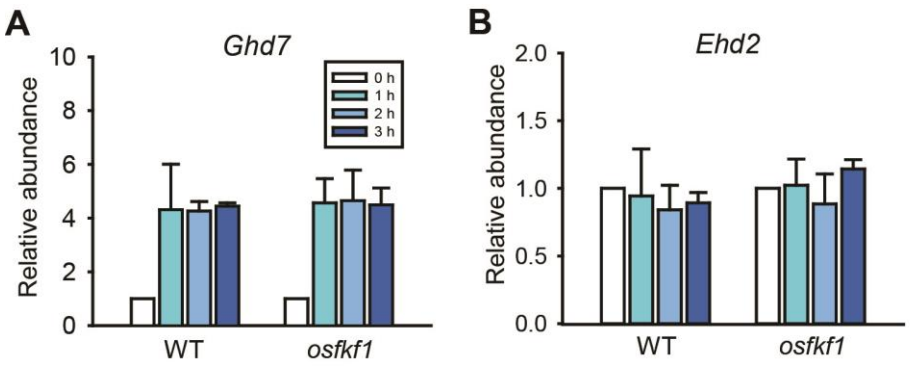


Figure 12. Relative transcript levels of *Ghd7* and *Ehd2* in WT and *osfkf1* under blue light.

Rice seedlings were first grown under day-neutral (12-hour light/12-hour dark) conditions for 5 weeks and then transferred to blue light (60 μmol m^{-2} s^{-1}) or darkness at dawn, and harvested at the indicated times. The relative abundance of the transcripts of *Ghd7* and *Ehd2* was calculated by dividing the transcript levels in the blue light-treated plants by those in the dark-treated plants. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations.

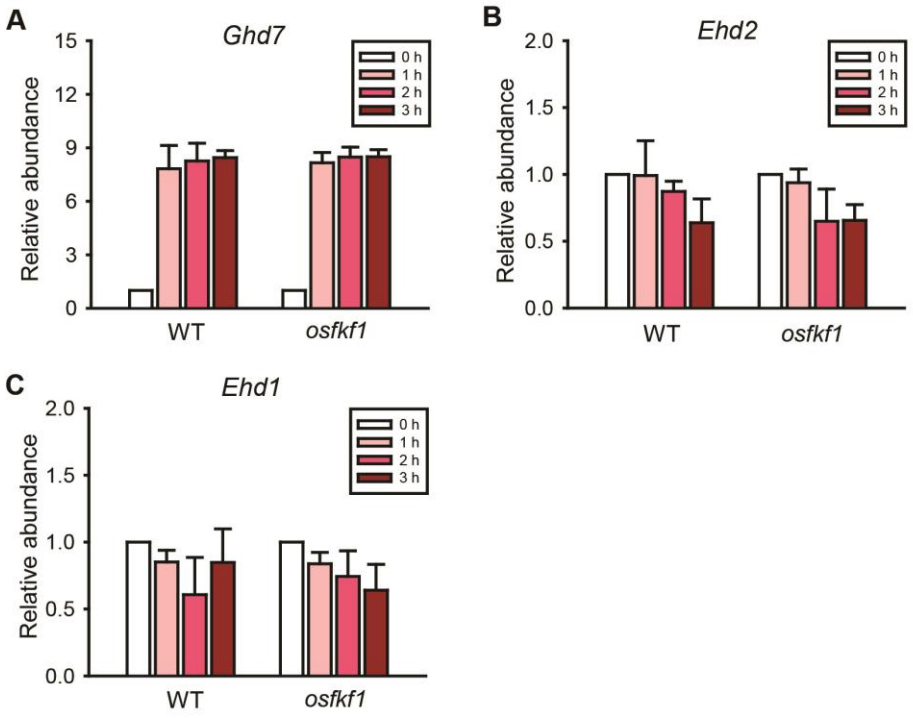


Figure 13. Relative transcript levels of *Ghd7*, *Ehd2* and *Ehd1* in WT and *osfkf1* under red light.

Rice seedlings were initially grown under day-neutral (12-hour light/12-hour dark) conditions for 5 weeks and then transferred to the red light (60 μmol m^{-2} s^{-1}) or darkness at dawn, and harvested at the indicated times. The relative mRNA abundance of *Ghd7*, *Ehd2* and *Ehd1* was calculated by dividing the mRNA levels of each gene in the red light-treated plants by those in the dark-treated plants. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations.

OsFKF1 interacts with OsGI and OsCDF1/OsDOF12

The blue light-mediated activation of *Ehd1* decreased in *osfkf1* mutants (**Figure 11**), strongly suggesting that, like AtFKF1, OsFKF1 perceives blue light for floral induction. *OsGI* shapes the sensitive phase set by the circadian clock in response to blue light signals that increase *Ehd1* expression at dawn (Itoh *et al*. 2010). Notably, both *OsFKF1* and *OsGI* are involved in the blue light-mediated induction of *Ehd1* and show similar diurnal expression patterns, peaking before dusk (**Figure 5D**, **5E**; **Figure 8C**, 8**D**). In addition, our expression analyses in SD and LD suggest that *OsGI* is not a downstream target of *OsFKF1* because we observed no significant difference in the *OsGI* mRNA levels between WT and *osfkf1* mutants (**Figure 8C**, 8**D**). Based on this idea, we hypothesized that OsFKF1 interacts with OsGI and forms an OsFKF1-OsGI complex, analogous to the *Arabidopsis* FKF1-GI complex.

To examine whether OsFKF1 interacts with OsGI, we performed yeast two-hybrid assays. As the bait vector containing the full-length *OsGI* self-activated, we used partial *OsGI* cDNA fragments (**Figure 14A**). We found that OsFKF1 interacts with the N-terminal region of OsGI (aa 1-398, OsGI-N), but not with the middle (aa 349-754, OsGI-

M) or C-terminal regions (aa 703-1160, OsGI-C) (**Figure 14A**). To further examine their *in vivo* interaction, we performed bimolecular fluorescence complementation (BiFC) assays. OsGI localizes to both the nucleus and cytosol of rice leaf sheath cells (Abe *et al*. 2008). Using a transient expression assay in onion (*Allium cepa*) epidermal layers, we detected the reconstituted YFP fluorescence only in the nucleus when nYFP-OsGI and OsFKF1-cYFP plasmids were co-transformed (**Figure 14B**). These results indicate that OsFKF1 interacts with OsGI in the nucleus.

The *Arabidopsis* FKF1-GI interaction promotes flowering only in LD conditions by mediating degradation of CDFs in a blue light-dependent manner (Imaizumi *et al.* 2005; Sawa *et al.* 2007; Fornara *et al.* 2009). Therefore, we hypothesized that OsFKF1 and OsGI interact with unidentified protein(s) to upregulate *Ehd1* transcription in a blue light-dependent manner. In addition, as the OsGI protein level was lowest at dawn and peaked before dusk, it appears that *OsGI* indirectly controls the morning gate for *Ehd1* by regulating the downstream gate components (Itoh *et al*. 2010). This unidentified downstream component could be an OsCDF, one of the rice orthologs of *Arabidopsis* CDFs. To investigate whether one of the OsCDFs interacts with OsFKF1 or OsGI,

we cloned the three *OsCDF*s, i.e. *OsCDF1* (also termed *OsDof12*; Os03g07360), *OsCDF2* (Os07g13260), and *OsCDF3* (Os07g48570), for yeast two-hybrid assays. We found that OsCDF1/OsDOF12 interacts with both OsFKF1 and OsGI, but not with OsCDF2 or OsCDF3 (**Figure 14A**). We further examined whether OsFKF1 or OsGI interacts with OsCDF1 *in vivo* by BiFC assays. OsCDF1 localizes to the nucleus (Li *et al*. 2009) and we detected the reconstituted YFP fluorescence in the nucleus of onion epidermal cells when nYFP-OsCDF1 plasmids were co-transformed with OsFKF1-cYFP or OsGI-cYFP plasmids (**Figure 14B**). Taken together, these results indicate that OsFKF1, OsGI, and OsCDF1 physically interact in the nucleus, thus indicating that the FKF1-GI-CDF1 interaction has been conserved in *Arabidopsis* and rice.

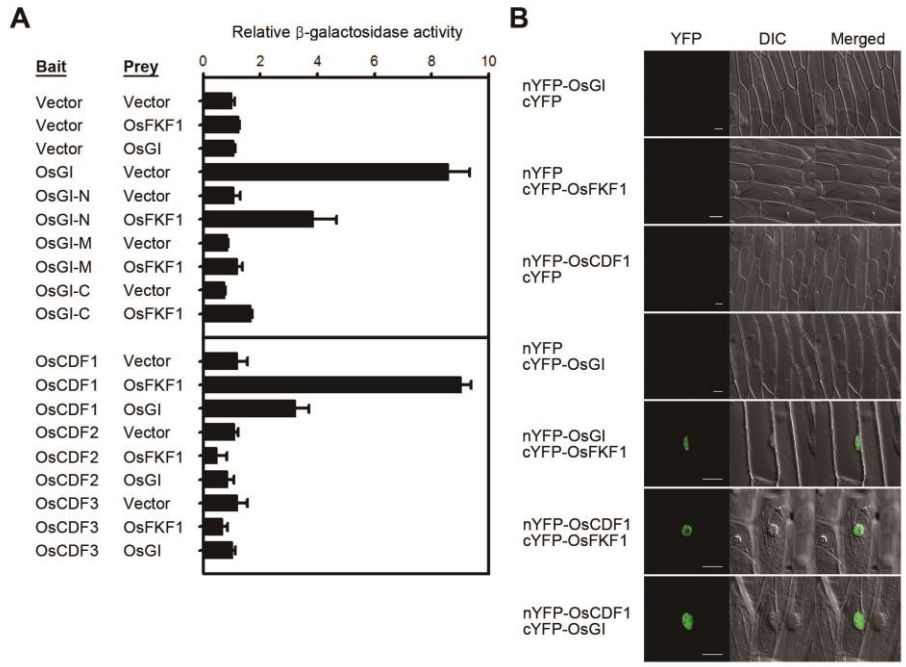


Figure 14. OsFKF1 interacts with OsGI and OsCDF1.

(**A**) Yeast two-hybrid assays among OsFKF1, OsGI, and OsCDF1. The upper panel shows the interaction between OsFKF1 and OsGI, and the lower panel shows the interaction of OsCDFs with OsFKF1 or OsGI in yeast two-hybrid assays. Baits were expressed as Gal4 DNA-BD (binding domain) fusion proteins in the pGBKT7 plasmid and preys were expressed as Gal4 AD (activation domain) fusion proteins in the pGADT7 vector. These vectors were co-transformed into the AH109 yeast strain. β-galactosidase activity was assayed by various combinations of bait and prey plasmids and is presented relative to that obtained for the interaction of empty pGBKT7 and empty pGADT7 plasmids (negative controls). OsGI-N, N-terminal partial cDNA of *OsGI*.

OsGI-M, middle region cDNA of *OsGI*. OsGI-C, C-terminal partial cDNA of *OsGI*. (**B**) BiFC assays revealed the *in vivo* interactions of OsGI-OsFKF1, OsCDF1-OsFKF1, and OsCDF1-OsGI in the nuclei of onion epidermal cells. Empty plasmids were used as a negative control. Each pair of recombinant plasmids encoding nYFP and cYFP fusions was mixed 1:1 (w/w), co-bombarded into onion (*Allium cepa*) epidermal cell layers. The transformed onion epidermal layers were incubated at 22℃ on Murashige and Skoog plates in the dark for 20 h. These experiments were repeated at least three times with similar results. Each bar indicates 50 μm. DIC, differential interference contrast.

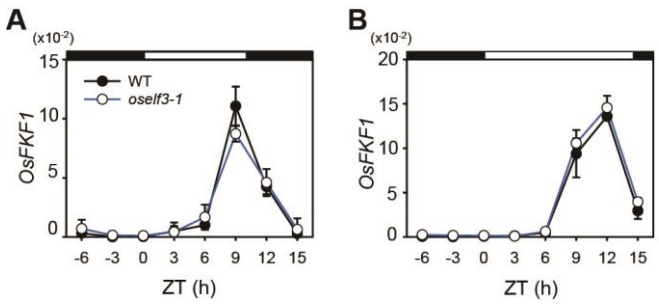


Figure 15. Diurnal expression of *OsFKF1* in *oself3-1* mutants under SD and LD.

Filled (●) and open (○) circles represent WT and *oself3-1*, respectively. Leaf samples were collected every 3 hours from 30 DAS plants in SD and LD. *Ubiquitin 5* (*UBQ5*) was used as an internal control. Values are shown as means of three biological replicates. Error bars indicate standard deviations. ZT, zeitgeber time; DAS, days after sowing.

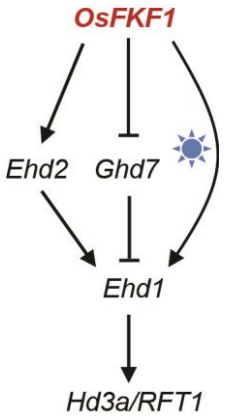


Figure 16. A working model of *OsFKF1* in rice floral induction.

To promote flowering, *OsFKF1* up-regulates *Ehd2* and down-regulates *Ghd7* to activate *Ehd1* expression in both SD and LD conditions. This suggests that *OsFKF1* acts as an autonomous floral inducer independent of photoperiod. *OsFKF1* also regulates *Ehd1* expression in a blue light-dependent manner, without modulating the expression of *Ehd2* or *Ghd7*. Arrows and bars indicate positive and negative regulation, respectively.

DISCUSSION

In this study, we first examined the effect of the *osfkf1* mutation on flowering time in rice and found that *OsFKF1* acts as an autonomous floral inducer, as *osfkf1* mutants flower later than WT, irrespective of day length (**Figure 2C**, **2D**). Expression analyses demonstrated that the *osfkf1* mutation causes down-regulation of *Ehd2* and up-regulation of *Ghd7* at the transcriptional level (**Figure 6I-6L**). Consequently, expression of their common downstream gene *Ehd1* decreases, which subsequently leads to decreases of *Hd3a* and *RFT1* expression in SD and LD (**Figure 6**). Although *Ghd7* expression increased significantly in *osfkf1* mutants under both SD and LD, it is unlikely that the enhanced expression of *Ghd7* affects *Ehd1* expression under SD, since *Ghd7* delays flowering only under LD by down-regulating *Ehd1* expression, but hardly affects *Ehd1* expression or flowering time under SD (Xue *et al.* 2008). Thus, the reduced expression of *Ehd1* under SD can be attributed to the significant decrease of *Ehd2* expression in the *osfkf1* mutants. Therefore, we concluded that *OsFKF1* promotes flowering by activation of the *Ehd1*-*Hd3a*/*RFT1* pathway through the regulation of

Ehd2 (the activator of *Ehd1*) and *Ghd7* (the repressor of *Ehd1*) under LD. Under SD, however, *OsFKF1* regulates flowering time mainly through the *Ehd2-Ehd1-Hd3a*/*RFT1* pathway.

Several molecular genetic approaches have identified additional regulators of *Ehd1*. Like *Ehd2*, both *Ehd3* (Matsubara *et al*. 2011) and *Ehd4* (Gao *et al*. 2013) act as positive regulators of *Ehd1* expression independent of photoperiod, whereas *DTH8* and *Ghd7* (Xue *et al*. 2008; Wei *et al.* 2010; Yan *et al.* 2011) act as negative regulators of *Ehd1* expression mainly under LD. Some MADS-box genes also affect *Ehd1* expression: *OsMADS51* acts as a SD-specific positive regulator (Kim *et al*. 2007), and *OsMADS50* and *OsMADS56* act as LD-specific positive and negative regulators, respectively (Lee *et al*. 2004; Ryu *et al*. 2009). However, our results showed that the *osfkf1* mutants do not affect the transcript levels of *Ehd3*, *Ehd4*, *DTH8*, *OsMADS51*, *OsMADS50*, and *OsMADS56*, suggesting that *OsFKF1* mainly regulates *Ehd1* expression by modulating the expression of *Ehd2* and *Ghd7* independently of other *Ehd1* regulators (**Figure 7**).

Recent work reported that *OsELF3*, the rice ortholog of *Arabidopsis ELF3*, promotes flowering by negatively regulating *Ghd7* expression in LD (Matsubara *et al.* 2012; Saito *et al.* 2012; Zhao *et al.* 2012; Yang *et*

al. 2013), and *OsELF3* is also important in the blue light-mediated activation of *Ehd1*. Thus, *OsELF3* and *OsFKF1* appear to have similar functions (**Figure 11**). Our analysis found no apparent difference in the abundance of *OsELF3* mRNA between WT and *osfkf1* mutants (**Figure 8A**, **8B**). Conversely, we also found no apparent difference in the abundance of *OsFKF1* mRNA between WT and *oself3-1* mutants (**Figure 15**). These results indicate that the *OsFKF1* regulatory module might function in repressing *Ghd7* expression and activating *Ehd1* expression independently of *OsELF3* (**Figure 8** and **Figure 15**). Further analysis is necessary to elucidate how *OsFKF1* regulates expression of *Ehd2* and *Ghd7* for floral induction under both photoperiods.

In addition to late flowering, *osfkf1* mutants produced more grains per panicle but fewer panicles per plant, and exhibited lower fertility and lower 500-grain weight under NLD conditions (**Figure 4**). *Ghd7* has important roles in regulating heading date and yield potential in rice, as enhanced expression of *Ghd7* delays flowering time and increases plant height, panicle size, and number of grains per panicle in LD (Xue *et al*. 2008). Similarly, *osfkf1* mutants had more grains per panicle and higher expression of *Ghd7*, although the plant height and panicle size of *osfkf1* mutants did not significantly differ from those of WT. The reduction in

percentage of ripened grains and 500-grain weight in *osfkf1* mutants is probably due to unfavorably low temperatures during fertilization and grain filling caused by late flowering in temperate regions (37°N latitude; Suwon, Korea). Moreover, *Ehd1* controls panicle formation and the combination of *Hd1* and *Ehd1* can reduce the number of primary branches in a panicle, resulting in fewer spikelets per panicle, independent of flowering-time regulation (Endo-Higashi & Izawa 2011). In contrast, *osfkf1* mutants showed lower *Ehd1* mRNA levels at the peak time-point, and *Hd1* expression was also slightly down-regulated, at least during the evening (**Figure 6E**, **6F**), compared with WT. These results may explain the increase in spikelets per panicle in *osfkf1* mutants. Thus, it would be worthwhile to evaluate the agronomic traits of *osfkf1* mutants in the *indica* rice cultivars at low latitudes, where late flowering will not cause exposure to unfavorable temperatures.

Mutation of *OsFKF1* causes late flowering under both SD and LD in rice (**Figure 2C**, **2D**), but *Arabidopsis fkf1* mutants exhibit late flowering in LD, not in SD (Nelson *et al*. 2000). This difference between rice and *Arabidopsis* indicated that these FKF1s might participate in different genetic networks of floral induction. Photoperiodic flowering in *Arabidopsis* is mainly determined by the *FKF1/GI-CO-FT* pathway.

Under inductive LD conditions, the peaks of *FKF1* and *GI* expression coincide in the afternoon, which enables the interaction of FKF1 and GI during daytime, leading to floral promotion via the induction of *CO* transcription in the late afternoon (Sawa *et al*. 2007; Fornara *et al*. 2009). Under non-inductive SD conditions, however, *GI* expression peaks earlier than *FKF1* expression, leading to very little FKF1-GI interaction, resulting in *CO* expression at the end of the day. Since CO protein is destabilized in the dark, *CO* induction in the light under LD is crucial for LD-dependent early flowering in *Arabidopsis* (Valverde *et al*. 2004; Jang *et al*. 2008). By contrast, rice has at least two independent flowering pathways, the *Hd1*-*Hd3a* and the *Ehd1*-*Hd3a*/*RFT1* pathways. In *osfkf1* mutants, the mRNA levels of *Hd1* were almost the same as those of WT, except for the slightly reduced expression during the evening (**Figure 6E**, **6F**). By contrast, *Ehd1* expression markedly decreased in both SD and LD (**Figure 6G**, **6H**). The slight reduction in *Hd1* expression during nighttime is likely associated with the reduced expression of *Ehd2* in *osfkf1* mutants, because in previous studies, *ehd2* mutants showed reduced *Hd1* mRNA levels, especially during the dark period (Matsubara *et al.* 2008; Park *et al.* 2008; Wu *et al.* 2008). Hd1 acts as a floral repressor under LD by suppressing *Hd3a* expression (Yano *et al.* 2000;

Tamaki *et al.* 2007); therefore, reduction of *Hd1* expression should promote flowering. However, *osfkf1* mutants showed late flowering with remarkably reduced *Hd3a* expression under LD. This indicates that the slightly reduced expression of *Hd1* did not considerably affect flowering time in *osfkf1* mutants. In addition, we observed no significant difference in the transcript levels of *Hd1* between WT and *osfkf1* mutants during development under SD and LD (**Figure 10**). Thus, we concluded that *OsFKF1* is mainly involved in the *Ehd1-Hd3a*/*RFT1* pathway to induce flowering, rather than in the *Hd1-Hd3a* pathway. In addition, *Arabidopsis* does not have well conserved homologs of *Ehd1*, *Ehd2*, and *Ghd7* whose expression is regulated by *OsFKF1* (Matsubara *et al.* 2008; Park *et al.* 2008; Wu *et al.* 2008; Xue *et al.* 2008). Together, our results indicate that the differences in *OsFKF1* and *AtFKF1* function may be due to the existence of two independent pathways downstream of *OsFKF1* and the regulation of rice-specific genes such as *Ehd2*, *Ghd7*, and *Ehd1* in the floral induction pathways in rice (**Figure 16**). Further molecular genetic and biochemical studies will be needed to reveal how OsFKF1 regulates these rice specific *Ehd1* regulators.

In *Arabidopsis*, the FKF1-GI interaction activates *CO* transcription in a blue-light dependent manner (Imaizumi *et al.* 2005; Sawa *et al.* 2007;

Fornara *et al.* 2009). In rice, however, based on transcriptional (**Figure 6**, **Figure 9**, and **Figure 11**) and interaction analyses (**Figure 14**), the OsFKF1-OsGI interaction might be involved in the blue light-mediated activation of *Ehd1*, not *Hd1*, the rice ortholog of *CO* (**Figure 11** and **Figure 14B**). These findings imply that the function of the OsFKF1-OsGI interaction likely differs from that of the *Arabidopsis* FKF1-GI interaction, since it depends on the function of downstream modules, *CO-FT* in *Arabidopsis* or *Hd1*/*Ehd1-Hd3a*/*RFT1* in rice. In other species, *GmFKF1*/*GmFKF2* (soybean homologs of *FKF1*) and *GmGI1*/*GmGI2* (soybean homologs of *GI*) have conserved functional domains with *Arabidopsis FKF1* and *GI*, and GmFKF1 interacts with GmGI1 and GmGI2 in yeast two-hybrid assays (Li *et al*. 2013). In addition, Kubota et al. (2014) reported that the orthologs of *FKF1* and *GI* are also present in the liverwort *Marchantia polymorpha*, and MpFKF1 interacts with MpGI in yeast two-hybrid assays. The MpFKF1-MpGI interaction is critical for photoperiodic growth-phase transition from vegetative to reproductive phase. These findings suggest that the FKF1-GI system had already been acquired in a common ancestor of land plants (Kubota *et al*. 2014). By contrast, *Brachypodium*, a model monocot for the temperate grasses, has no ortholog of *Ehd1*, although flanking genes have been well

conserved between *Brachypodium* and rice (Higgins *et al*. 2010). This strongly suggests that the FKF1-GI interaction is evolutionarily more ancient than the *Arabidopsis CO-FT* module or the rice *Hd1*/*Ehd1*-*Hd3a*/*RFT1* module, which has been recruited into one or more pathways depending on the plant lineage.

As the expression levels of *OsFKF1* and *OsGI* are low at dawn (**Figure 5D**, **5E** and **Figure 8C**, **8D**), the OsFKF1-OsGI interaction might regulate the blue light-mediated activation of *Ehd1* through unidentified protein(s) such as the *Arabidopsis* CDFs; indeed, both OsFKF1 and OsGI physically interact with OsCDF1/OsDOF12 (**Figure 14**). Interestingly, a previous study reported that the transgenic line overexpressing *OsDof12* flowered early under LD, but not SD, and *OsDof12* overexpression promotes flowering by controlling the expression of *Hd3a* and *OsMADS14*, the rice ortholog of *Arabidopsis APETALA 1* (Li *et al*. 2009). These results suggest that rice OsCDF1/ OsDOF12 might have different functions from the *Arabidopsis* CDFs, which repress *CO* transcription (Imaizumi *et al.* 2005; Sawa *et al.* 2007; Fornara *et al.* 2009). In contrast to the *Arabidopsis* CDFs, OsCDF1/ OsDOF12 may cooperate with OsFKF1 and OsGI to induce flowering, at least under LD. Previous studies reported that the expression of

OsCDF1 is strongly inhibited in the dark (Li *et al*. 2008; Li *et al*. 2009) and the expression levels of *OsFKF1* and *OsGI* peaked in the afternoon under both SD and LD (**Figure 5D**, **5E** and **Figure 8C**, **8D**). Thus, we speculate that the interaction of OsFKF1-OsGI-OsCDF1 likely occurs in the afternoon and remains stable in the light. Further molecular genetic and biochemical analyses will be needed to elucidate the role of other OsCDF homologs in the blue light-mediated pathway of *Ehd1* activation. Furthermore, comparative studies on the regulatory mechanisms downstream of OsFKF1-OsGI among species will provide molecular insights on how the photoperiodic pathways that regulate flowering have diverged in monocot and dicot plants.

REFERENCES

Abe M., Fujiwara M., Kurotani K., Yokoi S. & Shimamoto K. (2008) Identification of dynamin as an interactor of rice GIGANTEA by tandem affinity purification (TAP). *Plant and Cell Physiology* **49**, 420-432.

Andrade M.A., Gonzalez-Guzman M., Serrano R. & Rodriguez P.L. (2001) A combination of the F-box motif and kelch repeats defines a large Arabidopsis family of F-box proteins. *Plant Molecular Biology* **46**, 603-614.

Citovsky V., Lee L.Y., Vyas S., Glick E., Chen M.H., Vainstein A., ..., Tzfira T. (2006) Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. *Journal of Molecular Biology* **362**, 1120-1131.

Curtis M. & Grossniklaus U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**, 462-469.

Dai C. & Xue H.W. (2010) Rice early flowering1, a CKI, phosphorylates DELLA protein SLR1 to negatively regulate

gibberellin signalling. *EMBO Journal* **29**, 1916-1927.

Doi K., Izawa T., Fuse T., Yamanouchi U., Kubo T., Shimatani Z., Yano M. & Yoshimura A. (2004) Ehd1, a B-type response regulator in rice, confers short-day promotion of flowering and controls FT-like gene expression independently of Hd1. *Genes and Development* **18**, 926-936.

Endo-Higashi N. & Izawa T. (2011) Flowering time genes Heading date 1 and Early heading date 1 together control panicle development in rice. *Plant and Cell Physiology* **52**, 1083-1094.

Fornara F., Panigrahi K.C., Gissot L., Sauerbrunn N., Ruhl M., Jarillo J.A. & Coupland G. (2009) Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. *Developmental Cell* **17**, 75-86.

Gao H., Zheng X.M., Fei G., Chen J., Jin M., Ren Y., …, Wan J.M. (2013) Ehd4 encodes a novel and Oryza-genus-specific regulator of photoperiodic flowering in rice. *PLoS Genetics* **9**, e1003281.

Hayama R., Yokoi S., Tamaki S., Yano M. & Shimamoto K. (2003) Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature* **422**, 719-722.

Higgins J.A., Bailey P.C. & Laurie D.A. (2010) Comparative genomics of flowering time pathways using Brachypodium distachyon as a model for the temperate grasses. *PLoS One* **5**, e10065.

Hori K., Ogiso-Tanaka E., Matsubara K., Yamanouchi U., Ebana K. & Yano M. (2013) Hd16, a gene for casein kinase I, is involved in the control of rice flowering time by modulating the day-length response. *The Plant Journal* **76**, 36-46.

Huq E., Tepperman J.M. & Quail P.H. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 9789-9794.

Imaizumi T., Tran H.G., Swartz T.E., Briggs W.R. & Kay S.A. (2003) FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. *Nature* **426**, 302-306.

Imaizumi T., Schultz T.F., Harmon F.G., Ho L.A. & Kay S.A. (2005) FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. *Science* **309**, 293-297.

Imaizumi T. & Kay S.A. (2006) Photoperiodic control of flowering: not only by coincidence. *Trends in Plant Science* **11**, 550-558.

Itoh H., Nonoue Y., Yano M. & Izawa T. (2010) A pair of floral

regulators sets critical day length for Hd3a florigen expression in rice. *Nature Genetics* **42**, 635-638.

Itoh J.I., Hasegawa A., Kitano H. & Nagato Y. (1998) A recessive heterochronic mutation, plastochron1, shortens the plastochron and elongates the vegetative phase in rice. *The Plant Cell* **10**, 1511-1522.

Izawa T., Oikawa T., Sugiyama N., Tanisaka T., Yano M. & Shimamoto K. (2002) Phytochrome mediates the external light signal to repress FT orthologs in photoperiodic flowering of rice. *Genes and Development* **16**, 2006-2020.

Izawa T. (2007) Adaptation of flowering-time by natural and artificial selection in Arabidopsis and rice. *Journal of Experimental Botany* **58**, 3091-3097.

Jain M., Nijhawan A., Tyagi A.K. & Khurana J.P. (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochemical and Biophysical Research Communications* **345**, 646-651.

Jang S, Marchal V, Panigrahi KC, Wenkel S, Soppe W, Deng XW, …, Coupland G. (2008) Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *EMBO Journal* **27**, 1277-1288.

Jeon J.S., Chung Y.Y., Lee S., Yi G.H., Oh B.G. & An G. (1999) Isolation and characterization of an anther-specific gene, RA8, from rice (Oryza sativa L.). *Plant Molecular Biology* **39**, 35-44.

Jeon J.S., Lee S., Jung K.H., Jun S.H., Jeong D.H., Lee J, …, An G. (2000) T-DNA insertional mutagenesis for functional genomics in rice. *The Plant Journal* **22**, 561-570.

Jeong D.H., An S., Kang H.G., Moon S., Han J.J., Park S., Lee H.S., An K. & An G. (2002) T-DNA insertional mutagenesis for activation tagging in rice. *Plant Physiology* **130**, 1636-1644.

Kardailsky I., Shukla V.K., Ahn J.H., Dagenais N., Christensen S.K., Nguyen J.T., Chory J., Harrison M.J. & Weigel D. (1999) Activation tagging of the floral inducer FT. *Science* **286**, 1962-1965.

Kim S.L., Lee S., Kim H.J., Nam H.G. & An G. (2007) OsMADS51 is a short-day flowering promoter that functions upstream of Ehd1, OsMADS14, and Hd3a. *Plant Physiology* **145**, 1484-1494.

Kobayashi Y., Kaya H., Goto K., Iwabuchi M. & Araki T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960-1962.

Kobayashi Y. & Weigel D. (2007) Move on up, it's time for change--mobile signals controlling photoperiod-dependent flowering. *Genes*

and Development **21**, 2371-2384.

Kojima S., Takahashi Y., Kobayashi Y., Monna L., Sasaki T., Araki T. & Yano M. (2002) Hd3a, a rice ortholog of the Arabidopsis FT gene, promotes transition to flowering downstream of Hd1 under short-day conditions. *Plant and Cell Physiology* **43**, 1096-1105.

Komiya R., Ikegami A., Tamaki S., Yokoi S. & Shimamoto K. (2008) Hd3a and RFT1 are essential for flowering in rice. *Development* **135**, 767-774.

Komiya R., Yokoi S. & Shimamoto K. (2009) A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice. *Development* **136**, 3443-3450.

Koo B.H., Yoo S.C., Park J.W., Kwon C.T., Lee B.D., An G., …, Paek N.C. (2013) Natural variation in OsPRR37 regulates heading date and contributes to rice cultivation at a wide range of latitudes. *Molecular Plant* **6**, 1877-1888.

Kubota A., Kita S., Ishizaki K., Nishihama R., Yamato K.T. & Kohchi T. (2014) Co-option of a photoperiodic growth-phase transition system during land plant evolution. *Nature Communications* **5**, 3668.

Kwon C.T., Yoo S.C., Koo B.H., Cho S.H., Park J.W., Zhang Z., …,

Paek N.C. (2014) Natural variation in Early flowering1 contributes to early flowering in japonica rice under long days. *Plant, Cell and Environment* **37**, 101-112.

Lee S., Kim J., Han J.J., Han M.J. & An G. (2004) Functional analyses of the flowering time gene OsMADS50, the putative SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS-LIKE 20 (SOC1/AGL20) ortholog in rice. *The Plant Journal* **38**, 754-764.

Lee S.C., Jeon J.S., Jung K.H. & An G. (1999) Binary vector for efficient transformation of rice. *Journal of Plant Biology* **42**, 301-316.

Li D, Yang C, Li X, Ji G & Zhu L. (2008) Sense and antisense OsDof12 transcripts in rice. *BMC Molecular Biology* **9**, 80.

Li D., Yang C., Li X., Gan Q., Zhao X. & Zhu L. (2009) Functional characterization of rice OsDof12. *Planta* **229**, 1159-1169.

Li F., Zhang X., Hu R., Wu F., Ma J., Meng Y. & Fu Y. (2013) Identification and molecular characterization of FKF1 and GI homologous genes in soybean. *PLoS One* **8**, e79036.

Matsubara K., Yamanouchi U., Wang Z.X., Minobe Y., Izawa T. & Yano M. (2008) Ehd2, a rice ortholog of the maize INDETERMINATE1 gene, promotes flowering by up-regulating

Ehd1. *Plant Physiology* **148**, 1425-1435.

Matsubara K., Yamanouchi U., Nonoue Y., Sugimoto K., Wang Z.X., Minobe Y. & Yano M. (2011) Ehd3, encoding a plant homeodomain finger-containing protein, is a critical promoter of rice flowering. *The Plant Journal* **66**, 603-612.

Matsubara K., Ogiso-Tanaka E., Hori K., Ebana K., Ando T. & Yano M. (2012) Natural variation in Hd17, a homolog of Arabidopsis ELF3 that is involved in rice photoperiodic flowering. *Plant and Cell Physiology* **53**, 709-716.

Mizoguchi T., Wright L., Fujiwara S., Cremer F., Lee K., Onouchi H., …, Coupland G. (2005) Distinct roles of GIGANTEA in promoting flowering and regulating circadian rhythms in Arabidopsis. *The Plant Cell* **17**, 2255-2270.

Murakami M., Ashikari M., Miura K., Yamashino T. & Mizuno T. (2003) The evolutionarily conserved OsPRR quintet: Rice Pseudo-Response Regulators implicated in circadian rhythm. *Plant and Cell Physiology* **44**, 1229-1236.

Murakami M., Matsushika A., Ashikari M., Yamashino T. & Mizuno T. (2005). Circadian-associated rice Pseudo-Response Regulators (OsPRRs): Insight into the control of flowering time. *Bioscience*,

Biotechnology, and Biochemistry **69**, 410-414.

Murakami M., Tago Y., Yamashino T. & Mizuno T. (2007) Comparative overviews of clock-associated genes of Arabidopsis thaliana and Oryza sativa. *Plant and Cell Physiology* **48**, 110-121.

Murray M.G. & Thompson W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4325.

Nelson D.C., Lasswell J., Rogg L.E., Cohen M.A. & Bartel B. (2000) FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. *Cell* **101**, 331-340.

Ogiso E., Takahashi Y., Sasaki T., Yano M. & Izawa T. (2010) The role of casein kinase II in flowering time regulation has diversified during evolution. *Plant Physiology* **152**, 808-820.

Park D.H., Somers D.E., Kim Y.S., Choy Y.H., Lim H.K., Soh M.S., Kim H.J., Kay S.A. & Nam H.G. (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis GIGANTEA gene. *Science* **285**, 1579-1582.

Park S.J., Kim S.L., Lee S., Je B.I., Piao H.L., Park S.H., …, Han C.D. (2008) Rice Indeterminate 1 (OsId1) is necessary for the expression of Ehd1 (Early heading date 1) regardless of photoperiod. *The Plant Journal* **56**, 1018-1029.

Putterill J., Robson F., Lee K., Simon R. & Coupland G. (1995) The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* **80**, 847-857.

Ryu C.H., Lee S., Cho L.H., Kim S.L., Lee Y.S., Choi S.C., …, An G. (2009) OsMADS50 and OsMADS56 function antagonistically in regulating long day (LD)-dependent flowering in rice. *Plant, Cell and Environment* **32**, 1412-1427.

Saito H., Ogiso-Tanaka E., Okumoto Y., Yoshitake Y., Izumi H., Yokoo T., …, Tanisaka T. (2012) Ef7 encodes an ELF3-like protein and promotes rice flowering by negatively regulating the floral repressor gene Ghd7 under both short- and long-day conditions. *Plant and Cell Physiology* **53**, 717-728.

Sawa M., Nusinow D.A., Kay S.A. & Imaizumi T. (2007) FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science* **318**, 261-265.

Searle I. & Coupland G. (2004) Induction of flowering by seasonal changes in photoperiod. *EMBO Journal* **23**, 1217-1222.

Shibaya T., Nonoue Y., Ono N., Yamanouchi U., Hori K. & Yano M. (2011) Genetic interactions involved in the inhibition of heading by

heading date QTL, Hd2 in rice under long-day conditions. *Theoretical and Applied Genetics* **123**, 1133-1143.

Shrestha R., Gomez-Ariza J., Brambilla V. & Fornara F. (2014) Molecular control of seasonal flowering in rice, arabidopsis and temperate cereals. *Annals of Botany* **114**, 1445-1458.

Song Y.H., Smith R.W., To B.J., Millar A.J. & Imaizumi T. (2012) FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science* **336**, 1045-1049.

Suarez-Lopez P., Wheatley K., Robson F., Onouchi H., Valverde F. & Coupland G. (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature* **410**, 1116-1120.

Takase T., Nishiyama Y., Tanihigashi H., Ogura Y., Miyazaki Y., Yamada Y. & Kiyosue T. (2011) LOV KELCH PROTEIN2 and ZEITLUPE repress Arabidopsis photoperiodic flowering under non-inductive conditions, dependent on FLAVIN-BINDING KELCH REPEAT F-BOX1. *The Plant Journal* **67**, 608-621.

Tamaki S., Matsuo S., Wong H.L., Yokoi S. & Shimamoto K. (2007) Hd3a protein is a mobile flowering signal in rice. *Science* **316**, 1033-1036.

Thomas BaV-P, D. (1997) Photoperiodism in Plants. London: Academic Press.

Tsuji H., Tamaki S., Komiya R. & Shimamoto K. (2008) Florigen and the Photoperiodic Control of Flowering in Rice. *Rice* **1**, 25-35.

Tsuji H., Taoka K. & Shimamoto K. (2011) Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation. *Current Opinion in Plant Biology* **14**, 45-52.

Turck F., Fornara F. & Coupland G. (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573-594.

Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A & Coupland G. (2004) Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303**, 1003-1006.

Wei X., Xu J., Guo H., Jiang L., Chen S., Yu C., …, Wan J. (2010) DTH8 suppresses flowering in rice, influencing plant height and yield potential simultaneously. *Plant Physiology* **153**, 1747-1758.

Wu C., You C., Li C., Long T., Chen G., Byrne M.E. & Zhang Q. (2008) RID1, encoding a Cys2/His2-type zinc finger transcription factor, acts as a master switch from vegetative to floral development in rice. *Proceedings of the National Academy of Sciences of Sciences*

of the United States of America **105**, 12915-12920.

Xue W., Xing Y., Weng X., Zhao Y., Tang W., Wang L., …, Zhang Q. (2008) Natural variation in Ghd7 is an important regulator of heading date and yield potential in rice. *Nature Genetics* **40**, 761-767.

Yan W.H., Wang P., Chen H.X., Zhou H.J., Li Q.P., Wang C.R., …, Zhang QF. (2011) A major QTL, Ghd8, plays pleiotropic roles in regulating grain productivity, plant height, and heading date in rice. *Molecular Plant* **4**, 319-330.

Yang Y., Peng Q., Chen G.X., Li X.H. & Wu C.Y. (2013) OsELF3 is involved in circadian clock regulation for promoting flowering under long-day conditions in rice. *Molecular Plant* **6**, 202-215.

Yano M., Katayose Y., Ashikari M., Yamanouchi U., Monna L., Fuse T., …, Sasaki T. (2000) Hd1, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. *The Plant Cell* **12**, 2473-2484.

Yasuhara M., Mitsui S., Hirano H., Takanabe R., Tokioka Y., Ihara N., ..., Kiyosue T. (2004) Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein 2) in Arabidopsis. *Journal of Experimental Botany* **55**, 2015-2027.

Zhao J., Huang X., Ouyang X., Chen W., Du A., Zhu L., Wang S., Deng X.W. & Li S. (2012) OsELF3-1, an ortholog of Arabidopsis early flowering 3, regulates rice circadian rhythm and photoperiodic flowering. *PLoS One* **7**, e43705.

국문 초록

식물의 개화시기, 즉 출수기는 식물 재배에 있어 재배 지역이나 재배 시기를 결정하는 중요한 요소이다. 대표적인 장일식물인 애기장대의 *FLAVIN-BINDING, KELCH REPEAT, F-BOX1* (*FKF1*) 유전자는 청색 빛에 의해 활성화되어 장일조건 하에서 *CONSTANS* (*CO*) 유전자 의 전사와 번역 후 과정에 관여한다고 알려져 있다. 한편, 벼는 단일조건에서 개화가 촉진되며 장일 조건에서는 개화가 지연되는 단일 식물로 알려져 있으며, 이러한 벼에서 FKF1 유전자 (*OsFKF1*) 에 대한 연구는 아직 진행되지 않은 상태다. 본 논문에서는 *OsFKF1* 의 기능을 알아보고자 *OsFKF1* 돌연변이체인 *osfkf1* 의 표현형을 관찰하였고, *osfkf1* 의 개화시기가 단일조건, 장일조건, 자연 장일조건에서 모두 지연되는 것을 확인하였다. *osfkf1* 에서 개화시기 조절 유전자들의 발현량을 측정한 결과, 정상개체와 비교했을 때, 단일조건과 장일조건에서 모두 *Early heading date 1*

(*Ehd1*)의 전사를 촉진하는 *Early heading date 2* (*Ehd2*)의 발현이 감소하고 *Ehd1* 의 전사를 억제한다고 알려진 *Grain number, plant height and heading date 7* (*Ghd7*)의 발현이 증가함을 확인하였다. 따라서 *OsFKF1* 은 단일조건과 장일조건에서 *Ehd2* 의 전사를 촉진시키고 *Ghd7* 의 전사를 억제함으로써 하위 유전자인 *Early heading date 1* (*Ehd1*), *Heading date 3a* (*Hd3a*), *RICE FLOWERING LOCUS T 1* (*RFT1*)의 발현을 증가시켜 개화를 촉진한다는 것을 알 수 있었다. 또한, 실험을 통해 *Ehd2, Ghd7* 발현량 조절과는 별개로 청색 빛에 의한 *Ehd1* 의 발현 증가에도 *OsFKF1* 이 관여한다는 사실을 밝혔다. 장일조건 하에서만 개화시기를 촉진한다고 알려진 애기장대 *FKF1* 과는 달리 *OsFKF1* 은 광주기에 상관없이 개화를 촉진하는 차이를 보였는데 이는 *OsFKF1* 이 조절하는 유전자들이 애기장대에는 존재하지 않고 벼에 특이적으로 존재하는 개화시기 조절 기작에 해당되기 때문으로 사료된다. 한편, 애기장대 FKF1 이 GIGANTEA (GI), CYCLING DOF FACTOR (CDF)와의 상호작용을 통해 *CO* 의 발현량을 조절한다고 알려져 있기

때문에 벼에서도 OsFKF1 과 GI, CDF 의 상동유전자들 (OsGI, OsCDF1) 간의 상호작용 여부를 확인해 본 결과, 애기장대와 마찬가지로 OsFKF1 이 OsGI, OsCDF1 과 상호작용함을 확인하였다.

이 논문을 통해 *OsFKF1* 의 개화촉진자 역할에 대한 새로운 모델을 제시하였으며 장일식물인 애기장대와 단일식물인 벼에서 *FKF1* 유전자 기능의 유사점과 차이점에 대해 논의하였다.

주요어: *OsFKF1*, 돌연변이체, 개화시기, *Ehd2*, *Ghd7*, *Ehd1*, 청색 빛 반응, 벼

학번: 2012-30303