

Functional Diversification of FD Transcription Factors in Rice, Components of Florigen Activation Complexes

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Florigen, a protein encoded by the *FLOWERING LOCUS T (FT)* in *Arabidopsis* and *Heading date 3a (Hd3a)* in rice, is the universal flowering hormone in plants. Florigen is transported from leaves to the shoot apical meristem and initiates floral evocation. In shoot apical cells, conserved cytoplasmic 14-3-3 proteins act as florigen receptors. A hexameric florigen activation complex (FAC) composed of Hd3a, 14-3-3 proteins, and OsFD1, a transcription factor, activates *OsMADS15*, a rice homolog of *Arabidopsis APETALA1*, leading to flowering. Because FD is a key component of the FAC, we characterized the FD gene family and their functions. Phylogenetic analysis of FD genes indicated that this family is divided into two groups: (i) canonical FD genes that are conserved among eudicots and non-Poaceae monocots; and (ii) Poaceae-specific FD genes that are organized into three subgroups: Poaceae FD1, FD2 and FD3. The Poaceae FD1 group shares a small sequence motif, T(A/V)LSLNS, with FDs of eudicots and non-Poaceae monocots. Overexpression of *OsFD2*, a member of the Poaceae FD2 group, produced smaller leaves with shorter plastochrons, suggesting that *OsFD2* controls leaf development. In vivo subcellular localization of Hd3a, 14-3-3 and *OsFD2* suggested that in contrast to *OsFD1*, *OsFD2* is restricted to the cytoplasm through its interaction with the cytoplasmic 14-3-3 proteins, and interaction of Hd3a with 14-3-3 facilitates nuclear translocation of the FAC containing *OsFD2*. These results suggest that FD function has diverged between *OsFD1* and *OsFD2*, but formation of a FAC is essential for their function.

Keywords: FD • Florigen activation complex (FAC) • Flowering • Hd3a • Plant transcription factor • Rice.

Abbreviations: BiFC, bimolecular fluorescence complementation; bZIP, basic leucine zipper; CDPK, calcium-dependent protein kinases; CFP, cyan fluorescent protein; EST, expressed sequence tag; FAC, florigen activation complex; *FT*, *FLOWERING LOCUS T*; GFP, green fluorescent protein; *Hd3a*, *Heading date 3a*; NES, nuclear exclusion signal; NLS, nuclear localization signal; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; SD, short day; WT, wild type.

Introduction

Florigen is a mobile flowering signal in plants, produced in leaves, and is transported through phloem tissue to the shoot apex where it initiates flowering (Zeevaart 2008, Matsoukas et al. 2012). The molecular nature of florigen has been revealed to be a protein encoded by *Heading date 3a (Hd3a)* in rice and its ortholog *FLOWERING LOCUS T (FT)* in *Arabidopsis*, both of which have a globular structure with a molecular mass of 22 kDa (Tsuji et al. 2011, Andres and Coupland 2012). Hd3a/FT protein moves through leaf phloem tissues, reaches the shoot apical meristem and triggers expression of floral meristem identity genes (Corbesier et al. 2007, Tamaki et al. 2007, Notaguchi et al. 2008, Yoo et al. 2012). Rice has two florigen genes, *Hd3a* and *RFT1*, and expression of both genes is controlled by the complex genetic network that integrates light signaling and circadian clock information (Itoh et al. 2010, Ishikawa et al. 2011, Matsubara et al. 2012, Saito et al. 2012). When expression of both genes is knocked down, the plant does not flower, suggesting that florigen is essential for flowering in rice (Komiya et al. 2008, Komiya et al. 2009). More recently, the molecular mechanism of florigen function in shoot apical cells was revealed in rice. Hd3a florigen interacts with 14-3-3 proteins in the cytoplasm and forms a ternary complex with *OsFD1* in the nucleus. The ternary complex is known as the florigen activation complex (FAC), which activates *OsMADS15*, a MADS-domain transcription factor that regulates flowering (Taoka et al. 2011, Kobayashi et al. 2012).

FD is a basic leucine zipper (bZIP)-containing transcription factor, first identified in *Arabidopsis*, and its loss-of-function mutants are late flowering (Abe et al. 2005, Wigge et al. 2005). The C-terminus of FD contains a short motif targeted by calcium-dependent protein kinases (CDPKs), and an alanine substitution of a serine/threonine residue within this motif disrupts FD function (Abe et al. 2005). This phosphorylation motif is required for interaction of 14-3-3 proteins with FD in rice, supporting the importance of the phosphorylation-dependent 14-3-3 protein interaction for FD function. The crystal structure of the FAC suggests that FD acts to tether the protein complex on the target promoter DNA (Taoka et al. 2011). The FD

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function seems to be conserved among higher plants. Maize DELAYED FLOWERING1 (DLF1) and wheat FDL2/FDL6, which are homologs of Arabidopsis and rice FDs, can interact with maize ZCN8 and wheat TaFT florigen proteins, respectively (Muszynski et al. 2006, Li and Dubcovsky 2008, Meng et al. 2011). Interestingly, all these FD homologs share 14-3-3 protein interaction motifs at their C-terminus, suggesting that participation of FD in the FAC is conserved.

The FAC model provides insight into the conserved nature of florigen function because the three proteins comprising the FAC are conserved among seed plants (Ferl et al. 2002, Karlgren et al. 2011, Taoka et al. 2011). This model also raises the possibility that transcription factors such as FD are exchangeable in the FAC because the transcription factors containing the 14-3-3 protein interaction motif can potentially interact with 14-3-3 proteins through a canonical mode of 14-3-3–phosphoserine interaction. Thus, an interesting hypothesis is that FAC function can be modulated depending on the transcription factor bound to the 14-3-3 protein in the FAC. FD homologs are potential candidates for testing this hypothesis; however, detailed characterization of FD homologs is limited for all species (Hanano and Goto 2011). Here, we describe the molecular analysis of the rice FD genes to answer three questions arising from the FAC model. (i) Are FD genes conserved among plants? (ii) Do FD homologs form FACs? (iii) Does the function of the FAC change depending on the FDs incorporated in the FAC? Our results suggest that OsFD2, a rice FD homolog, potentially forms a FAC and regulates leaf development. These results suggest the functional diversification of OsFD2 compared with the role of OsFD1 in flowering, and supports the hypothesis that FAC activity can be modified by FDs.

Results

Phylogenetic analysis of the FD gene family

We identified five new members of the FD gene family in rice, designated *OsFD2–OsFD6*, whose protein products share homology with the bZIP motif and C-terminal phosphorylation motif (SAP motif) of OsFD1 and other known FD proteins (**Supplementary Table S1**). The *OsFD2* gene (*Os06g0720900*) is triplicated in the genome, giving rise to *OsFD5* (*Os06g0724000*) and *OsFD6* (*Os06g0195000*), the latter encoding a mutant bZIP protein that has a truncated C-terminal region and is thus inferred to be a pseudogene.

To analyze the phylogenetic relationship of the FD gene family, we first identified 47 FD genes from diverse plant species by searching public databases of genomic sequences and expressed sequence tags (ESTs) (**Supplementary Table S1, Supplementary text**). Conserved amino acid motifs and their combinations in the predicted proteins were identified from these sequences using the SALAD database (Mihara et al. 2010) and by visual inspection. Finally we created a phylogenetic tree using the region spanning the bZIP motif to the C-terminal SAP motif. The structure of this phylogenetic tree matched

the classification of proteins according to the combinations of amino acid motifs (**Fig. 1**). From this phylogenetic analysis, we found three interesting features about the FD gene family. First, eudicot and non-Poaceae monocots share FD genes that contain a conserved motif arrangement, whereas genes encoding this type of FD are absent from Poaceae genomes. Eudicot FDs share a conserved motif arrangement comprised of motif A [(M/V)EEVWKDINLSSLHD], LSL [T(A/V)LSLN], bZIP and SAP [(S/T)LXR(X/S/T)(A/T)(P/Q)F] (**Fig. 1; Supplementary Figs. S1–S3**). The name of the SAP motif follows according to the definition from our previous characterization of OsFD1 (Taoka et al. 2011). This group of FD genes is found in two monocot species, banana (*Musa acuminata*) and date palm (*Phoenix dactylifera*), whose genome sequences were recently reported, suggesting conservation of FD (Paterson et al. 2009, Wei et al. 2009, Al-Dous et al. 2011, D'Hont et al. 2012). In contrast, this type of FD was not identified from Poaceae genomes. Genomic sequences of rice, maize, brachypodium and foxtail millet, and EST databases of barley and wheat do not contain this type of FD sequence (Tanaka et al. 2008, Paterson et al. 2009, Schnable et al. 2009, Wei et al. 2009, International Brachypodium Initiative 2010, Zhang et al. 2012). Secondly, three groups of Poaceae-specific FD genes were identified. The three groups were designated as Poaceae FD1, FD2 and FD3, respectively, according to their phylogenetic relationships and motif arrangements. The Poaceae FD1 group includes rice OsFD1 and maize DLF1, both of which were shown to participate in the activation of *AP1/FUL* homologs and promotion of flowering (Muszynski et al. 2006, Taoka et al. 2011). The characteristic feature of the motif combination in this group is the presence of motif 1 [MEDD(E/D)DMW(A/G)XTSSPSASPP], LSL, bZIP and SAP. The Poaceae FD2 group contains motif 2 [NYHHYQMAV(A/H)AA] and motif 3 [(L/M/V)SGCSSLFSIS(S/T)] with bZIP and a partially modified SAP motif. Motif 3 and SAP are partially shared with Poaceae FD3, suggesting that these two groups had the same evolutionary origin. Thirdly, the Poaceae FD1 group and eudicot/non-Poaceae monocot FDs share the LSL motif at the N-terminus of the sequences, although neither group shows strong similarity in the entire arrangement of motifs.

Organ-specific expression of rice FDs

The accumulation of *OsFD1*, *OsFD2* and *OsFD3* mRNAs in various organs was examined by reverse transcription–PCR (RT–PCR) (**Fig. 2**). All three transcripts accumulated in all of the organs tested: leaf blades, leaf sheaths, lamina joint regions connecting the leaf blade and leaf sheath, stems of vegetative phase plants, crown roots, tiller buds and shoot apices of vegetative phase plants. Lamina joints included ligules and auricles. *Hd3a* was specifically expressed in the leaf blade, with faint expression in lamina joints, tiller buds and shoot apices. *OsMADS15* was weakly expressed in shoot apices and stems.

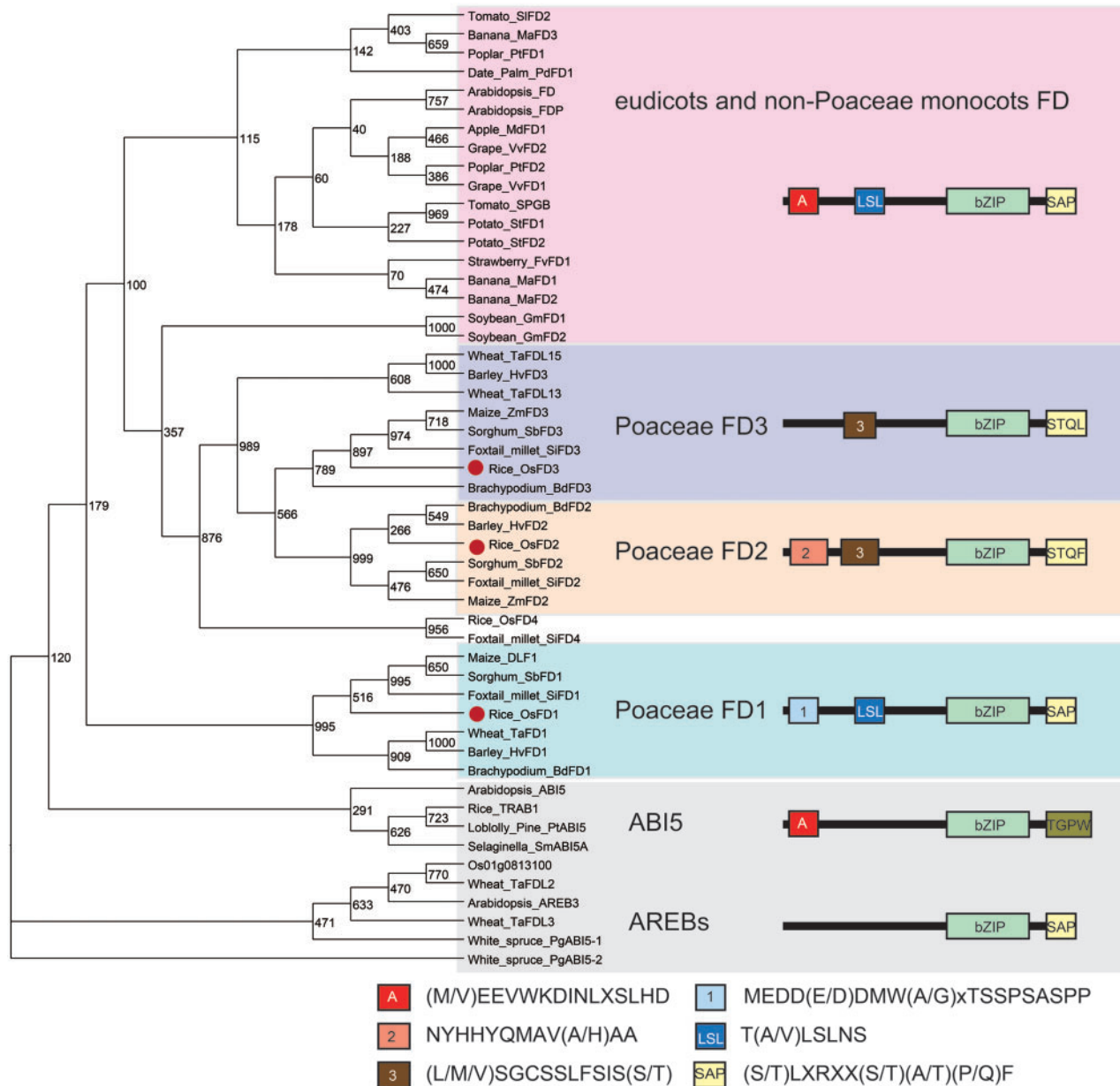


Fig. 1 Phylogenetic tree of predicted FD proteins and arrangements of amino acid motifs in each FD group. The phylogenetic tree was constructed with Neighbor-Joining methods using regions from bZIP to the C-terminus of the deduced amino acid sequences of FDs. AREBs/ABI5s are included as an outgroup. Red dots beside the protein name denote rice FD1, FD2 or FD3. The motif arrangement of FD proteins is schematically presented, with boxes and lines representing the conserved motifs identified in this study and other protein regions, respectively. The consensus amino acid sequences are presented below the phylogenetic tree.

Effect of *OsFD2* overexpression on plant development

To study the function of *OsFD2* in plants, we generated transgenic plants overexpressing *OsFD2* or the *OsFD2* S164A construct, in which an alanine residue was introduced into the putative phosphorylation site within the SAP motif to disrupt interaction with 14-3-3 proteins (Supplementary Fig. S4A; see Figs. 5 and 6) under the constitutive ubiquitin promoter (*pUbiq*). In the vegetative stage, no obvious phenotypes were

observed among three genotypes (Fig. 3A–C). Flowering time was not affected in these transgenic plants (Fig. 3D–F, J). In the reproductive stage, about 10% of the panicles that emerged from *pUbiq:OsFD2* plants showed a dense panicle phenotype, and S164A mutation suppressed this phenotype (Fig. 3G–I). These results suggest that *OsFD2* can delay the transition from inflorescence branch meristem to floral (or spikelet) meristem in the panicle branch, because the number of lateral organs in the inflorescence branch is determined by the timing of the

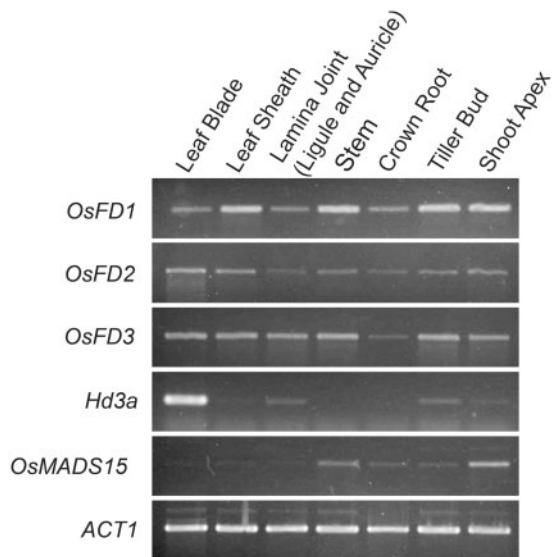
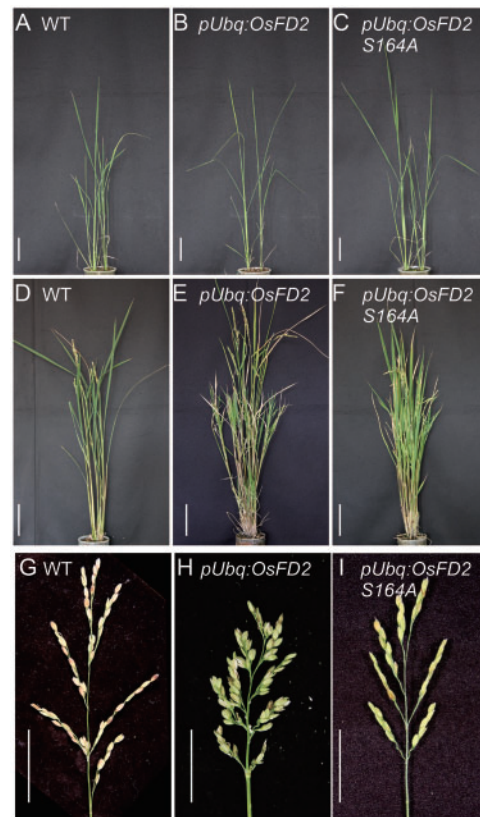


Fig. 2 Expression of rice *FD* genes in various organs. Rice *FD* gene family members are expressed in all organs tested, whereas *Hd3a* and *OsMADS15* are expressed specifically in leaf blades and shoot apices, respectively. The *ACT1* gene was used as the control for cDNA amplification.

transition from the inflorescence branch meristem to the spikelet meristem. The inflorescence meristem develops lateral spikelet meristems or branch meristems until the inflorescence meristem itself turned into the spikelet meristem (Nakagawa et al. 2002). The delay in this transition allows the longer period of lateral meristem development, and in consequence the more plentiful spikelets or secondary branches, to produce the dense panicle phenotype (Nakagawa et al. 2002).

At the reproductive stage, *pUbq:OsFD2* plants showed a striking phenotype in the leaves of branch shoots (Fig. 4A–D; Supplementary Fig. S4B, C). *pUbq:OsFD2* plants elongated abnormal branch shoots from several branch buds that are dormant in the wild-type (WT) plants (Fig. 4A; Supplementary Fig. S4B, C). The majority of these abnormal shoots never developed panicles and iterated leaf development, occasionally producing panicles similar to the main culm (Fig. 3H). These shoots can be detached, replanted in soil and grown for several weeks to develop leaves or, occasionally, panicles (Fig. 4B; Supplementary Fig. S4B, C). The abnormal shoots iterated the development of small leaves and short internodes (Fig. 4D–F). The rate of leaf initiation (plastochron) is shortened in *pUbq:OsFD2* plants compared with the WT shoots (Fig. 4G), contributing to the generation of many phytomers that can elongate at the internodes.

The SAP-like motif of *OsFD2* at its C-terminus is similar to the canonical mode-I type binding motif of 14-3-3 proteins (RXXSTQF in *OsFD2*, compared with the RXXSAPF in *OsFD1*), previously shown to be required for *OsFD1* to interact with 14-3-3 proteins in rice cells. The serine residue within the SAP motif is probably phosphorylated by unknown CDPK(s)



J

Genotype	Days to flowering	n	P
WT	54.6 ± 6.5	10	
<i>pUbq:FD2</i>	55.2 ± 5.1	10	0.82
<i>pUbq:FD2 S164A</i>	57.8 ± 9.6	6	0.58

Fig. 3 The effect of overexpressing *OsFD2* on flowering and inflorescence development. (A–C) Gross morphology of WT (A), *pUbq:OsFD2* (B) and *pUbq:OsFD2 S164A* (C) plants in the vegetative stage, showing no apparent difference among the three genotypes. (D–F) Gross morphology of WT (D), *pUbq:OsFD2* (E) and *pUbq:OsFD2 S164A* (F) plants in the reproductive stage. *pUbq:OsFD2* develops abnormal branch shoots with small leaves (E), whereas *pUbq:OsFD2 S164A* shows normal development (F). (G–I) Panicles of WT (G) and *pUbq:OsFD2* (H). *pUbq:OsFD2* produces more spikelets to form a dense panicle architecture (H). Scale bars are 5 cm in (A, B, C, G and H) and 10 cm in (D, E and F). (J) Flowering times of transgenic plants of the T₀ generation under SD conditions. WT indicates plants regenerated from non-transformed calli. Statistical significance compared with the WT was calculated using Student's *t*-test.

(Abe et al. 2005), and this phosphorylation is essential for the recognition of *OsFD1* by 14-3-3 proteins (Taoka et al. 2011). Consistent with the role of the SAP motif in *OsFD1* function, an alanine substitution in S164, the putative phosphorylation site within the SAP motif of *OsFD2*, attenuated the interaction of *OsFD2* with a 14-3-3 isoform GF14b in rice cells and yeast (see Figs. 5, 6; Supplementary Fig. S5). None of the typical leaf phenotypes observed in *pUbq:OsFD2* WT plants was observed in *pUbq:OsFD2 S164A* plants, indicating that the

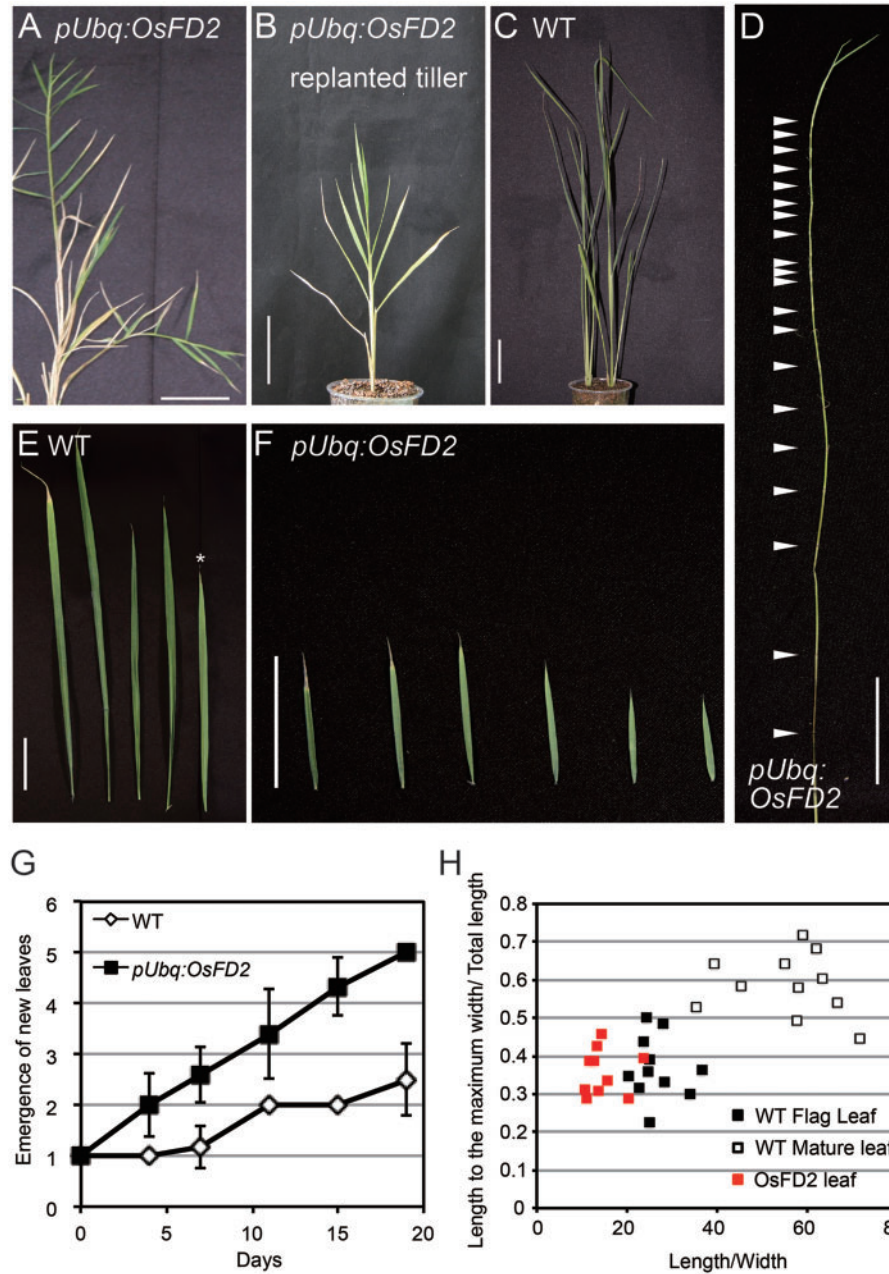


Fig. 4 The effect of overexpressing *OsFD2* on leaf development. (A) Branch shoots developing small leaves grew out from nodes on the elongating stem internodes in *pUbq:OsFD2* plants at the late reproductive stage. (B) A branch shoot detached from a *pUbq:OsFD2* plant replanted in soil. (C) WT plants transplanted at the same timing of replantation of the *pUbq:OsFD2* branch shoot in (B). (D) Stem of a growing branch shoot from *pUbq:OsFD2*. Reiteration of leaf development and internode elongation produced numerous nodes (arrowheads). (E and F) Leaves of a WT plant (E) and *pUbq:OsFD2* branch shoot (F). The asterisk indicates a flag leaf, the last leaf that develops before flowering. Scale bars in (A–F) = 5 cm. (G) Emergence of new leaves from tillers of WT (open diamonds) and branch shoots of *pUbq:OsFD2* plants (filled squares). Leaf number was counted after 90 d since transplantation, when the *pUbq:OsFD2* plants produced abnormal shoots. (H) A comparison of leaf morphology among flag leaves (filled squares) and mature leaves (open squares) of WT plants and the small leaves that developed in the branch shoots of *pUbq:OsFD2* plants (red squares). The x-axis indicates the length/width ratio and the y-axis indicates the ratio of the positions at which the leaves reached the maximum width, to the total length of the leaves.

OsFD2 C-terminal serine residue in the SAP motif was required for *OsFD2* function. These data suggest that the interaction of *OsFD2* with 14-3-3 proteins plays a role in *OsFD2* function in leaf development (**Supplementary Fig. S4D, E**).

We next characterized the small leaves produced by *pUbq:OsFD2* plants. In rice, the last leaf produced before panicle formation is called the flag leaf, and its shape is different from that of the other leaves when evaluated by the length/width

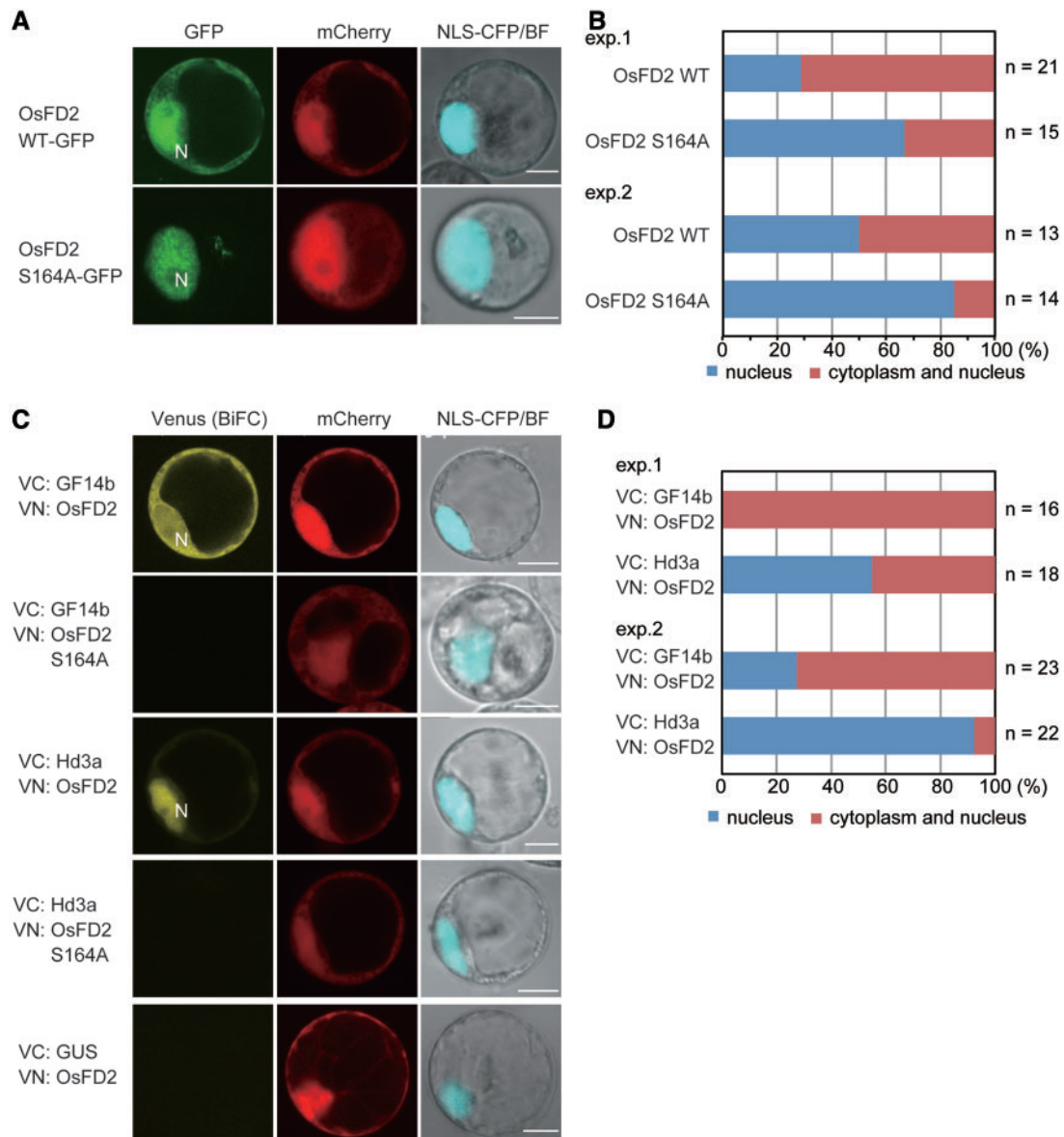


Fig. 5 Subcellular localization of OsFD2 and interaction among OsFD2, GF14b and Hd3a in rice cells. (A) Confocal images of cells expressing GFP–OsFD2 and GFP–OsFD2 S164A. Nuclear marker proteins (NLS–CFP) and mCherry protein were co-expressed. (B) Quantification of the subcellular localization of GFP–OsFD2 and GFP–OsFD2 S164A. (C) BiFC assays showing interactions of GF14b–OsFD2, GF14b–OsFD2 S164A, Hd3a–OsFD2 and Hd3a–OsFD2 S164A. Venus fluorescence in cells expressing the indicated proteins tagged with the N- or C-terminal halves of Venus is shown. Nuclear marker proteins (NLS–CFP and/or mCherry protein) were co-expressed. (D) Quantification of subcellular localization of the BiFC signal arose from interactions of GF14b–OsFD2 and Hd3a–OsFD2.

ratio and the position where the leaf reaches its maximum width (Fig. 4H). Because the abnormal leaves of *pUbiq:OsFD2* plants were produced at and after flag leaf development, we measured leaf morphology parameters for the abnormal leaves and compared them with those for the WT flag leaves and mature leaves. We found that the morphology of *pUbiq:OsFD2* leaves was more similar to that of flag leaves than to that of mature leaves (Fig. 4H), suggesting that the abnormal leaves share characteristics with flag leaves. These results suggest that OsFD2 controls leaf development.

Although we tried to produce OsFD2 suppression lines by RNA interference (RNAi) and artificial microRNA methods, we were not able to obtain transgenic plants with significant reductions in OsFD2 expression among >50 independent transgenic plants (data not shown).

Subcellular localization and in vivo interaction of Hd3a, 14-3-3 and OsFD2

Previous work indicated that OsFD1 accumulated predominantly in the nuclei of rice cells (Taoka *et al.* 2011). In contrast,

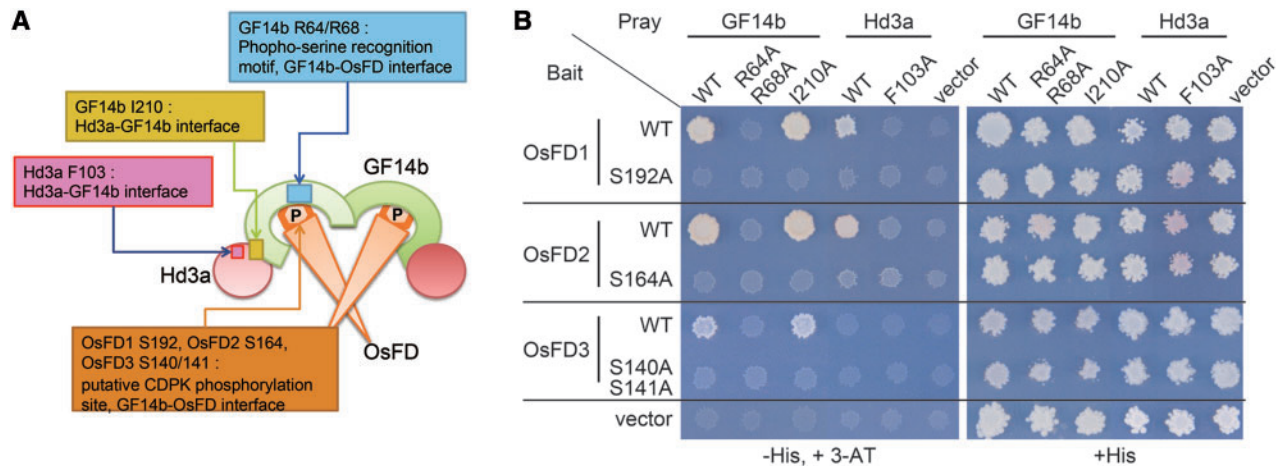


Fig. 6 Yeast two-hybrid assays. (A) A model for the FAC composed of Hd3a–GF14–OsFD, highlighting the locations of residues critical for protein–protein interactions. **P** represents phosphorylation at the SAP motif of OsFDs. (B) Yeast two-hybrid assay of interactions between OsFD proteins and GF14b or Hd3a. The effects of alanine substitutions for the amino acids essential for the formation of FAC were examined.

when the green fluorescent protein (GFP)–OsFD2 fusion protein was expressed in rice cells, clear GFP fluorescence was observed in nuclei and the cytoplasm (Fig. 5A, B). Because some transcription factors are anchored in the cytoplasm through 14-3-3 protein binding (Igarashi et al. 2001, Ishida et al. 2004, Bai et al. 2007, Gampala et al. 2007, Wang et al. 2011), and a 14-3-3 protein recognition motif within the SAP motif of OsFD2 was present (Fig. 1), we hypothesized that accumulation of OsFD2 in the cytoplasm was changed by its interaction with 14-3-3 proteins. To test this hypothesis, we introduced a construct containing the S164A mutation to disrupt interaction between OsFD2 and its corresponding 14-3-3 protein (see Figs. 5C, 6; Supplementary Fig. S5B). GFP–OsFD2 S164A was localized exclusively in nuclei (Fig. 5A), and the ratio of nuclear localization was much higher with OsFD2 S164A than in OsFD2 WT (Fig. 5B), suggesting that OsFD2 was excluded from nuclei through 14-3-3 protein binding. This result was in contrast to OsFD1 that normally accumulates in nuclei. The alanine substitution for the serine residue within the SAP motif of OsFD1 had no effect on its nuclear accumulation (see Discussion) (Taoka et al. 2011).

Next, the interaction between OsFD2 and a 14-3-3 protein (GF14b) was monitored by bimolecular fluorescent complementation (BiFC) assays (Fig. 5C). When constructs encoding N-terminal or C-terminal halves of Venus (VN or VC, respectively) were used to tag GF14b and OsFD2 and were expressed in rice cells, a Venus signal was detected mainly in the cytoplasm and very weakly in nuclei (Fig. 5C, D; Supplementary Fig. S5). This result is consistent with the hypothesis that 14-3-3 proteins bind OsFD2 and anchor it in the cytoplasm. The OsFD2 S164A mutant could not interact with a 14-3-3 protein (GF14b) *in vivo*, indicating the importance of the SAP motif for 14-3-3–FD interaction (Fig. 5C; Supplementary Fig. S5B). We then tested the interaction of Hd3a and OsFD2, which is possibly mediated by an endogenous 14-3-3 protein to form the

FAC (FAC-OsFD2). Interestingly, the Hd3a–OsFD2 BiFC signal was mainly detected in nuclei and very weakly in the cytoplasm, whereas the GF14b–OsFD2 interaction was predominantly detected in the cytoplasm (Fig. 5C, D). OsFD2 S164A could not interact with Hd3a (Fig. 5C; Supplementary Fig. S5C), indicating the 14-3-3 binding to OsFD2 is essential for the interaction of OsFD2 with Hd3a. Collectively, these results suggest that OsFD2 can potentially form a FAC in rice cells. Furthermore, FAC-OsFD2 may be a nuclear–cytoplasmic shuttling complex, and its localization may be controlled by 14-3-3 protein and Hd3a.

Protein interactions among Hd3a, 14-3-3 protein and OsFDs

To test for the formation of a FAC containing OsFD2, we analyzed interactions among Hd3a, 14-3-3 protein and OsFD2 by yeast two-hybrid analysis. The outline of protein–protein interactions among the three proteins of the FAC is shown in Fig. 6A, taking Hd3a, GF14b (a 14-3-3 protein) and OsFD1 as an example (Taoka et al. 2011). The FAC is a hetero-hexameric complex composed of two molecules each of Hd3a, 14-3-3 protein and OsFD1. Two Hd3a proteins cover both sides of a 14-3-3 protein dimer, and the OsFD1 dimer is located at the center of the complex through the interaction with the 14-3-3 protein's phosphoserine-binding pocket.

The 14-3-3 protein has a phosphoserine-binding pocket (Fig. 6A, blue box in the center of GF14b) that recognizes the phosphoserine in R/K-X-X-pS/pT-X-P, and the phosphorylated form of the SAP motif of OsFD1 is inserted into this pocket (Fig. 6A, orange hexagon labeled as **P** at the center of the complex). GF14b R64 and R68 contribute to the structure of the phosphoserine-binding pocket, and R64 forms a hydrogen bond with the phosphorylated S192 in the OsFD1 SAP motif. Thus, GF14b R64/R68 and OsFD1 S192 are essential for GF14b–OsFD1 interaction. Alanine substitutions of GF14b

R64/R68 or OsFD1 S192 abolished GF14b–OsFD1 interaction (Fig. 6B). Hd3a does not contact OsFD1 directly, and these two proteins interact with each other through interaction with the 14-3-3 protein.

The 14-3-3 protein interacts with Hd3a through the wide surface of the C-terminal region of the 14-3-3 protein (Taoka *et al.* 2011) (Fig. 6A, magenta and yellow boxes inside Hd3a and GF14b, respectively), which is composed of a hydrophobic cavity and an acidic loop on the surface of GF14b. GF14b I210 is located within this hydrophobic cavity and interacts with the hydrophobic side chain of Hd3a F103, indicating that both GF14b I210 and Hd3a F103 are essential for Hd3a–GF14b interaction (Taoka *et al.* 2011) (Fig. 6A, magenta and yellow boxes inside Hd3a and GF14b, respectively). On the other hand, GF14b I210 is dispensable for interaction of GF14b with OsFD1 because GF14b I210 is distantly located from the site of interaction with OsFD1 (Fig. 6A, compare yellow and blue boxes in GF14b). Thus, the GF14b I210A mutation specifically disrupts the interaction with Hd3a, but does not affect the interaction with OsFD1 (Fig. 6B, upper right panel). The 14-3-3 protein bridges the interaction between Hd3a and OsFD1; thus Hd3a F103A lost its ability to interact with the 14-3-3 protein and, in consequence, with OsFD1 (Fig. 6B) (Taoka *et al.* 2011). OsFD1 S192A also disrupts the interaction with the 14-3-3 protein, and, consequently, with Hd3a (Fig. 6B) (Taoka *et al.* 2011). In our yeast two-hybrid assays, Hd3a and OsFD1 interaction can be bridged by endogenous 14-3-3 proteins in yeast cells since yeast 14-3-3 proteins have conserved the structural requirements for Hd3a–14-3-3 and 14-3-3–OsFD1 interactions (Taoka *et al.* 2011).

The above information indicates that the yeast two-hybrid assay using mutations at essential amino acids can be used to examine whether OsFD2 and OsFD3 can form a FAC (Fig. 6B). OsFD2 interacted with GF14b, and alanine substitutions of GF14b R64/R68 abolished GF14b–OsFD2 interaction, suggesting that this interaction is phosphoserine dependent (Fig. 6B). Consistent with this result, an alanine substitution in OsFD2 S164, located at the putative phosphorylation site in the OsFD2 C-terminal SAP-like motif, abolished interaction between OsFD2 and GF14b. On the other hand, the GF14b I210A mutation, known to disrupt specifically the interaction of GF14b with Hd3a, did not affect the interaction of GF14b with OsFD2. This finding suggests that GF14b interacts with Hd3a and OsFD2 via distinct regions, similar to the model for OsFD1 interaction. Next, we performed yeast two-hybrid assays using Hd3a and OsFD2. OsFD2 interacted with Hd3a and, when the Hd3a–14-3-3 interaction was disrupted by the Hd3a F103A mutation (Taoka *et al.* 2011), Hd3a lost its ability to interact with OsFD2. The OsFD2 S164A mutation disrupted the interaction of OsFD2 with GF14b and, in consequence, the interaction with Hd3a (Fig. 6B). These data suggest that OsFD2 can form a FAC with Hd3a and GF14b in a similar manner to OsFD1 (Taoka *et al.* 2011).

In contrast to OsFD2, we could not detect any interaction between Hd3a and OsFD3, whereas we observed an interaction

between GF14b and OsFD3 by the canonical 14-3-3 protein and phosphoserine binding system. These results suggest that there may be technical difficulties in detecting interactions in yeast, or the presence of an unknown mechanism inhibiting FAC formation by OsFD3 in yeast cells. To examine the function of OsFD3, we generated *OsFD3* RNAi plants; however, these plants showed no changes in morphology or flowering time (Supplementary Fig. S6).

Discussion

Evolution of FD in plants

Phylogenetic analysis of FD in plants suggested unique evolutionary aspects of FD genes in the Poaceae family (Fig. 1). Three groups of Poaceae-specific FD genes were identified, but canonical FD genes are absent from the Poaceae genome. Although the entire sequence context is not strongly conserved, at least two deduced proteins, OsFD1 and OsFD2, can form FACs (Fig. 6), suggesting conservation of FAC formation in different groups of FD proteins. We found that the small LSL motif was well conserved between the Poaceae-specific OsFD1 group and the eudicot/non-Poaceae monocot FD group (Fig. 1). Interestingly, both groups contribute to the promotion of flowering; thus, the presence of the LSL motif may define the FD proteins capable of activating the *AP1/FUL* clade of MADS box genes for flowering (Abe *et al.* 2005, Wigge *et al.* 2005, Li and Dubcovsky 2008, Taoka *et al.* 2011).

We found FD genes from diverse species of angiosperm plants, but not from the moss (bryophyte) *Physcomitrella patens* (Rensing *et al.* 2008, Hauser *et al.* 2011) or the spike moss (lycophyte, basal vascular plant) *Selaginella moellendorffii* (Banks *et al.* 2011), suggesting that FACs containing FD proteins may not have occurred before the emergence of seed plants and evolved after the emergence of angiosperms. Although searches of the available databases did not detect FD genes in gymnosperms, further genome sequence analysis may help our understanding of the evolution of FACs in land plants.

Diversification of FD functions in rice

Phylogenetic analysis of FDs in plants suggested that the FD gene family is divided into two groups, the eudicot/non-Poaceae FDs and the Poaceae-specific FDs (Fig. 1). We studied the functions of OsFD1 and OsFD2, two members of the Poaceae-specific FDs in rice, and found that the function of FD has diverged between these homologs. OsFD1 functions in the activation of *AP1/FUL* genes and promotion of flowering (Taoka *et al.* 2011), and OsFD2 functions in leaf development (Figs. 3, 4). Although the precise mechanism for the functional difference between OsFD1 and OsFD2 is unclear, modifications of the motif arrangement and DNA-binding domains could contribute to these differences (Supplementary Fig. S7). Two amino acids in the basic regions of OsFD1 and OsFD2 are different, and this slight difference may change the target genes that are regulated by these transcription factors.

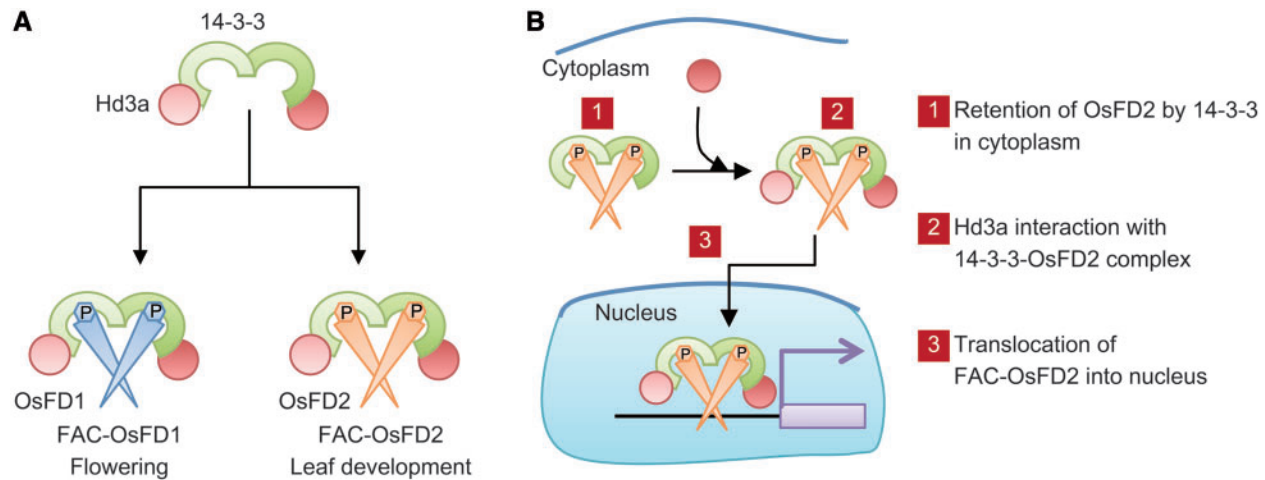


Fig. 7 Model of FAC function converted by OsFD1 or OsFD2 (A) and the mechanism of FAC formation including OsFD2 (B). (A) The Hd3a–14-3-3 protein subcomplex serves as the basic component of the FAC. When OsFD1 enters the complex, the resultant FAC-OsFD1 promotes flowering. OsFD2 is the proposed component of FAC to form FAC-OsFD2 that putatively controls leaf development. In this model, the function of FAC can be converted, depending on the function of the OsFDs recruited into the FAC. (B) A part of the OsFD2 protein is localized in the cytoplasm by the interaction with 14-3-3 proteins. When Hd3a interacts with the 14-3-3–OsFD2 complex in the cytoplasm to form a FAC, the complex enters the nucleus to regulate gene expression.

OsMADS15 is not the target of OsFD2, because OsFD2 and Hd3a co-expression could not induce OsMADS15 expression in our transient assay using protoplasts (data not shown).

The recent discovery of FAC explains the molecular mechanism by which florigen Hd3a, 14-3-3 protein and OsFD1 promote flowering through activation of downstream target genes (Taoka et al. 2011). Hd3a interacts with 14-3-3 proteins and OsFD1 to form the ternary transcriptional complex, FAC-OsFD1, to activate OsMADS15 expression. Here, we show that OsFD2 can form a FAC with Hd3a and the 14-3-3 isoform GF14b (Figs. 5, 6). The OsFD2 S164A mutation disrupted the interaction of OsFD2 with GF14b and Hd3a, and abolished the function of OsFD2 in leaf development. These data suggest that OsFD2 is a potential component of the FAC, and FAC formation is essential for OsFD2 function to control leaf development in rice.

Our model of FAC that includes OsFD1 and OsFD2 suggests that FAC function is modified depending on the transcription factors recruited through the 14-3-3 protein (Fig. 7A). In this model, the Hd3a–14-3-3 subcomplex constitutes a common backbone of the FACs, and transcription factors interacting with 14-3-3 proteins determine the function of the FAC. Our results suggest that if OsFD1 is recruited, FAC-OsFD1 acts in the promotion of flowering, and if OsFD2 is recruited, FAC-OsFD2 functions in rice leaf development (Figs. 3, 4, 7A). Our model offers a molecular basis for the participation of florigen in multiple developmental processes other than flowering, including stomatal opening in Arabidopsis (Kinoshita et al. 2011), panicle morphology in rice (Endo-Higashi and Izawa 2011), leaf morphology and inflorescence architecture in Arabidopsis and tomato (Teper-Bamnolker and Samach 2005, Lifschitz et al. 2006, Krieger et al. 2010, Hiraoka et al. 2013), tuber formation in

potato (Navarro et al. 2011) and growth cessation in tree species (Bohlenius et al. 2006, Hsu et al. 2011). In potato tuberization, for example, SP6A, a Hd3a homolog, moves from the leaves to the stolon to initiate tuber formation. SP6A may form a FAC with tuberization-specific transcription factors to activate downstream gene expression in potato (Navarro et al. 2011). Exploring the participation of FACs in developmental processes other than flowering is a promising direction for further study of the multifunctionality of florigen.

FAC formation and nuclear translocation

Subcellular localization analysis and *in vivo* interaction studies suggested a novel mechanism for FAC-OsFD2 formation in cell nuclei (Fig. 7B). OsFD2 can shuttle between the cytoplasm and the nucleus, and 14-3-3 proteins are involved in this shuttling by facilitating the cytoplasmic translocation of OsFD2 (Fig. 5A, B). A mutant version of OsFD2 that had lost its ability to interact with a 14-3-3 protein, as a result of alanine substitution of OsFD2 S164 in the conserved 14-3-3 binding motif, localized exclusively to nuclei. However, the nuclear translocation of OsFD2 is not sufficient for OsFD2 function because overexpression of this mutant version in plants did not show any phenotype (Figs. 3, 4; Supplementary Fig. S4), suggesting that FAC formation is necessary for OsFD2 function. The BiFC experiments indicated interaction between 14-3-3 protein and OsFD2 in the cytoplasm, whereas interaction between Hd3a and OsFD2, which is mediated by 14-3-3 protein, occurred in the nuclei (Fig. 5C, D). This finding suggested that the presence of Hd3a in the FAC changed its subcellular localization. In our model, OsFD2 is localized in the cytoplasm through its binding with 14-3-3 protein, and Hd3a interacts with the C-terminal region of a 14-3-3 protein to form a FAC. This interaction

initiates the nuclear translocation of the FAC into the nucleus (Fig. 7B).

The molecular mechanism for this nuclear translocation is an open question, but inhibition of the nuclear exclusion signal (NES) within the 14-3-3 protein by Hd3a could contribute to this mechanism. The crystal structure of FACs indicated that the interface for Hd3a and 14-3-3 interaction is located in the region that overlaps with the NES region within the 14-3-3 protein, and Hd3a binding on the 14-3-3 protein covers the entire NES (Taoka *et al.* 2011). This binding may inhibit the interaction of the evolutionarily conserved nuclear exclusion machinery including exportin proteins onto the 14-3-3 NES (Rittinger *et al.* 1999). Subsequently, the activity of the OsFD2 nuclear localization signal (NLS) can cause the translocation of the entire FAC complex into the nucleus. Detailed observations about the cellular processes during FAC formation will be valuable for understanding this mechanism. In contrast, GFP–OsFD1 localized to nuclei exclusively in the presence of 14-3-3 protein interaction (Taoka *et al.* 2011), suggesting that different FACs may behave differently in their formation and nuclear translocation. The precise mechanisms generating these differences remain unknown, but differences in important amino acids in the NLS in OsFD1 and OsFD2 may contribute to these differences (Supplementary Fig. S7). OsFD1 and OsFD2 contain a bipartite NLS in the basic region of their bZIP motifs, but the more N-terminal region of the bipartite NLS contains different amino acids: RRKR in OsFD1 and RTIR in OsFD2. This slight difference in OsFD2 may affect nuclear accumulation because the corresponding region of the NLS in opaque2 (O2), the bZIP transcription factor of maize, is essential for its NLS activity (Varagona and Raikhel 1994). RRKR in WT O2 accumulates in nuclei, whereas a mutated sequence RTNR, which is similar to the OsFD2 NLS, accumulates both in the cytoplasm and in the nucleus (Varagona and Raikhel 1994).

Materials and Methods

Plant materials and growth conditions

Rice (*Oryza sativa* L. subspecies *japonica*) variety Norin 8 was used as the WT. *pUbiq:OsFD1* transgenic rice plants were described previously (Taoka *et al.* 2011). *pUbiq:OsFD1* Δ L5L, *pUbiq:OsFD2* and *pUbiq:OsFD2 S164A* rice plants were generated using *Agrobacterium*-mediated transformation of rice calli, as previously described (Hiei *et al.* 1994). Hygromycin-resistant plants were regenerated from the transformed callus. Transgene integration was further confirmed by PCR amplification of the hygromycin phosphotransferase gene in genomic DNA extracted from regenerated plants. Plants were grown in climate chambers at 70% humidity, under short-day (SD) conditions with daily cycles of 10 h of light at 30°C and 14 h of dark at 25°C. Light was provided by fluorescent white lights. Flowering time was measured as the number of days to the heading stage after T₀ transgenic plants were transferred to SD

conditions. For flowering time measurement, the tillers were removed to save space (Ohnishi *et al.* 2011). Rice suspension-cultured cells were maintained as described previously (Taoka *et al.* 2011). The leaf morphology and plastochron were measured at 90 d after transplantation when the majority of the *pUbiq:OsFD2* plants showed characteristic leaf phenotypes on their abnormally outgrowing branch shoots.

Phylogenetic analysis

Databases listed in Supplementary Table S1 were searched for DNA sequences encoding FD proteins using the Arabidopsis and rice FDs as the queries.

Predicted FD amino acid sequences were used for phylogenetic and motif analyses. Conserved motifs and their arrangements were extracted from FDs with interactive SALAD analysis from the SALAD database (Mihara *et al.* 2010), with the parameters of 10 for 'maximum number of motifs to find' and 1e-2 for 'expect threshold'. The extracted motifs were then manually curated and aligned with the T-Coffee program and displayed with Boxshade software (Di Tommaso *et al.* 2011). Phylogenetic trees using the regions spanning bZIP to the C-terminal SAP motif were constructed based on the alignment from CLUSTALW using the Neighbor-Joining method. We obtained a phylogenetic tree with a similar shape from the T-Coffee program using the same bZIP-SAP region and interactive SALAD analysis using the entire motif architecture of FDs.

Protoplast transformation

Transformation of rice Oc protoplasts was performed as described previously (Taoka *et al.* 2011, Kim *et al.* 2012). For transient expression analysis, 8 μ g of *Hd3a* expression vectors and 16 μ g of *OsFD1* expression vectors were introduced into 500 μ l of a protoplast suspension at a concentration of 2×10^7 protoplasts ml⁻¹ by the polyethylene glycol (PEG)-mediated transformation method. After 16 or 48 h incubations at 30°C, the protoplast suspension was centrifuged and the cell pellet was frozen at -80°C for RNA extraction.

RNA extraction and real-time RT-PCR analysis

Total RNA from protoplasts was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 0.1–1.0 μ g of total RNA, using a 21 nucleotide oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). cDNA (1 μ l) was used for quantitative analysis of gene expression using SYBR Green PCR master mix (Life Technologies). Data were collected using the StepOnePlus sequence detection system in accordance with the manufacturer's instruction manual. The sequences of primers used in this study are listed in Supplementary Table S2.

Subcellular localization and bimolecular fluorescent complementation

The OsFD2 and OsFD2 S164A coding regions were cloned into fluorescent protein expression vectors or BiFC vectors and

purified using the Purelink Plasmid Midiprep kit (Invitrogen). We co-transformed 5 µg of GFP–OsFD2 or GFP–OsFD2 S164A expression plasmids with both 5 µg of mCherry and 10 µg of NLS–cyan fluorescent protein (CFP) expression plasmids. For BiFC experiments, 5 µg of VN- or VC-tagged protein expression vector was co-transformed with both 5 µg mCherry and 10 µg of NLS–CFP expression plasmids as markers. Protein–protein interactions from BiFC experiments were quantified as described previously, with some modifications (Taoka et al. 2011). Briefly, we calculated the ratio of Venus/mCherry from each of cells in the BiFC experiment and could recognize reliable BiFC signals in cells showing Venus/mCherry ratios >0.83 (experiment 1), 0.53 (experiment 2) for the OsFD2–GF14b and OsFD2–Hd3a interaction, 0.37 (experiment 3) and 0.38 (experiment 4) for the OsFD2–GF14b and OsFD2 S164A–GF14b interaction, and 0.29 (experiment 5) and 0.34 (experiment 6) for the OsFD2–Hd3a and OsFD2 S164A–Hd3a interaction. The number of cells showing ratios exceeding these values was recorded.

We examined the degree of nuclear accumulation of GFP fusion proteins and the BiFC signal arising from GFP–OsFD2, OsFD2–GF14b and OsFD2–Hd3a. First, we measured the fluorescence intensities of Venus and mCherry in the nuclei and cytoplasm of transformed cells. Next, we calculated values for (Venus in nucleus/mCherry in nucleus)/(Venus in cytoplasm/mCherry in cytoplasm) that indicates a measure of nuclear accumulation of Venus. Finally, we compared these values and the corresponding confocal images from each cell to determine the nuclear accumulation of the fluorescent proteins.

Yeast two-hybrid assay

Gateway destination vectors pBTM116-GW and pVP16-GW were used to construct the bait and prey vectors by LR recombination reactions. Yeast cells were grown at 30°C for 5 d on SC medium without uracil, tryptophan, leucine and histidine, or containing added histidine or 1–10 mM 3-amino-1,2,4-triazole (3-AT). The concentration of 3-AT was determined by the bait–prey combination (Purwestri et al. 2009).

Supplementary data

Supplementary data are available at PCP online.

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References

- Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y. et al. (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* 309: 1052–1056.
- Al-Dous, E.K., George, B., Al-Mahmoud, M.E., Al-Jaber, M.Y., Wang, H., Salameh, Y.M. et al. (2011) De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). *Nat. Biotechnol.* 29: 521–527.
- Andres, F. and Coupland, G. (2012) The genetic basis of flowering responses to seasonal cues. *Nat. Rev. Genet.* 13: 627–639.
- Bai, M.Y., Zhang, L.Y., Gampala, S.S., Zhu, S.W., Song, W.Y., Chong, K. et al. (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. *Proc. Natl Acad. Sci. USA* 104: 13839–13844.
- Banks, J.A., Nishiyama, T., Hasebe, M., Bowman, J.L., Gribskov, M., dePamphilis, C. et al. (2011) The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. *Science* 332: 960–963.
- Bohlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A.M., Jansson, S., Strauss, S.H. et al. (2006) CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science* 312: 1040–1043.
- Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I. et al. (2007) FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science* 316: 1030–1033.
- D'Hont, A., Denoeud, F., Aury, J.M., Baurens, F.C., Carreel, F., Garsmeur, O. et al. (2012) The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 488: 213–217.
- Di Tommaso, P., Moretti, S., Xenarios, I., Orobítg, M., Montanyola, A., Chang, J.M. et al. (2011) T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res.* 39: W13–W17.
- Endo-Higashi, N. and Izawa, T. (2011) Flowering time genes Heading date 1 and Early heading date 1 together control panicle development in rice. *Plant Cell Physiol.* 52: 1083–1094.
- Ferl, R.J., Manak, M.S. and Reyes, M.F. (2002) The 14-3-3s. *Genome Biol.* 3: REVIEWS3010.
- Gampala, S.S., Kim, T.W., He, J.X., Tang, W., Deng, Z., Bai, M.Y. et al. (2007) An essential role for 14-3-3 proteins in brassinosteroid signal transduction in Arabidopsis. *Dev. Cell* 13: 177–189.
- Hanano, S. and Goto, K. (2011) Arabidopsis TERMINAL FLOWER1 is involved in the regulation of flowering time and inflorescence development through transcriptional repression. *Plant Cell* 23: 3172–3184.
- Hauser, F., Waadt, R. and Schroeder, J.I. (2011) Evolution of abscisic acid synthesis and signaling mechanisms. *Curr. Biol.* 21: R346–R355.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6: 271–282.

- Hiraoka, K., Yamaguchi, A., Abe, M. and Araki, T. (2013) The florigen genes FT and TSF modulate lateral shoot outgrowth in *Arabidopsis thaliana*. *Plant Cell Physiol.* 54: 352–368.
- Hsu, C.Y., Adams, J.P., Kim, H., No, K., Ma, C., Strauss, S.H. et al. (2011) FLOWERING LOCUS T duplication coordinates reproductive and vegetative growth in perennial poplar. *Proc. Natl Acad. Sci. USA* 108: 10756–10761.
- Igarashi, D., Ishida, S., Fukazawa, J. and Takahashi, Y. (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell* 13: 2483–2497.
- International Brachypodium Initiative. (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463: 763–768.
- Ishida, S., Fukazawa, J., Yuasa, T. and Takahashi, Y. (2004) Involvement of 14-3-3 signaling protein binding in the functional regulation of the transcriptional activator REPRESSION OF SHOOT GROWTH by gibberellins. *Plant Cell* 16: 2641–2651.
- Ishikawa, R., Aoki, M., Kurotani, K.-i., Yokoi, S., Shinomura, T., Takano, M. et al. (2011) Phytochrome B regulates Heading date 1 (Hd1)-mediated expression of rice florigen Hd3a and critical day length in rice. *Mol. Genet. Genomics* 285: 461–470.
- Itoh, H., Nonoue, Y., Yano, M. and Izawa, T. (2010) A pair of floral regulators sets critical day length for Hd3a florigen expression in rice. *Nat. Genet.* 42: 635–638.
- Karlgen, A., Gyllenstrand, N., Kallman, T., Sundstrom, J.F., Moore, D., Lascoux, M. et al. (2011) Evolution of the PEBP gene family in plants: functional diversification in seed plant evolution. *Plant Physiol.* 156: 1967–1977.
- Kim, S.H., Oikawa, T., Kyojuka, J., Wong, H.L., Umemura, K., Kishi-Kaboshi, M. et al. (2012) The bHLH Rac immunity1 (RAI1) is activated by OsRac1 via OsMAPK3 and OsMAPK6 in rice immunity. *Plant Cell Physiol.* 53: 740–754.
- Kinoshita, T., Ono, N., Hayashi, Y., Morimoto, S., Nakamura, S., Soda, M. et al. (2011) Flowering locus T regulates stomatal opening. *Curr. Biol.* 21: 1232–1238.
- Kobayashi, K., Yasuno, N., Sato, Y., Yoda, M., Yamazaki, R., Kimizu, M. et al. (2012) Inflorescence meristem identity in rice is specified by overlapping functions of three AP1/FUL-like MADS box genes and PAP2, a SEPALLATA MADS box gene. *Plant Cell* 24: 1848–1859.
- Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S. and Shimamoto, K. (2008) Hd3a and RFT1 are essential for flowering in rice. *Development* 135: 767–774.
- Komiya, R., Yokoi, S. and Shimamoto, K. (2009) A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice. *Development* 136: 3443–3450.
- Krieger, U., Lippman, Z.B. and Zamir, D. (2010) The flowering gene SINGLE FLOWER TRUSS drives heterosis for yield in tomato. *Nat. Genet.* 42: 459–463.
- Li, C.X. and Dubcovsky, J. (2008) Wheat FT protein regulates VRN1 transcription through interactions with FDL2. *Plant J.* 55: 543–554.
- Lifshitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z. et al. (2006) The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proc. Natl Acad. Sci. USA* 103: 6398–6403.
- Matsoukas, I.G., Massiah, A.J. and Thomas, B. (2012) Florigenic and antiflorigenic signaling in plants. *Plant Cell Physiol.* 53: 1827–1842.
- Matsubara, K., Ogiso-Tanaka, E., Hori, K., Ebana, K., Ando, T. and Yano, M. (2012) Natural variation in Hd17, a homolog of Arabidopsis ELF3 that is involved in rice photoperiodic flowering. *Plant Cell Physiol.* 53: 709–716.
- Meng, X., Muszynski, M.G. and Danilevskaia, O.N. (2011) The FT-Like ZCN8 gene functions as a floral activator and is involved in photoperiod sensitivity in maize. *Plant Cell* 23: 942–960.
- Mihara, M., Itoh, T. and Izawa, T. (2010) SALAD database: a motif-based database of protein annotations for plant comparative genomics. *Nucleic Acids Res.* 38: D835–D842.
- Muszynski, M.G., Dam, T., Li, B., Shirbroun, D.M., Hou, Z., Bruggemann, E. et al. (2006) delayed flowering1 encodes a basic leucine zipper protein that mediates floral inductive signals at the shoot apex in maize. *Plant Physiol.* 142: 1523–1536.
- Nakagawa, M., Shimamoto, K. and Kyojuka, J. (2002) Overexpression of RCN1 and RCN2, rice TERMINAL FLOWER 1/CENTRORADIALIS homologs, confers delay of phase transition and altered panicle morphology in rice. *Plant J.* 29: 743–750.
- Navarro, C., Abelenda, J.A., Cruz-Oro, E., Cuellar, C.A., Tamaki, S., Silva, J. et al. (2011) Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature* 478: 119–122.
- Notaguchi, M., Abe, M., Kimura, T., Daimon, Y., Kobayashi, T., Yamaguchi, A. et al. (2008) Long-distance, graft-transmissible action of Arabidopsis FLOWERING LOCUS T protein to promote flowering. *Plant Cell Physiol.* 49: 1645–1658.
- Ohnishi, T., Yoshino, M., Yamakawa, H. and Kinoshita, T. (2011) The biotron breeding system: a rapid and reliable procedure for genetic studies and breeding in rice. *Plant Cell Physiol.* 52: 1249–1257.
- Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H. et al. (2009) The Sorghum bicolor genome and the diversification of grasses. *Nature* 457: 551–556.
- Purwestri, Y.A., Ogaki, Y., Tamaki, S., Tsuji, H. and Shimamoto, K. (2009) The 14-3-3 protein GF14c acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a. *Plant Cell Physiol.* 50: 429–438.
- Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H. et al. (2008) The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. *Science* 319: 64–69.
- Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L.C., Smerdon, S.J. et al. (1999) Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol. Cell* 4: 153–166.
- Saito, H., Ogiso-Tanaka, E., Okumoto, Y., Yoshitake, Y., Izumi, H., Yokoo, T. et al. (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 Cell Physiol.* 53: 717–728.
- Schnable, P.S., Ware, D., Fulton, R.S., Stein, J.C., Wei, F., Pasternak, S. et al. (2009) The B73 maize genome: complexity, diversity, and dynamics. *Science* 326: 1112–1115.
- Tamaki, S., Matsuom, S., Wong, H.L., Yokoi, S. and Shimamoto, K. (2007) Hd3a protein is a mobile flowering signal in rice. *Science* 316: 1033–1036.
- Tanaka, T., Antonio, B.A., Kikuchi, S., Matsumoto, T., Nagamura, Y., Numa, H. et al. (2008) The Rice Annotation Project Database (RAP-DB): 2008 update. *Nucleic Acids Res.* 36: D1028–D1033.
- Taoka, K.-i., Ohki, I., Tsuji, H., Furuita, K., Hayashi, K., Yanase, T. et al. (2011) 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. *Nature* 476: 332–335.
- Teper-Bamnolker, P. and Samach, A. (2005) The flowering integrator FT regulates SEPALLATA3 and FRUITFULL accumulation in Arabidopsis leaves. *Plant Cell* 17: 2661–2675.

- Tsuji, H., Taoka, K.-i. and Shimamoto, K. (2011) Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation. *Curr. Opin. Plant Biol.* 14: 45–52.
- Varagona, M.J. and Raikhel, N.V. (1994) The basic domain in the bZIP regulatory protein Opaque2 serves two independent functions: DNA binding and nuclear localization. *Plant J.* 5: 207–214.
- Wang, H., Yang, C., Zhang, C., Wang, N., Lu, D., Wang, J. et al. (2011) Dual role of BK11 and 14-3-3s in brassinosteroid signaling to link receptor with transcription factors. *Dev. Cell* 21: 825–834.
- Wei, F., Zhang, J., Zhou, S., He, R., Schaeffer, M., Collura, K. et al. (2009) The physical and genetic framework of the maize B73 genome. *PLoS Genet.* 5: e1000715.
- Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U. et al. (2005) Integration of spatial and temporal information during floral induction in Arabidopsis. *Science* 309: 1056–1059.
- Yoo, S.J., Hong, S.M., Jung, H.S. and Ahn, J.H. (2012) The cotyledons produce sufficient FT protein to induce flowering: evidence from cotyledon micrografting in Arabidopsis. *Plant Cell Physiol.* 54: 119–128.
- Zeevaart, J.A. (2008) Leaf-produced floral signals. *Curr. Opin. Plant Biol.* 11: 541–547.
- Zhang, G., Liu, X., Quan, Z., Cheng, S., Xu, X., Pan, S. et al. (2012) Genome sequence of foxtail millet (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nat. Biotechnol.* 30: 549–554.

