OsFD4 promotes the rice floral transition via Florigen Activation Complex formation in the shoot apical meristem

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SUMMARY

- In rice, the florigens Heading Date 3a (Hd3a) and Rice Flowering Locus T 1 (RFT1), OsFD-like bZIP transcription factors, and Gf14 proteins assemble into Florigen Activation/Repressor Complexes (FACs/FRCs), which regulate transition to flowering in leaves and apical meristem. Only OsFD1 has been described as part of complexes promoting flowering at the meristem, and little is known about the role of other bZIP transcription factors, the combinatorial complexity of FAC formation, and their DNA binding properties.
- Here, we used mutant analysis, protein-protein interaction assays and DAP-sequencing coupled to *in silico* prediction of binding syntaxes, to study several bZIP proteins that assemble into FACs or FRCs.
- We identified OsFD4 as component of a FAC promoting flowering at the shoot apical meristem, downstream of *OsFD1*. The *osfd4* mutants are late flowering and delay expression of genes promoting inflorescence development. Protein–protein interactions indicate an extensive network of contacts between several bZIPs and Gf14 proteins. Finally, we identified genomic regions bound by bZIPs with promotive and repressive effects on flowering.
- We conclude that distinct bZIPs orchestrate floral induction at the meristem and that FAC formation is largely combinatorial. While binding to the same consensus motif, their DNA binding syntax is different, suggesting discriminatory functions.

Key words: bZIP, florigen activation complex, photoperiodic flowering, rice, shoot apical meristem.

INTRODUCTION

When external and internal conditions are favourable, plants switch their life cycle from the vegetative to the reproductive phase. This process is called floral transition and is determined by events taking place in both leaves and shoot apical meristem (SAM). In rice (*Oryza sativa*), transition to the reproductive phase is controlled by changes in the photoperiod and is accelerated when day length falls under a critical threshold. Because of this response, rice is classified as short day (SD) plant. Yet, it can flower also under non-inductive long day (LD) conditions, after a prolonged vegetative phase. As such, its photoperiodic response is facultative.

The molecular events that guide the floral transition start in the leaves when transcription of *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) takes place in response to inductive photoperiods. *Hd3a* and *RFT1* are homologs of Arabidopsis *FLOWERING LOCUS T* (*FT*) and members of the phosphatidyl ethanolamine-binding protein (PEBP) family (Kojima *et al.*, 2002; Komiya *et al.*, 2008, 2009). Similarly to *FT*, they are transcribed in companion cells of leaves; subsequently, their cognate proteins can move through the phloem to reach the SAM, where they promote expression of genes necessary to activate the inflorescence development program (Tamaki *et al.*, 2007; Komiya *et al.*, 2009). Analysis of RNA interference lines and CRISPR mutants demonstrated that a delay in flowering occurs upon downregulation of *Hd3a* or *RFT1* (Komiya et al., 2008, Liu et al., 2019). However, the phenotypic effects of reduced expression of *Hd3a* or *RFT1* depend on the photoperiod. Under SD, *Hd3a* RNAi lines delay flowering and double *RFT1-Hd3a* RNAi plants completely block it, indicating that both florigens can redundantly promote the floral transition (Komiya *et al.*, 2008; Tamaki *et al.*, 2015). Conversely, *RFT1* RNAi lines delay flowering under LD only, indicating that *RFT1* can promote flowering also under non-inductive photoperiods (Komiya *et al.*, 2009). This difference correlates with the expression of *Hd3a* and *RFT1* in leaves: when plants grow under SD conditions, both florigens are transcribed, whereas growth under non-inductive LD conditions promotes *RFT1* expression only (Komiya *et al.*, 2008, 2009). Differential sensitivity of *Hd3a* and *RFT1* transcription to day length is partly explained by distinct promoter architectures (Komiya *et al.*, 2008, 2009).

Several positive and negative regulators control expression of the florigens in leaves. Among them, the zinc finger transcription factor *Heading date 1* (*Hd1*) , homologous of Arabidopsis CONSTANS, promotes expression of *Hd3a* and *RFT1* under SD, while it represses it under LD (Yano *et al.*, 2000; Hayama *et al.*, 2003). Hd1 functions as transcriptional regulator through physical interaction with Grain yield, plant height and heading date 8 (Ghd8), Ghd7 and OsNF-YC7. Ghd8 and OsNF-YC7 are the B and C subunits of a heterotrimeric NF-Y complex, respectively, that assemble with Hd1, bind the promoter of *Hd3a* and repress its transcription (Goretti *et al.*, 2017; Zhu *et al.*, 2017; Du *et*

103 *al.*, 2017). *Ghd7* encodes for a CCT domain protein that represses flowering (Xue *et al.*, 2008;
104 Nemoto *et al.*, 2016) .
105 The B-type response regulator *Early Heading date 1* (*Ehd1*) is a major flowering promotor that shares
106 no homology with other genes of *Arabidopsis thaliana* (Doi *et al.*, 2004). *Ehd1* is expressed at higher
107 levels under SD conditions and preferentially promotes *Hd3a* transcription (Zhao *et al.*, 2015). In
108 agreement with its role as a flowering activator, *ehd1* mutants show a late flowering phenotype
109 compared to wild type, whereas *Ehd1* overexpression triggers early flowering under both SD and LD
110 conditions (Doi *et al.*, 2004; Itoh *et al.*, 2010; Cho *et al.*, 2016). Hd1 can repress *Ehd1* mRNA
111 expression under LD, by directly binding to its promoter (Nemoto *et al.*, 2016). These genetic
112 interactions are at the core of the photoperiodic flowering pathway in rice, which exhibits a different
113 architecture compared to the one of Arabidopsis (Andrés & Coupland, 2012).
114 When florigenic proteins are produced, they influence downstream gene expression by assembling
115 into Florigen Activation or Repressor Complexes (FACs/FRCs), higher-order complexes containing
116 PEBP molecules and FD-like bZIP transcription factors (Tsuji *et al.*, 2013; Brambilla *et al.*, 2017;
117 Kaneko-Suzuki *et al.*, 2018). The latter allow the complex to bind DNA at consensus sites
118 characterized by the core sequence ACGT (Izawa *et al.*, 1993). Interactions between florigens and
119 bZIPs can occur either directly or indirectly via bridging proteins called Gf14s, members of the 14-
120 3-3 protein family (Taoka *et al.*, 2011). In leaves, FACs/FRCs regulate expression of the florigens,
121 generating positive and negative feedback loops on *Ehd1* production (Brambilla *et al.*, 2017). More
122 specifically, FRCs composed by the florigens and the bZIP transcription factor Hd3a BINDING
123 REPRESSOR FACTOR 1 (HBF1) delay the floral transition, as rice loss-of-function *HBF1* mutants
124 flower earlier compared to the wild type. HBF1 can bind the promoter of *Ehd1* to reduce its expression
125 in leaves, thus generating a negative feedback loop on *Hd3a* and *RFT1* production, that likely
126 modulates the amount of florigens being produced (Brambilla *et al.*, 2017).
127 Another bZIP transcription factor, *OsFD1*, encodes an interactor of the florigens and is part of a
128 flowering-promoting FAC (Taoka *et al.*, 2011). *OsFD1* is expressed in both leaves and SAM and
129 interacts with Hd3a and RFT1 only indirectly via Gf14 proteins. In leaves, OsFD1 promotes the
130 expression of *Ehd1* generating a positive feedback loop on florigen production, whereas in the SAM,
131 OsFD1-containing FACs promote transcription of *OsMADS14* and *OsMADS15*, two key activators
132 of flower development in rice (Taoka *et al.*, 2011; Kobayashi *et al.*, 2012; Zhao *et al.*, 2015; Wu *et*
133 *al.*, 2017). Thus, the florigens not only connect leaves and SAM to convey seasonal information to
134 the apex, but their movement throughout the plant allows formation of distinct florigen-containing
135 complexes in different tissues and with diversified functions.

136 In the SAM, OsFD1 can further switch from activating to repressing flowering by forming FRCs. In
137 FRCs, OsFD1 interacts with four different CENTRORADIALIS-like proteins, *RICE*
138 *CENTRORADIALIS 1* (*RCN1*), *RCN2*, *RCN3* and *RCN4* which are PEBP and members of the TFL1-
139 like subclade (Kaneko-Suzuki *et al.*, 2018). They are expressed in the rice vascular tissue and,
140 similarly to Hd3a and RFT1, can move through the phloem and reach the SAM where they bind
141 OsFD1 via Gf14s, to regulate production of secondary branches of the panicle (Kaneko-Suzuki *et al.*,
142 2018).
143 Despite its central position in molecular complexes controlling flowering, *osfd1* RNA interference
144 plants showed almost no flowering delay compared to wild type plants (Taoka *et al.*, 2011). Its
145 interaction in both FACs and FRCs with opposite effects on flowering could partially explain such
146 phenotype. However, it cannot be excluded that additional bZIP transcription factors could operate
147 in parallel or redundantly with OsFD1.
148 Here, we functionally characterize *OsFD4*, an FD-like bZIP transcription factor promoting the floral
149 transition under both inductive and non-inductive photoperiodic conditions. We determine the extent
150 of OsFD4 interactions in FACs and its genetic relationship with OsFD1. Using Positional Weight
151 Matrices (PWM) gathered from DNA Affinity Purification sequencing (DAP-seq), we interrogate the
152 binding of OsFD1, OsDF4 and HBF1 to promoters of target genes, starting to define their distinct
153 modes of DNA binding.
154

155 MATERIALS AND METHODS

156 Plant growth conditions, RNA preparation and quantification of gene expression

157 The Nipponbare and Dongjin ecotypes were used in this study. Plants were grown in phytotrons under
158 long-day conditions (14,5h light/9,5h dark) or short-day conditions (10h light/14h dark). For
159 quantification of gene expression using qRT-PCR, SAM samples were collected at *Zeitgeber* (ZT) 0
160 in time courses of several days. Three biological replicates were performed for *osfd4-1* time-courses.
161 Two biological replicates were sampled for *osfd1-1* time courses. SAMs were manually dissected
162 under a stereomicroscope and the samples included the meristem proper as well as some small
163 meristematic leaves. For leaf samples, the distal part of mature leaves was used. RNA was extracted
164 with *TRIzol*® (Termofisher Scientific) following manufacturer's instructions. The cDNA was
165 retrotranscribed with Im-Prom-II RT (Promega) using a polyT primer and 1 µg of total RNA as
166 starting template. Quantification of transcripts was performed with the primers listed in Table S2
167 using a RealPlex^2 thermocycler. The Maxima SYBR qPCR master mix (Termofisher Scientific) was
168 used in qRT-PCR experiments. Three technical replicates were performed for each qRT-PCR.
169

170 **Protein-protein interaction assays**

171 Yeast two hybrid was performed cloning the coding sequences into the vectors pGADT7 and
172 pGBKT7 (Clontech) and transformed into AH109 and Y187 yeast strains, respectively. Interactions
173 were tested by mating and growth on selective drop out media without the aminoacid -L-W-H-A
174 according to Brambilla et al., 2017. All mating controls are shown in Fig. S3.
175 BiFC experiments were performed using the vectors pBAT TL-B sYFP-N and pBAT TL-B sYFP-C.
176 Clones were transformed in tobacco epidermal cells by Agro-infiltration. Infiltrated leaves were
177 observed at the confocal 24 h after infiltration. Each interaction was tested in three different replicates.
178

179 **CRISPR-Cas9 cloning and Rice transformation**

180 The CRISPR-Cas9 vectors previously described by Miao et al., 2013 were used. We designed one
181 single-guide RNA for OsFD1, OsFD4 and OsFD3 (Table S2). We obtained edited *osfd4*, *osfd3* and
182 *osfd1* mutants starting from Nipponbare calli produced according to the protocol of Sahoo et al., 2011.
183 Rice calli were transformed using EHA105 *Agrobacterium tumefaciens* strain. Transformed calli
184 were selected on two selection media containing 50 mg/L and 100 mg/L of hygromycin, respectively.
185 After regeneration, we genotyped transformant plants via by sequencing PCR products amplified
186 across the single-guide RNA targeted region.
187

188 **DAP seq assay**

189 pENTR clones containing the *OsBZIP42*, *OsBZIP69*, and *OsBZIP77* ORFs were recombined into the
190 pIX-HALO vector and processed according to Bartlett et al., 2017. We used 1 ug of pIX-HALO-TF
191 plasmid DNA for protein expression in the TNT rabbit reticulocyte expression system (Promega).
192 For DNA binding reactions, 500 ng of adapter-ligated library prepared from genomic DNA extracted
193 from Nipponbare leaves was used. Libraries were sequenced on a NextSeq500 with 75bp single end
194 reads. A GST-HALO negative control sample was also processed in parallel and used for background
195 subtraction in peak calling.
196

197 **DAP-seq analysis**

198 Raw reads were trimmed using Trimmomatic (Bolger *et al.*, 2014) with the following parameters:
199 ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15
200 MINLEN:50. Trimmed reads were mapped to the *Osativa* v7 reference genome (release 40;
201 https://plants.ensembl.org/Oryza_sativa/) using Bowtie2 v2.2.8 (Langmead & Salzberg, 2012) with
202 default parameters. Mapped reads were filtered for reads aligning only to a single location in the
203 genome. Peaks were called using the GEM peak caller (Guo et al., 2012) as described in Galli et al.,

204 2018. PAVIS (Huang et al. 2013) was used to identify putative target genes. Target genes were
205 assigned based on the gene located closest to each peak and were restricted to those peaks located
206 5kb upstream of the TSS, 3kb downstream of the TES, or located within the gene. The 2000 interval
207 relative to the TSS was surveyed for detecting the position of peaks and distribution of them using a
208 bin size of 25 bp.
209

210 **Motif enrichment analysis and spacing/syntax analysis**

211 Peaks identified by the GEM (Guo et al., 2012) peak caller were used to compute motif enrichment,
212 using the MEME-suite (default arguments). Generated motifs were evaluated using ROC analysis to
213 estimate their prediction power, i.e. their capability to differentiate between bound and not bound
214 regions (Fig. S4). For this, a negative set of non-bound regions with similar features (same length,
215 same %GC, same genomic origin [exonic, promoter,...] as the bound sequences) was generated as
216 previously described (Stigliani *et al.*, 2019). Detection of preferred spacings between motifs was done
217 using the called peaks and the MEME-generated position weight matrix (PWM). Predicted individual
218 binding sites were detected for a given threshold, for sets of both bound and unbound regions. Spacing
219 between predicted binding sites were computed for both sets. Enrichment was calculated by
220 comparing bound and not bound sets of regions. The PWM threshold values were empirically chosen:
221 we used a range of threshold values to ensure robustness of the result. Detailed methods are available
222 in Stigliani et al., 2019.
223

224 **RESULTS**

225 **OsFD4 promotes flowering in rice**

226 In leaves, HBF1- and OsFD1-containing complexes control production of the florigens by regulating
227 *Ehd1* expression (Brambilla *et al.*, 2017). In the SAM, the OsFD1-containing FAC is known to
228 promote the floral transition by directly inducing expression of *OsMADS14* and *OsMADS15* (Taoka
229 *et al.*, 2011). Yet, *OsFD1* RNA interference plants showed only a marginally significant flowering
230 delay compared to the wild type, in sharp contrast to the phenotype shown by *Hd3a*-RNAi, *RFT1*-
231 RNAi and *Hd3a*/*RFT1* double RNAi plants (Komiya *et al.*, 2008). This suggests that other bZIP
232 transcription factors might share similar functions and compensate for reduced *OsFD1* expression.
233 We previously explored the bZIP family, searching for members having positive or negative effects
234 on the floral transition (Brambilla *et al.*, 2017). Here, we describe the functional characterization of
235 *bZIP69*/*OsFD4* (*LOC_Os08g43600*).
236 After searching the PFG rice T-DNA collection we identified line 2D_41663 in the Dongjin variety
237 that harboured a T-DNA insertion in the 3’-UTR of *OsFD4* (Fig. S1). The mutant line was renamed

238 *osfd4-1*. We could not detect *OsFD4* mRNA expression in *osfd4-1* (Fig. S1). Heading dates of the
239 mutant plants were assessed under both SD and LD, alongside Dongjin wild type controls. Mutant
240 plants flowered late under both conditions (Fig. 1a).
241 To validate the *osfd4-1* mutant phenotype, we generated additional *osfd4* mutant alleles using
242 CRISPR-Cas9 site-directed mutagenesis in the Nipponbare background (Miao *et al.*, 2013). We also
243 generated novel *osfd1* mutants to compare single mutant phenotypes. Finally, since we previously
244 showed that OsFD4 strongly interacts with bZIP24/OsFD3 (Brambilla et al., 2017 and Fig. 4), we
245 also generated *osfd3* mutants (Fig. S1). Homozygous plants of the T1 generation were grown for two
246 months under non-inductive LD conditions and then shifted to inductive SD to score heading dates.
247 As shown in Fig. 1b and Fig. S1, *osfd4-3* showed delayed flowering compared to the wild type,
248 confirming the *osfd4-1* phenotype. The *osfd1-1* mutant flowered later than both wild type and *osfd4-*
249 *3*, showing a strong delay (Fig. 1b-d). The difference compared to published *OsFD1*-RNAi mutants
250 could be due to the different conditions used between studies, to the different genetic backgrounds
251 used, or to a limited reduction of *OsFD1* mRNA in RNAi lines. No effect on heading could be
252 observed in *osfd3-1* mutants (Fig. 1b).
253 To investigate the possible functional redundancy between *OsFD4* and *OsFD1*, we crossed *osfd4-1*
254 and *osfd1-2* mutants to generate an *osfd4-1 osfd1-2* double mutant (note that the genetic background
255 is mixed between Nipponbare and Dongjin). Flowering time experiments under inductive conditions
256 demonstrated that no additive effect could be detected in the *osfd4-1 osfd1-2* double mutant relative
257 to *osfd1-2* (Fig. 1d), i.e. the phenotypic effect observed was that of *osfd1-2* single (Fig. 1b-d). These
258 data indicate that *OsFD4* encodes a promotor of flowering in rice and that the effect of *osfd4* and
259 *osfd1* mutants is not additive.
260

261 ***OsFD4* is expressed in the shoot apical meristem and is regulated by *OsFD1***

262 *OsFD1* is expressed in both leaves and SAM. It promotes flowering, having distinct functions in both
263 tissues during the floral transition (Taoka *et al.*, 2011; Tamaki *et al.*, 2015; Jang *et al.*, 2017;
264 Brambilla *et al.*, 2017). We quantified the expression of *OsFD4* mRNA in leaves and apical
265 meristems, using qRT-PCR. Transcripts of *OsFD4* were detected in apical meristems but not in leaves
266 during the transition to flowering (Fig. 2a,b). Quantifications showed that *OsFD1* and *OsFD3*
267 expression was higher relative to *OsFD4* expression in both tissues.
268 To verify the epistatic interaction between *OsFD1* and *OsFD4*, observed in the *osfd4-1 osfd1-2*
269 double mutant, we analysed *OsFD4* and *OsFD1* mRNA levels in *osfd1-1* and *osfd4-1* mutants,
270 respectively. We observed that *OsFD4* expression was decreased in *osfd1-1* compared to the wild
271 type (Fig. 2c), whereas *OsFD1* expression was unchanged in *osfd4-1* mutants compared to Dj wild

272 type (Fig. 2d). These data indicate that *OsFD1* is an upstream promotor of *OsFD4* expression, in
273 agreement with the phenotype of double *osfd4-1 osfd1-2* mutants (Fig. 1d).
274

275 **Network of interactions among FAC components**

276 The rice florigens interact with bZIP transcription factors either directly or through Gf14 proteins.
277 Interactions are likely dynamic and combinatorial, giving rise to several complexes, some also with
278 repressive functions (Zhao *et al.*, 2015; Jang *et al.*, 2017; Brambilla *et al.*, 2017; Kaneko-Suzuki *et*
279 *al.*, 2018). Dimers between Gf14 proteins form a W-shaped scaffold that bridges interactions with
280 bZIPs and with the florigens. We found that extensive combinatorial interactions are possible between
281 Gf14s expressed at the apex, giving rise to homo- and heterodimers (Fig. S2). Interactions between
282 Gf14s and bZIPs occur via the C-terminal SAP-domain, which is common among several bZIPs,
283 including OsFD4 and OsFD3 (Fig. S1). We tested whether OsFD4 could interact with components
284 of FACs. We first tested the binding between OsFD4 and the six Gf14s expressed in the SAM,
285 including Gf14A to Gf14F (Purwestri *et al.*, 2009). All six Gf14 proteins interacted with OsFD4 in
286 Yeat-2-Hybrid (Y2H), but the strongest interactions were detected between OsFD4 and Gf14A and
287 Gf14F (Fig. 3a). Weaker yeast growth on -L-W-H-A selective media was observed with other
288 OsFD4-Gf14 combinations. Note that in a previous report we did not detect the OsFD4-Gf14C
289 interaction, using -L-W-H +3AT media (Brambilla *et al.*, 2017), consistent with the findings of Kim
290 et al., 2016. Thus, interactions between OsFD4 and Gf14B, C, D, and E are likely weak. Also, OsFD3
291 could interact with all Gf14s tested (Fig. S2).
292 We next asked if all Gf14 proteins expressed in the SAM could interact with RFT1 and Hd3a. Both
293 florigens interacted with all the Gf14s tested, suggesting that in the SAM, OsFD4, OsFD3 and OsFD1
294 can potentially form FACs indirectly via all 14-3-3 proteins (Fig. 3b). To confirm Y2H findings, we
295 performed BiFC with OsFD4 and Gf14B and observed reconstituted YFP expression in the nucleus
296 indicating a positive interaction (Fig. S2).
297 Since HBF1 and HBF2 can directly contact Hd3a without the bridging function of Gf14s (Brambilla
298 *et al.*, 2017), we tested direct interactions between OsFD4 and Hd3a or RFT1. Using Y2H, we found
299 that OsFD4 can directly associate with RFT1, but no interaction was detected between OsFD4 and
300 Hd3a, whereas OsFD3 could interact with both florigens (Fig. 3c). These data are consistent with a
301 recent independent study, indicating that OsFD4 preferentially binds to one florigen only (Jang *et al.*,
302 2017). We then used BiFC to further test the OsFD4-RFT1 interaction, as well as to determine its
303 subcellular localization. We detected fluorescence in nuclei of *Nicotiana benthamiana* leaves
304 indicating nuclear localization of the heterodimer (Fig. 3d). No fluorescent signals could be detected
305 for the OsFD4–Hd3a interaction (Fig. 3e and Fig. S2).

306

307 **OsFD4 can homodimerize or heterodimerize with OsFD3**

308 bZIP transcription factors must homo- or heterodimerize to bind DNA (Dröge-Laser *et al.*, 2018).
309 We thus tested combinatorial interactions between bZIPs. We previously showed that OsFD4 and
310 OsFD3 can form homo- and heterodimers, whereas no dimerization was found with OsFD1
311 (Brambilla et al., 2017; Fig. 3f,g). Here, we corroborated those results using BiFC, further showing
312 that OsFD4 and OsFD3 homo- and heterodimers are localized in the nucleus. We did not detect homo-
313 or heterodimerization of OsFD1 in BiFC assays (Fig. 3h,i).

314

315 **OsFD4 regulates expression of *AP1/FUL*-like genes**

316 The rice genome encodes four different *AP1*/*FUL*-like genes, *OsMADS14*, *OsMADS15*, *OsMADS18*
317 and *OsMADS20* which control flower development (Wu *et al.*, 2017). When plants are exposed to
318 inductive photoperiods and the meristem undergoes phase change, *OsMADS14*, *OsMADS15* and
319 *OsMADS18* increase their expression, whereas *OsMADS20* is downregulated (Kobayashi *et al.*, 2012;
320 Gómez-Ariza *et al.*, 2019). In the SAM, OsFD1 promotes the expression of *OsMADS14* and
321 *OsMADS15*. This regulation has been shown to be direct at least for *OsMADS15,* as the OsFD1-
322 containing FAC is able to bind to its promoter (Taoka *et al.*, 2011). To assess if an OsFD4-containing
323 FAC could also regulate the expression of *OsMADS* genes we measured transcription of *AP1*/*FUL*-
324 like genes in apical meristems of *osfd4-1*. We performed time course experiments shifting plants from
325 non-inductive LD conditions to inductive SD conditions and sampled SAMs at 0, 6, 12 and 18 Days
326 After Shifting (DAS). These time points were chosen based on the progression of floral transition in
327 rice which becomes irreversible at 12 SDs, after which a fully committed SAM develops into a
328 branched inflorescence (Kobayashi *et al.*, 2012; Gómez-Ariza *et al.*, 2019). Comparing *OsMADS*
329 gene expression between wild type Dongjin and *osfd4-1* plants, we observed that *OsMADS14* and
330 *OsMADS15* levels could increase in the mutant but at slower rates, whereas expression of *OsMADS18*
331 and *OsMADS20* was not changed compared to the wild type (Fig. 4a-d).

332 Rice *AP1*/*FUL*-like genes work together with *OsMADS34*/*PAP2*, a *SEPALLATA* (*SEP*)-like gene, to
333 promote the floral transition (Kobayashi *et al.*, 2010, 2012). Expression of *OsMADS34*/*PAP2*
334 increased in both wild type and *osfd4-1* but was significantly delayed in the mutant (Fig. 4e). To
335 complete our analysis, we also quantified the expression of *AP1*/*FUL*-like genes and of
336 *OsMADS34*/*PAP2* in the *osfd1-1* mutant. We observed that their expression had similar dynamics in
337 both *osfd1-1* and *osfd4-1* (Fig. 4f-j). Taken together, these data suggest that OsFD4-FAC, as well as
338 OsFD1-FAC, act upstream of *OsMADS* genes to promote the rice floral transition.

339

340 **Genome-wide identification of OsFD4, OsFD1 and HBF1 binding sites**

341 In the SAM, changes in the photoperiod regulate the transcription of several genes (Furutani *et al.*, 342 2006; Kobayashi *et al.*, 2012; Tamaki *et al.*, 2015; Gómez-Ariza *et al.*, 2019). Yet, only a few genes 343 are known to be under control of FACs/FRCs. As a first screen to identify DNA binding sites of bZIP 344 transcription factors and FAC-regulated genes, we performed DNA Affinity Purification sequencing 345 (DAP-seq) using OsFD4, OsFD1 and HBF1 (O'Malley *et al.*, 2016; Bartlett *et al.*, 2017; Galli *et al.*, 346 2018). Peak calling analysis identified 1107 DAP-peaks for OsFD4, 2059 DAP-peaks for OsFD1, 347 and 28323 DAP-peaks for HBF1 (Fig. 5a). The top enriched motif for OsFD1, OsFD4 and HBF1 348 contained the core consensus motif CACGT, the known binding site for many bZIP transcription 349 factors known as G-box (Fig. 5a) (O'Malley *et al.*, 2016). At the flanks of this core motif, two 350 nucleotides -GC- were enriched with different frequencies, depending on the bZIP tested. For 351 example, OsFD4 peaks were most enriched for the motif GCCACGT (Fig. 5a).

352 To identify putative target genes regulated by these binding events, we assigned all DAP-peaks lying 353 within 5000 bp upstream of an ATG and 3000 bp downstream of a STOP codon (including regions 354 within exons and introns) to the closest gene. Based on this analysis, HBF1 peaks were associated 355 with 15,937 putative target genes, and OsFD1 and OsFD4 were associated with 1,717 and 925 356 putative target genes, respectively (Fig. 5a; Table S1). Most of the binding sites fell in putative 357 promoter regions (Fig. 5b). The highest frequency of peaks (30% for OsFD4, 27% for OsFD1 and 358 17% for HBF1) was in the 500 bp upstream of the transcription start site (TSS; Fig. 5c), consistent 359 with these sites being highly represented in the core promoter sequences. We next examined the 360 overlap between genes bound by OsFD1, OsFD4 and HBF1 and observed that 698 putative target 361 genes were shared by all tested bZIPs; 220 genes were shared by OsFD4 and HBF1, and 991 were 362 shared by OsFD1 and HBF1. All genes common to OsFD1 and OsFD4 were also common to HBF1, 363 thus no gene was found at the intersection between OsFD1 and OsFD4 datasets only (Fig. 5d and 364 Table S1). Finally, 7, 28 and 14,028 genes were uniquely targeted by OsFD4, OsFD1 or HBF1 (Fig. 365 5d).

366 The DAP-seq method uses the entire naked genome to identify transcription factor binding sites. 367 Thus, many bound regions might not be functionally relevant for regulation of gene expression *in* 368 *vivo* (O'Malley *et al.*, 2016; Bartlett *et al.*, 2017). To focus our analysis on those tissues where OsFD4, 369 OsFD1 and HBF1 are expressed (Fig. 2a), we filtered for genes expressed specifically in the SAM 370 and mature leaves. To this end, we first compared the lists of genes bound by OsFD1 and OsFD4 to 371 those expressed in the shoot apex, based on recently published RNA-seq data (Fig. 5e) (Gómez-Ariza 372 *et al.*, 2019). The lists of genes bound by HBF1 and OsFD1 were compared to the list of genes 373 expressed in mature leaves, again as determined by published RNA-seq data (Galbiati *et al.*, 2016)

374 (Fig. 5f). This analysis identified 240 genes expressed in shoot apices that were putative targets of
375 both OsFD4 and OsFD1, 58 genes that were putative targets of OsFD4, and 320 genes that were
376 putative targets of OsFD1 (Fig. 5d). Of those genes showing expression in mature leaves, 566 were
377 putative targets of both HBF1 and OsFD1, 3691 were putative targets of HBF1, and seven were
378 putative targets of OsFD1.

379

380 ***OsMADS62* and *OsARF19* are targets of both the OsFD4-FAC and OsFD1-FAC**

381 To determine if binding of OsFD1 and OsFD4 to genomic regions correlated with transcriptional
382 regulation of the neighboring genes, we quantified transcript abundance of selected target genes in
383 *osfd1-1*, *osfd4-1* and wild type. For this analysis, we focused on several transcription factors
384 expressed in the apical meristem and whose promoters contained OsFD1 or OsFD4 binding peaks.
385 These included two genes bound by both *OsFD1* and *OsFD4*, *LOC_Os08g38590* (*OsMADS62*) and
386 *LOC_Os06g48950* (*OsARF19*), three genes bound only by *OsFD1*, *LOC_Os01g14440* (*WRKY*),
387 *LOC_Os01g64360* (*MYB*), and *LOC_Os04g51000* (*RICE FLORICAULA/LEAFY*), and two genes
388 bound by *OsFD4* only, *LOC_Os04g31730* (*B3*) and *LOC_Os07g41580* (*NF-YB*) (Fig. 5g-h and Fig.
389 S5). Expression of both *OsMADS62* and *OsARF19* was reduced in *osfd4-1* and *osfd1-1* compared to
390 the wild type, suggesting that OsFD1 and OsFD4 may be direct activators of both genes (Fig. 5g-h).
391 The other genes tested showed reduced expression in both mutants relative to wild type probably
392 because of the epistatic effect of *OsFD1* to *OsFD4* (Fig. S5). These data suggest that OsFD4 and
393 OsFD1 regulate several common target genes, but their transcriptional effects are not completely
394 overlapping.

395

396 **OsFD4, OsFD1 and HBF1 show preferential DNA binding configurations**

397 OsFD1, OsFD4 and HBF1 bind overlapping but also distinct sets of genes in DAP-Seq experiments,
398 yet they recognize a very similar motif. Because bZIP TFs are known to bind to tandem motifs, we
399 asked if spacing between CACGT consensus motifs found in OsFD1, OsFD4 and HBF1 DAP-seq
400 peaks varied. We used a Position Weight Matrix (PWM) derived from DAP-seq data to locate bZIP
401 binding sites and test whether there are some preferences in terms of binding syntaxes (spacing and
402 configuration between two consensus motifs (Fig. 6a) (Stigliani *et al.*, 2019). We first evaluated the
403 predictive power of the generated PWM which resulted exceptionally high, with ROC values of
404 0.985, 0.971 and 0.9 respectively (Fig. S4). Next, we analyzed the distribution of spacings for each
405 bZIP, quantifying overrepresented configurations against a set of negative regions (unbound), and we
406 found specific profiles for each bZIP.

407 Direct Repeats (DR) 0, Everted Repeats (ER) 1, 5, ER18-20, ER44, Inverted Repeats (IR) 41
408 configurations showed at least a 4-fold overrepresentation among OsFD4 bound regions, with the
409 ER44 configuration being highly overrepresented (Fig. 6b). Regions bound by OsFD1 showed a
410 robust enrichment of at least 3-fold in the DR30, ER4, ER16, ER32-34, ER41-2 and ER46, and IR38
411 configurations (Fig. 6c), with ER16 and ER32-34 as the most overrepresented. Configuration
412 enrichments for HBF1 included DR29, DR40, ER3, ER16-18, ER42 and IR10 with at least 2-fold
413 enrichment compared to the negative set of regions (Fig. 6d). These data identify different spacing
414 configurations between consensus motifs, which are preferred by OsFD4, OsFD1 and HBF1 and
415 likely contribute to the specificity of target recognition.
416
417 **DISCUSSION**
418 Transcription factors of the bZIP family have important roles during phase transitions in different
419 plant species (Abe *et al.*, 2005; Wigge *et al.*, 2005; Taoka *et al.*, 2011; Tsuji *et al.*, 2013; Park *et al.*,
420 2014; Tylewicz *et al.*, 2015; Brambilla *et al.*, 2017; Teo *et al.*, 2017). However, those controlling
421 flowering time and for which functional studies have been carried out, appear insufficient to account
422 for all aspects of phase change, suggesting a certain level of redundancy, or the existence of additional
423 transcriptional regulators. In rice, the role of OsFD1 as a promoter of flowering has been supported
424 by overexpression studies, but the analysis of RNAi mutants failed to demonstrate phenotypic effects
425 on flowering (Taoka *et al.*, 2011; Jang *et al.*, 2017; Brambilla *et al.*, 2017). Other *OsFD*-like genes
426 have been investigated previously, however they harbour functions distinct from those of OsFD1
427 (Tsuji *et al.*, 2013; Brambilla *et al.*, 2017). Here, to identify additional components of florigen
428 signalling, we reported the characterization of OsFD4 and OsFD3, as well as further insights into
429 OsFD1 function based on CRISPR-mediated mutagenesis. While describing such additional FD-like
430 components integrating florigen signalling, it became clear that despite similar modes of action,
431 differences in their expression profiles, specific combinatorial interactions with Gf14 proteins and
432 florigens, and distinct DNA binding syntaxes distinguish different members of the family.
433
434 **The FAC paradigm across Angiosperms**
435 Formation of a FAC requires that florigenic proteins that have reached the SAM first interact in the
436 cytosol with Gf14 proteins and then enter the nucleus, where phosphorylated bZIP transcription
437 factors contact the Gf14 and contribute a DNA-recognition function. This model of interaction
438 between florigens and bZIP transcription factors was first proposed in Arabidopsis and later supported
439 by studies in rice, where the role of Gf14s as bridges was identified. Subsequently, it has been

440 extended to many other species including dicots and monocots and shown to control a wide range of
441 developmental processes.
442 In rice, the functional homolog of Arabidopsis FD is encoded by *OsFD1*. When overexpressed using
443 either a constitutive or phloem-specific promoter, *OsFD1* can accelerate flowering (Jang et al., 2017,
444 Brambilla et al., 2017). However, RNAi lines flower similarly to wild type, possibly *OsFD1*
445 transcripts are not completely absent in these knock-down lines (Taoka *et al.*, 2011). Thus, the
446 functional significance of OsFD1 as a flowering regulator has remained unclear. Knock-out of *OsFD1*
447 using CRISPR-Cas9 showed that the gene has indeed a prominent effect in promoting flowering. This
448 is likely the result of a more complex molecular function that includes (i) induction of *OsMADS14*
449 and *15* gene expression in the SAM and in leaves, (ii) induction of *Ehd1*, *Hd3a* and *RFT1* mRNA
450 expression in leaves, and (iii) interaction with RCNs in repressor complexes that antagonize FAC-
451 dependent activation of *OsMADS* gene expression. Thus, OsFD1 and Gf14 proteins are central to
452 both activating and repressive flowering pathways, the switch between them being likely controlled
453 by the relative abundance of FT-like and TFL1-like proteins at the apex.
454 Still, while double *Hd3a* and *RFT1* RNAi mutants do not flower for up to 300 days under inductive
455 SDs, single *osfd1* loss-of-function mutants are delayed in flowering but they can flower, an apparent
456 inconsistency justified by functional redundancy between bZIPs (Komiya *et al.*, 2008). In
457 Arabidopsis, FD is redundant with FD PARALOG (FDP). Double *fd fdp* mutations flower later than
458 *fd* single, and almost completely suppress precocious flowering of *35S:FT* (Jaeger *et al.*, 2013). In
459 rice, by mutating additional bZIPs, including *OsFD3* and *OsFD4*, we have identified floral promoting
460 factors. Mutations in *osfd4*, but not in *osfd3*, can delay flowering and activation of *OsMADS* targets.
461 However, flowering was not delayed as much as in *osfd1* mutants, indicating a less prominent role
462 compared to OsFD1. Also, *osfd1 osfd4* double mutants did not further delay flowering compared to
463 *osfd1* single mutants. Based on expression data, we propose that *OsFD1* activates *OsFD4*
464 transcription. Thus, in *osfd1* mutants, the levels of both regulators are low. This arrangement does not
465 fully exclude redundancy but suggests a complex regulatory network.
466 Considering the interaction patterns with the florigens, we found that OsFD4 can interact directly
467 with RFT1, but only via Gf14s with Hd3a, suggesting different preferences for FAC architectures.
468 The SAM of rice is induced to flower by both florigens under SD, but only by RFT1 under LD
469 (Komiya *et al.*, 2008, 2009). Since the flowering delay observed in *osfd4* mutants is much stronger
470 under LD than under SD, we propose that different bZIPs perform distinct functions in the
471 photoperiod, and that the OsFD4-RFT1 module is key to promote flowering mostly under non-
472 inductive conditions.
473

474 **DAP-Seq identifies genome-wide OsFD1, OsFD4 and HBF1 binding sites**
475 Application of DAP-seq using purified OsFD1, OsFD4 and HBF1 proteins resulted in the
476 identification of hundreds of putative direct binding sites for these bZIPs. Consistent with a function
477 in core promoters, binding sites were enriched in the proximity of the TSS. It should be noted that the
478 *in vitro* nature of DAP-seq allows for the identification of direct binding events by a single TF,
479 specifically capturing those mediated by individual homodimers. Given that OsFD1, OsFD4 and
480 HBF1 formed heterodimers, these events may represent only a fraction of all *in vivo* binding events
481 resulting from additional protein interactions. Integrating DAP-seq data with expression data allowed
482 us to focus specifically on those genes that were expressed in relevant tissues and on those
483 differentially expressed in the *osfd4-1* and *osfd1-2* mutants. Our data provide a first examination of
484 the direct genome-wide binding landscape of bZIP flowering regulators in rice. This approach needs
485 now to be refined for several reasons.
486 First, we used a single purified bZIP transcription factor in DAP-seq reactions. Being disengaged
487 from its florigen-containing complex, its DNA binding capacity might be biased. Arabidopsis FD can
488 bind functional sites without assembling into a FAC and with no need for FT or its closest homologue,
489 TWIN SISTER OF FT (TSF) (Collani *et al.*, 2019). Also purified OsFD1 alone can bind a fragment
490 of the *AP1* promoter containing a C-box element (GACGTC) in gel shift assays (Taoka *et al.*, 2011).
491 We previously showed that HBF1 can bind *in vitro* an ABRE/G-box element in the *Ehd1* promoter
492 region, a finding that we further corroborated and extended in this study (Brambilla *et al.*, 2017).
493 Thus, bZIPs are likely to conserve their basal DNA binding ability even when not combined in ternary
494 complexes, but despite these examples, affinity for DNA is likely potentiated by binding with other
495 interactors, including members of the PEBP family (Collani *et al.*, 2019).
496 Second, since bZIPs bind the DNA as homo or heterodimers, and given their combinatorial
497 interactions and overlapping expression in different plant tissues, including the SAM, it cannot be
498 excluded that DNA binding specificity changes when assessing heterodimeric configurations.
499 Third, bZIPs might be incorporated into higher order complexes that include other transcriptional
500 regulators with different DNA binding properties. In Arabidopsis, SQUAMOSA PROMOTER-
501 BINDING PROTEIN-LIKE 3 (SPL3), SPL4 and SPL5 interact with FD to enhance its transcriptional
502 activation of target genes and its specificity for target promoters (Jung *et al.*, 2016). Since SPLs are
503 produced at the SAM in response to plant aging, the SPL-FD complex has been proposed to integrate
504 photoperiodic and aging signals to activate the *AP1* promoter (Jung *et al.*, 2016). The study further
505 suggested that the proximal C-box in the *AP1* promoter is dispensable for FD-mediated induction,
506 and that a distinct SPL-binding site might contribute to transcription of *AP1*. A similar case has been
507 described for Class II TCP transcription factors, including TCP5, 13 and 17. These proteins can

508 physically interact with FD, and facilitate its binding to the *AP1* promoter. Also in this case, binding
509 of FD to the *AP1* promoter was not observed at the C-box, but was dependent upon a TCP binding
510 motif located between the SPL-binding site and the C-box (Li *et al.*, 2019).
511 Finally, post transcriptional modifications have a major role *in vivo*. Many FD-like proteins harbour
512 at their C terminus an SAP domain containing serine, threonine or both, which needs to be
513 phosphorylated to interact with Gf14s (Taoka *et al.*, 2011; Park *et al.*, 2014; Li *et al.*, 2015; Collani
514 *et al.*, 2019). When phosphorylated, Arabidopsis FD has a higher affinity for DNA. Expression of
515 phosphomimic versions of FD or OsFD1 can accelerate flowering in Arabidopsis and rice,
516 respectively. The kinases responsible for phosphorylation in Arabidopsis include Calcium-dependent
517 Protein Kinase 6 (CPK6) and CPK33, which interact with FD and, if mutated, slightly delay flowering
518 (Kawamoto *et al.*, 2015). Thus, optimal DNA binding and floral promoting activity of FAC
519 complexes likely relies on this modification of the bZIP components. Future DNA-binding
520 experiments either in vivo or using multiple complex components could help elucidate the individual
521 contributions of each component.
522
523 **Direct targets of OsFD1 and OsFD4 and alternative binding syntaxes**
524 Despite these caveats, DAP-seq led to the identification of several putative direct targets of the bZIPs
525 studied here, out of which *OsMADS62* and *ARF19* were validated as transcriptionally regulated by
526 both OsFD1 and OsFD4. *OsMADS62* controls pollen maturation and germination, partially
527 redundantly with *OsMADS63* and *OsMADS68* (Liu *et al.*, 2013). OsFD1 and OsFD4 promoted
528 *OsMADS62* expression, a function they likely perform after meristem commitment, during
529 inflorescence and flower development. *OsARF19* encodes for an AUXIN RESPONSE FACTOR
530 broadly expressed in the plant but with higher levels of transcription in the shoot, which is further
531 elevated by auxin treatments (Zhang et al., 2015).
532 We did not find OsFD1 or OsFD4 bound to the *OsMADS14*, *OsMADS15* or *OsMADS34/PAP2*
533 promoter. *OsMADS15* has been proposed as direct target of an OsFD1-containing FAC (Taoka *et al.*,
534 2011). Other proteins could possibly stabilize OsFD4 and OsFD1 *in vivo* and allow binding to these
535 *loci*. Alternatively, the rice OsFD1-*OsMADS* connection might be indirect. Data in support of a direct
536 connection have not been as thoroughly repeated and validated in rice as they have been in
537 Arabidopsis, in which direct contacts between FD and the C-box element in the *AP1* promoter were
538 disproved (Benlloch *et al.*, 2011; Jung *et al.*, 2016; Collani *et al.*, 2019; Li *et al.*, 2019). Further
539 assessment of *in vivo* binding will be necessary.
540 Genome scanning by DAP-seq identified identical core bZIP-binding motifs that were highly
541 enriched in peaks of all three bZIP datasets. The same 5'-CACGT-3' core motif was identified as

542 bound by OsFD1, OsFD4 and HBF1. The same 5’-CACGT-3’ core motif was identified as enriched
543 among FD binding regions *in vivo*, suggesting conservation between rice and Arabidopsis, and indeed
544 many other bZIPs from diverse organisms (Dröge-Laser et al., 2018).
545 We found that despite identical core DNA binding sites, spacing among tandem motifs was different
546 for each of the three bZIPs tested in our assay. OsFD1 and OsFD4 which are activators of the rice
547 floral transition prefer to bind the DNA with ER conformations, whereas HBF1, a repressor, binds
548 more frequently ER and DR conformations. These results indicate distinct and specific binding
549 syntaxes for each transcription factor. The method was recently applied to define binding syntaxes of
550 MONOPTEROS (MP) and AtARF2, which have opposing transcriptional functions but share the
551 same consensus motif (Stigliani *et al.*, 2019). The method can capture DNA binding features of
552 proteins that interact as either homo or heterodimers, but the ER or DR arrangements might suggest
553 the possibility of more elaborated structures, including tetramers.

FIGURE LEGENDS

Figure 1. The *osfd4* and *osfd1* mutants delay rice flowering.

(a) Days to heading of *Dongjin* wild type (Dj) and *osfd4-1* under inductive short day conditions (SD) and non-inductive long day conditions (LD). (b) Days to heading of *Nipponbare* wild type (Nb), *osfd1-1*, *osfd3-1* and *osfd4-3* under SD conditions. (c) Representative pictures of *osfd1-1* (left) and Nb (right) plants. White arrows indicate emerging panicles. (d) Days to heading of Nb, *osfd1-2*, *Dj*, *osfd4-1* and double *osfd4-1 osfd1-2* mutants under SD conditions. Data are represented as mean ± StDev. Asterisks indicate the p-value calculated using ANOVA, ****=p<0.0001, ***=p<0.001, **=p<0.01, ns=non significant. All flowering time experiments were repeated at least twice, and one representative experiment is shown in (a) and (b). The graph in (d) includes data from three independent experiments.

Figure 2. Temporal expression pattern of *OsFD1*, *OsFD3* and *OsFD4* in the SAM and genetic interaction between *OsFD1* and *OsFD4*.

Quantification of *OsFD1*, *OsFD3* and *OsFD4* transcripts in the SAM (a) and in leaves (b) of Nb wild type. DAS, Days After Shift from LD to SD conditions. (c) Quantification of *OsFD4* transcripts in SAMs of *osfd1-1* mutants. (d) Quantification of *OsFD1* transcripts in SAMs of *osfd4-1* mutants. Each time point represents the mean ± StDev of three technical replicates. The experiments were repeated twice with similar results. Ubiquitin was used to normalize gene expression.

Figure 3. Interactions between OsFD1, OsFD3, OsFD4 and components of FACs.

(a) Interactions between AD-OsFD1, AD-OsFD4 and BD-Gf14A-F. (b) Interactions between AD-Gf14A-F and BD-Hd3a and BD-RFT1. (c) Interactions between AD-OsFD4, AD-OsFD3 and Hd3a and RFT1 fused to the BD. Bimolecular fluorescence complementation (BiFC) between OsFD4 fused with N-terminus of YFP (N-YFP) and (d) RFT1 fused with the C-terminus of YFP (C-YFP) or (e) Hd3a fused with the C-YFP. (f) Interactions between OsFD4 and OsFD1 fused with the AD or the BD. (g) Interactions between OsFD3, OsFD4 and OsFD1 fused to the BD and OsFD3 fused to the AD. (h) Assessment of OsFDs homodimerization by BiFC. From left, N-YFP:OsFD4/C-YFP:OsFD4, N-YFP:OsFD1/C-YFP:OsFD1 and N-YFP:OsFD3/C-YFP:OsFD3. (i) Assessment of OsFDs heterodimerization by BiFC. From left N-YFP:OsFD4/C-YFP:OsFD1, N-YFP:OsFD3/C-YFP:OsFD4 and N-YFP:OsFD1/C-YFP:OsFD3. Interactions were determined in yeast on selective drop out media -L-W-H-A. AD, Activation Domain. BD, Binding Domain. The yeast experiments were repeated three times with identical results. AD and BD clones containing empty vectors were

588 used as negative controls. BiFC experiments were repeated three times with identical results. N-YFP
589 and C-YFP clones containing empty vectors were used as negative controls.
590

591 **Figure 4. *OsFD4* and *OsFD1* promote *OsMADS14* and *15* transcription.**
592 Quantification of (a), (f) *OsMADS14*, (b), (g) *OsMADS15*, (c), (h) *OsMADS18*, (d), (i) *OsMADS20*,
593 and (e), (j) *OsMADS34/PAP2* transcription in *Dj* vs. *osfd4-1* mutants (a)-(e) and in *Nb* vs. *osfd1-1*
594 mutants (f)-(j). Expression was quantified in plants grown for two months under LD and then shifted
595 to SD conditions. Apical meristems were sampled at 0, 6, 12 and 18 Days After Shifting (DAS). Each
596 time point represents the mean ± St Dev of three technical replicates. The experiments were repeated
597 twice with similar results. Ubiquitin was used to normalize gene expression.
598

599 **Figure 5. Identification of OsFD1, OsFD4 and HBF1 binding sites.**
600 (a) Summary of DAP-seq results of OsFD1, OsFD4 and HBF1, including number of peaks, putative
601 target genes and consensus motives. (b) Distribution of peaks within gene features. (c) Distribution
602 of peaks near the TSS of putative target genes. (d) Venn diagrams showing the overlap between
603 putative target genes of OsFD1, OsFD4 and HBF1. (e) Venn diagrams showing the overlap between
604 targets of OsFD1 and OsFD4 and genes expressed at the apical meristem. (f) Venn diagrams showing
605 the overlap between targets of OsFD1 and HBF1 and genes expressed in leaves. Genome browser
606 view of OsFD1 and OsFD4 binding peaks in the 3'UTR of *OsMADS62* (g) and in the putative
607 promoter of *OsARF19* (h), and quantification of transcripts in *osfd4-1* and *osfd1-1* mutants.
608

609 **Figure 6. Analysis of OsFD1, OsFD4 and HBF1 binding syntax.**
610 (a) Scheme of Everted Repeats (ER), Direct Repeats (DR) and Inverted Repeats (IR) of "ACGTGGC"
611 motives. (b)-(d) Spacing analysis of absolute and normalized enrichment of OsFD4 (b), OsFD1 (c)
612 and HBF1 (d) binding sites**.** The numbers on top of the graphs represent the distance between two
613 consensus motives.
614

615 **Supplemental figures and tables**
616 **Fig. S1** Genotype and phenotype of *OsFD4* and *OsFD1* mutants
617 **Fig. S2** OsFD3 and Gf14s Y2H assays and OsFD4-Hd3a BiFC
618 **Fig. S3** Y2H mating controls
619 **Fig. S4** Spacing analysis control: OsFD4, OsFD1 and HBF1 ROC curves
620 **Fig. S5** Expression analysis of some DAP-seq targets in *osfd4-1* and *osfd1-1*
621 **Table S1** List of OsFD4, OsFD1 and HBF1 DAP-seq bound genes

622 **Table S2** Sequences of oligonucleotides used in this study

623

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628

629 **Author Contributions**

630 MC, MG, JL, FPa, AG, VB and FF designed research; MC, FG, MG, BK, JL and FPo performed
631 research; MC, FG, MG, BK, JL, ET, VB and FF analysed and interpreted data; MC and FF wrote the
632 manuscript.

633

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