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Rice bZIP transcription factors regulate the floral transition via formation of Florigen Activation Complexes (FACs)

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1 ABSTRACTS

1.1 ITALIAN ABSTRACT

La transizione fiorale è un processo che ha luogo quando la pianta smette di produrre foglie e inizia a sviluppare fiori. In riso (*Oryza sativa*) due molecole, chiamate florigeni, regolano la fioritura, *Heading Date 3a* (*Hd3a*) e *RICE FLOWERING LOCUS T 1* (*RFT1*). Questi geni vengono espressi e tradotti nelle foglie e le loro proteine sono in grado di spostarsi tramite il floema e raggiungere il Meristema Apicale del Germoglio dove avviene la transizione vera e propria. In questo tessuto Hd3a e RFT1 interagiscono con OsFD1, un fattore di trascrizione di tipo bZIP, tramite l'ausilio di Gf14c, una proteina di tipo 14-3-3 che funge da ponte molecolare tra i florigeni ed OsFD1. Il complesso così formato si chiama Florigen Activation Complex (FAC) e regola la trascrizione di *OsMADS14* e *OsMADS15*, due geni coinvolti nello sviluppo del fiore.

Durante il mio dottorato mi sono focalizzata sullo studio di altri fattori di trascrizione di tipo bZIP coinvolti nella fioritura. Abbiamo scoperto che il complesso OsFD1-FAC ha un ruolo anche nelle foglie dove promuove la produzione dei florigeni. Inoltre, abbiamo caratterizzato OsHBF1, un altro bZIP che ha funzione di reprimere l'espressione di *Hd3a* e *RFT1* nelle foglie. Abbiamo infine isolato OsFD4 un bZIP che promuove la transizione fiorale nel meristema in parallelo con OsFD1. OsFD4 interagisce con i florigeni e il complesso OsFD4-FAC attiva l'espressione di *OsMADS14* e *OsMADS15*. Infine, abbiamo condotto DAP-sequencing su OsFD1, OsFD4 e OsHBF1 per trovare i geni regolati da queste proteine e capire il motivo della loro diversa funzione. Abbiamo constatato che i bZIP analizzati legano lo stesso motivo consenso, ma che la distanza tra due motivi non è conservata tra di loro. Questo dato suggerisce che la distanza tra due motivi e non la sequenza contribuisce a determinare la diversa funzione dei bZIP.

1.2 ENGLISH ABSTRACT

When external and internal cues are favorable, rice plants stop to produce leaves and start to make flowers. This process is called floral transition and occurs in leaves and at the Shoot Apical Meristem (SAM). In rice, *Heading Date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) are two signal molecules, called florigens, which are expressed and translated in leaves. Florigens are small and globular proteins with non-cell autonomous functions, as they can move via the vascular tissue of rice and arrive in the SAM, where they promote the transition from the vegetative to the reproductive phase. In this tissue they bind the FD-like bZIP transcription factor OsFD1 thanks to the help of Gf14c, which bridges their interaction, and they form the Florigen Activation Complex (FAC). *OsMADS14* and *OsMADS15* are FAC targets and they are required for proper flower development.

Because the florigens cannot bind the DNA alone, FD-like bZIPs have a crucial role during the floral transition. For this reason, we focused on FACs formation and we characterized different bZIPs involved in the floral transition. We found that in leaves OsFD1-FAC regulates flowering time via positive feedback loops on *Hd3a* and *RFT1* expression. We then characterized *OsHBF1*, a FD-like bZIP, which generates a negative feedback loop on florigens production via OsHBF1-FAC formation. We isolated the bZIP *OsFD4* which promotes the floral transition in the SAM in parallel to *OsFD1*. OsFD4 interacts with Hd3a and RFT1 in FACs and regulates the expression of *OsMADS14* and *OsMADS15*, such as OsFD1-FAC does. Finally, we performed DAP-sequencing with OsFD1, OsFD4 and OsHBF1 to analyze differences among these bZIPs and to find their targets. Results indicate that bZIPs bind the same consensus motif, but the distance between two motives varies depending on the bZIP considered, suggesting that it could be causal to their different role during the floral transition.

2 AIM OF THE THESIS

The floral transition is one of the most important moment during a plant life cycle, and is regulated in two different tissues, leaves and Shoot Apical Meristem (SAM). In rice (*Oryza sativa*) this process is promoted by *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*), two Phosphatidyl-Ethanolamide Binding proteins (PEBP), which are called florigens. These genes are expressed and translated in leaves and then their proteins move via the vascular tissue and arrive in the SAM where they promote floral development instead of leaves production. In rice, to allow this switching, the florigens bind indirectly OsFD1, a FD-like bZIP transcription factor, via Gf14c which is a 14-3-3 protein that bridges this interaction. These proteins together form the Florigen Activation Complex (FAC) which regulates the expression of two floral promoters, *OsMADS14* and *OsMADS15*, in the SAM.

During my PhD I studied rice FACs formed by OsFD1 and by other bZIP transcription factors, such as OsFD4 and OsHBF1. Initially, the reason of this choice was that *osfd1* RNA interference plants showed a very mild flowering phenotype respect to the wild type. Successively, I developed an increasing interest on FD-like bZIP transcription factors and their spatial regulation of floral transition. Two tissues are involved in flowering regulation leaves and SAM. We discovered that more than one FAC exists in rice and that they can be expressed in leaves, in the SAM or in both tissues. Understanding the way in which these complexes regulate the floral transition was the aim of my thesis.

To study the role of bZIPs in this process, I used different approaches. First, thanks to the use of the CRISPR-Cas9 technology, I performed reverse genetic approaches generating FD-like bZIPs mutants and I also crossed them to assess the phenotype of double mutants. I transformed rice to generate lines which overexpressed genes under *pACTIN* ubiquitous promoter, *pOSH1* tissue specific promoter or under the control of a Dexamethasone inducible system. A big part of my work was done using protein-protein interaction assays. I used Yeast Two Hybrid, Bimolecular Fluorescence Complementation and FRET Acceptor Photobleaching to test FACs proteins interactions. Using qRT-PCR I performed quantification of transcripts to analyze genes expression profiles and to verify changes in gene regulation between mutants and wild type plants, or during different time points of floral induction. Finally, I performed high throughput analysis to find new targets regulated during floral transition. To do that I used RNA-sequencing experiments to evaluate genes that change expression in different conditions, and I performed DNA Affinity Purification sequencing (DAP-seq) to find in vitro targets of bZIP transcription factors involved in the floral transition.

3 INTRODUCTION

3.1 Plant models *Arabidopsis thaliana* and *Oryza sativa*

Since many years and in several labs around the world the study of plants floral transition is one of the main research topics. There are a lot of reasons why to focus research on plants flowering, but particularly relevant ones include the fact that floral transition incorporates agronomically important traits, such as cycle length and yield. Additionally, studying flowering also implies understanding developmental patterning and phase transitions of meristems, and the related regulatory circuitry.

Many years ago, *Arabidopsis thaliana* was successfully established as one of the first plant models. Arabidopsis is a plant of no agronomical interest. Yet, it has interesting characteristics that fit well with the research requirements, such as small size, short life cycle, small genome and malleability for transformations and experimental procedures. Thanks to this little plant nowadays a lot of data about many genes involved in floral transition are available.

Another important plant model is rice (*Oryza sativa*) which is a monocot with a strong agronomical importance: about 20% of daily calories for the world population is provided by rice which is a major staple[1]. Rice is a tropical plant with a life cycle of about 4-5 months from seed to seed. In crops research rice is thus a reasonably quick model. It has a diploid genome of about 430Mb which is amenable to transformation and to several experimental procedures. Thanks to these characteristics, rice is one of the easiest crops to use for research purposes, differently from other crop models which have larger or polyploid genomes or longer life cycles, such as maize, barley or wheat.

3.2 Flowering and Photoperiod Perception

"We are survival machines, but 'we' does not mean just people. It embraces all animals, plants, bacteria, and viruses" (Richard Dawkins, "The Selfish Gene")

From a biological point of view, the goal of every individual is its own reproduction and plants are not an exception. In the life cycle of Angiosperms, flowering corresponds to the reproductive phase, in which flowers produce reproductive structures that upon fertilization of specific cells produce seeds which

represent the plant's progeny. To be successful in this process plants must perceive which is the right moment to flower. Because plants cannot move, they developed different ways to understand when favorable conditions occur. They can measure both external and internal cues with different strategies evaluating the level of water or nutrients in soil, the pathogens presence, the temperature and the photoperiod from the environment, but also their own age or the hormones production from their tissues. When conditions are advantageous, plants produce flowering signals that allow the transition from vegetative stage to the reproductive one.

During my PhD I focused my attention on photoperiod perception and the promotion of the floral transition. The photoperiod defines the number of hours of light during the day. For many plant species, photoperiod perception is crucial in order to understand in which period of the year they are, because the yearly variation of the photoperiod is constant and predictable at any given latitude. Plants can be classified based on two responses to the photoperiod. Some plants prefer to bloom under long-day conditions, such as *Arabidopsis thaliana*, when the number of hours of darkness falls below a critical threshold. Other plants prefer flowering under short-day conditions, such as *Oryza sativa*, when the number of hours of darkness exceeds a critical threshold. Long and short-day behaviors depend on their original habitat, but a large variation in the responses to the photoperiod can be observed even within the same species. The variation in the response to day length is even more extreme in cultivated species, as rice, because latitudinal adaptation has been artificially manipulated by breeders during cultivation. These differences could be explained thanks to the different behavior of some regulatory proteins, some of which are conserved in both Arabidopsis and rice, whereas others are specific to some species. In the following paragraphs, I will better explain the components of the photoperiodic pathways of both plant species.

3.2.1 Photoperiod Perception in *Arabidopsis thaliana*

Arabidopsis is a facultative long-day plant which flowers preferentially when the hours of light exceed a critical threshold during the day. In *A. thaliana* the main player in photoperiod perception is *CONSTANS* (*CO*). *co* mutants show flowering delay only in inductive long day conditions suggesting that it has a role in the photoperiod perception[2]. CO is a zinc finger transcription factor which contains a b-BOX domain and a CTT-domain[2,3.] It is supposed that these two domains are implicated in DNA-binding ability and protein-protein interactions. In particular, the CCT domain allows the formation of the trimeric complex NF-YB/NF-YC/CO which binds "CCACA" sequences and in this way genes promotes expression[3,4,5]. This gene is expressed in a circadian-clock dependent manner with a specific cycling pattern during the day. It shows two peaks of expression under inductive long-day conditions, one at the end of the day and the other

at the dawn, and only one peaks between 12 h and 20 h under non-inductive short day condition[2,6]. This distinctive expression is important to accumulate the CO protein during the light phase under long days. Conversely, CO is degraded during the hours of dark thanks to different post-translational regulation pathways[7,6], such as via COP1 regulation[8]. These results indicate that in short day conditions during which there are more hours of dark, CO is absent and cannot promote the floral transition via the production of the florigen FLOWERING LOCUS T (FT), which is a major promotor of flowering (see 3.3.1). Under long-day conditions, during which there are more hours of light, the CO protein is stabilized and promotes the expression of *FT* mRNA and in this way floral transition[7].

3.2.2 Photoperiod Perception in *Oryza sativa*

Contrary to Arabidopsis, *Oryza sativa* flowers preferentially under short day conditions, and the photoperiodic pathway is the major one controlling flowering. Several parallelisms can be drawn between the two species. In rice, different genes are involved in photoperiod perception, but two are the main regulators of photoperiod-dependent flowering induction, *Heading date 1* (*Hd1*) and *Early Heading date 1* (*Ehd1*).

Heading date 1 (Hd1)

Heading date 1 (*Hd1*) is the rice *CO* homolog, which encodes a zinc finger transcription factor with a double role in photoperiod perception in rice. Similarly to *CO*, *Hd1* has diurnal expression profile with a higher mRNA level during the night and weak expression during the day[9]. *hd1* mutants show different flowering behavior based on the photoperiod. In fact, in inductive short-day conditions, lack of *Hd1* causes a delay in flowering time, but under non-inductive long-day conditions, *hd1* mutants show an early flowering phenotype[10,9]. These data suggest a bivalent role of Hd1 in flowering regulation: in inductive short-day conditions Hd1 promotes the floral transition, but in non-inductive long-day conditions it represses this process. The molecular mechanism of Hd1 action is unclear. Like CO, also Hd1 contains two b-BOX zinc finger domains and a CCT-domain, but their role is not completely understood[10]. Evidences show that in short-day conditions, Hd1 promotes directly the expression of the florigen *Heading date 3a* (*Hd3a)* which is a master activator of flowering in rice (see 3.3.2), whereas in long-day conditions it represses the transcription of *Hd3a* and *Ehd1*, which is a major promoter of rice florigens production[9,10].

Early Heading date 1 (*Ehd1*)

Ehd1 is the other major gene controlling flowering in rice, which acts in a photoperiod dependent manner. *Ehd1* is expressed preferentially in short-day conditions and encodes for a B-type response regulator with no homolog in *Arabidopsis thaliana*[11]. Its expression shows a peak in the morning, which is promoted by the presence of blue light[12], indicating that light quality as well as the photoperiod play a crucial role in the regulation of this gene. In leaves, Ehd1 promotes the expression of the florigens, *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*), which are two master activators of flowering in rice, and in this way allow the transition from vegetative to reproductive phase[11,12](see 3.3.2). It was published that *ehd1* mutants show a late flowering phenotype respect to the wild type, whereas overexpression of this gene causes an early flowering phenotype in short day conditions[11,12]. The mode of action of Ehd1 was for many years unknown, but recently it was demonstrated that Ehd1, as member of the Response Regulator (RR) protein family, is involved in the cytokinin signaling[13]. There are two protein types in the RR family, Type-A RRs which negatively regulate the cytokinin signaling and Type-B RRs which are transcriptional activators. Both types contain the R domain which allows dimerization when it is phosphorylated, whereas only the Type-B RRs have the G-domain which is the DNA binding domain. Ehd1 contains both the R domain and the G-domain and seems to be a type-B RRs. Currently, it is supposed that Ehd1 homodimers promote flowering in rice activating Ehd1 targets, unless the OsRR1 protein is present. OsRR1 is a Type-A RR that causes late flowering when overexpressed. It is known that in vitro OsRR1 can bind Ehd1 and it is thought that in vivo OsRR1 can sequester Ehd1, antagonizing its activity and repressing the rice floral transition[13].

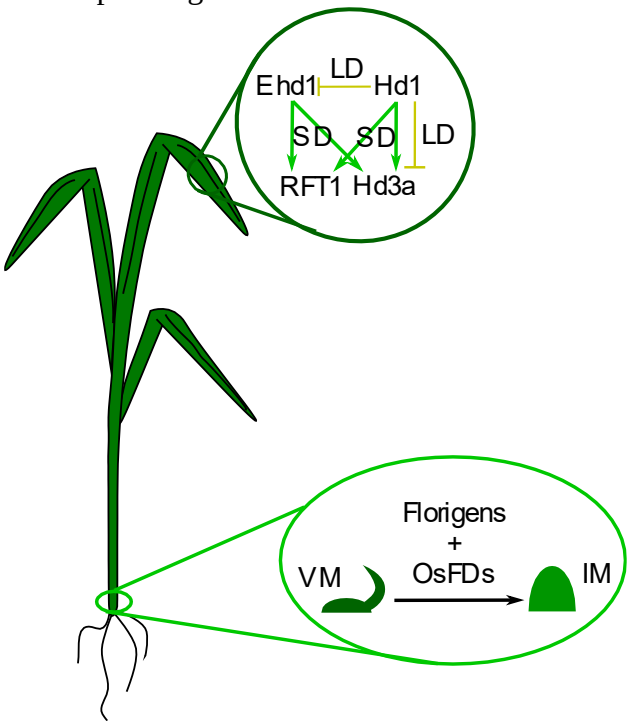


Figure 3.1: Floral transition scheme

Floral transition in rice occurs both in leaves and in SAM thanks to the florigens, *Hd3a* and *RFT1* which connect the two tissues.

In the picture you can appreciate:

a) In leaves the photoperiod regulation of the florigens via *Ehd1* and *Hd1*

b) In the SAM the effect of florigens which allow the transition from the Vegetative Meristem (VM) to the Inflorescence Meristem (IM) thanks to FD-like proteins.

3.3 Flowering signals: Florigens

The floral transition is a complex mechanism which allows the shoot apical meristem (SAM) to change its identity from the vegetative stage to the reproductive one. It is composed by some totipotent cells that during the vegetative phase produce leaves (Vegetative Meristem, VM), but, when floral transition occurs, it changes its developmental program stopping to produce leaves and starting to make flowers (Inflorescence Meristem, IM)[14]. However, flowering is a process that involves different tissues of the plant because the SAM receives signals produced elsewhere, and only integrates spatial and temporal information to generate a panicle. In several plant species, the floral transition is regulated by the expression of genes, called florigens, which are conserved in most flowering plants. Florigens are members of the phosphatidylethanolamine-binding protein (PEBP) family. These proteins are conserved in most organisms, from bacteria to plants and mammals, and play important roles in different processes. They are little globular proteins of about 20 KDa and usually they are non-cell autonomous signals. In plants, measurement of external and internal conditions can regulate florigens expression, promoting it when environmental conditions are favorable. When enough florigenic proteins are produced, they can move through the phloem from the leaves to reach cells of the SAM, where the floral transition occurs. The little and globular structure of these molecules allows the movement through the phloem and through plasmodesmata between cells.

3.3.1 *Arabidopsis thaliana* florigens

FLOWERING LOCUS T (FT) from Arabidopsis thaliana was the first florigen to be cloned[15]. *ft* mutants show a late flowering phenotype compared to the wild type, whereas *FT* overexpression promotes the floral transition, suggesting its role as flowering activator in Arabidopsis[15]. FT is a PEBP which is expressed and translated in leaves thanks to different regulators, most prominently CO. After its production, FT can move from the leaves to the SAM, where it regulates genes important for floral transition[16].

Even though FT was the first florigen discovered, Arabidopsis contains five additional PEBP family members, *TWIN SISTER OF FT* (*TSF*), *TERMINAL FLOWER1* (*TFL1*), *BROTHER OF FT AND TFL1* (*BFT*), *MOTHER OF FT AND TFL1* (*MFT*), and *Arabidopsis thaliana CENTRORADIALIS homologue* (*ATC*), which are grouped in three major clades.

The first clade is composed by *FT* and *TSF* which promote the floral transition in Arabidopsis. *TSF* has a similar function to *FT* and single *tsf* mutants delay flowering only in short-day conditions, while double

tsf/ft mutant shows additive effects in both short- and long-day conditions respect to the single *ft* mutant[17]. Both proteins can move from leaves to SAM, even though TSF is less mobile than FT suggesting that TSF could have a major role in leaves[18,17].

The second clade contains the TFL1-like proteins which are the flowering repressors *TFL1, ATC* and *BFT*. *terminal flower1* (*tfl1*) mutants owe their name to the phenotype which shows a terminal flower with defects in floral organs formation[19, 20]. *TFL1* is expressed in the SAM but not in leaves and has the role to maintain the indeterminate state of the SAM during the transition. It was demonstrated that TFL1 can move cell to cell in the SAM in order to maintain the meristem identity[21]. Contrary to *TFL1* but similarly to *FT, ATC* is expressed in the vascular tissue of leaves and then moves to arrive in the SAM[22]. *ATC* is expressed preferentially in short-day conditions and its function is to repress the floral transition in Arabidopsis[22]. The last TFL1-like is *BFT* which shows an expression pattern similar to FT but has TFL1-like function[23]. In fact, *BFT* delays the onset of flower identity and represses axillary inflorescence development[24]. It was shown that BFT represses flowering competing with FT for FD binding (see 3.4.1) especially in conditions of high salinity concentration in the soil[24].

The last clade contains only *MFT* which has a completely different role than members of the other two groups. *mft* has no effect on flowering time and genetic analysis demonstrated that no additive effects can be found neither in *ft-1/mft* nor in *tfl1-1/mft* nor in *atc-1/mft* double mutants. Nevertheless, MFT has a role in seed germination and its expression is promoted by the ABA signaling during this process.

3.3.2 *Oryza sativa* florigens

In rice, as in Arabidopsis, there are many PEBP proteins with different functions during floral transition and flower development.

Heading date 3a (*Hd3a*) and its homolog *RICE FLOWERING LOCUS T 1* (*RFT1*) are two rice FT homologs [10,25,26]. They are produced in leaves and then their proteins can move through the vascular tissue of leaves and arrive in the SAM where they promote the floral transition[27,25]. RNA interference lines for Hd3a or RFT1 show that in absence of these genes, plants bloom later than the wild type[26]. However, they show distinct roles and expression depending on the photoperiod. In fact, Hd3a RNAi plants show a late flowering phenotype only in inductive short-day conditions[26], whereas RFT1 RNAi lines delay flowering time only in not inductive long-day conditions[25]. These data suggest a different role of these two genes based on specific photoperiods. The double Hd3a RNAi and RFT1 RNAi mutant never flowers under short day, indicating that both florigens are redundant under inductive conditions[26]. This difference

is related to expression of Hd3a and RFT1. When plants are grown under short days expression of both florigens is activated, whereas growth under long days promotes expression of RFT1 only. This differential sensitivity to day length depends on distinct promoter set ups[25,26].

In rice there are 4 different CENTRORADIALIS-like proteins, *RICE CENTRORADIALIS 1* (*RCN1*), *RCN2*, *RCN3* and *RCN4*[28]. These genes are classified as members of the *TFL1* subclade and are expressed in rice vascular tissue[28]. RCNs can move through the phloem and arrive in the SAM where they regulate floral transition. RNAi plants for *RCNs* show several defects in panicle development such as a reduced number of spikelets and branches respect to the wild type[28], whereas when *RCN1* (*35S::RCN1*) is overexpressed, it causes an increase in secondary branches production[29].

Recently, *OsMFT1*, the homolog of *AtMFT*, was published. OsMFT1 has a double role in rice floral transition and is expressed in both leaves and SAM[30]. In leaves this gene represses the expression of *Ehd1* delaying flowering, instead in SAM it regulates late stages of panicle formation activating the production of primary and secondary branches regulating the expression of rice sepallata-like genes, such as *OsMADS34* (see 3.6.2).

3.4 FLORIGEN REGULATOR COMPLEXES

Internal and environmental conditions regulate florigens production which are the main triggers of floral transition. Because florigens are PEBP, they lack a DNA binding domain and for this reason they cannot regulate gene expression directly. To do so, they interact with some bZIP transcription factors to regulate gene expression. In plants bZIPs are one of the largest family of transcription factors. These proteins contain a conserved sequence which encodes for basic DNA-binding domain and an adjacent leucine-zipper domain[31]. This last domain allows bZIPs to homo- or hetero-dimerize. Their number and combinatorial mode of action give them the ability to be very adaptable, covering a wide range of functions during the plant life cycle. bZIPs have been so far implicated in flowering onset and flower development, pathogen defense, ABA signaling, seed maturation and light signaling[32]. Usually bZIP transcription factors bind specific DNA consensus motives which have a central core composed by "ACGT" sequence[33]. Here, I will focus my attention in the description of FD-like bZIPs which are usually involved in flowering regulation.

3.4.1 bZIP transcription factors controlling flowering in *Arabidopsis*

In *Arabidopsis thaliana FD* encodes for a bZIP transcription factor that directly interacts with the florigens[34]. This gene is expressed in the SAM and *fd* mutants are late flowering compared to the wild type indicating that *FD* is an activator of the floral transition in *Arabidopsis*[34,35]. FD can interact with FD PARALOGUE (FDP) which is another bZIP transcription factor expressed in the SAM and which has a role in floral transition in Arabidopsis[34].Using in situ hybridization *FD* expression could be detected in all the SAM, instead *FDP* mRNA is present only at SAM flanks[36]. *fdp* mutants show a mild flowering delay respect to single *fd* mutants, whereas the double *fd/fdp* mutant is very late flowering. When the double mutant is crossed with very early flowering *FT* overexpressing plants, it can suppress the effect of *FT* overexpression, meaning that both genes are important to mediate the FT florigenic signal in promoting flowering[36].

The interaction between FD and PEPB family members was well studied. In the C-terminus FD contains a conserved SAP domain which is necessary to allow the interaction between FD and FT[34,37]. It was shown that FD can also directly interact with TFL1[20], BFT[24] and ATC[22] via its C-teminus and in this way represses floral transition. The FT-FD interaction is supposedly taking place in the SAM, where FD provides DNA-binding activity to the complex and allows FT to regulate gene expression. It was demonstrated that FD can bind the promoter and activate expression of some important genes involved in floral development such as *AP1* and *FUL* (see 3.6.1)[34,35,37]. Recently, direct and indirect targets of FD were found using a combination of ChIP-seq and RNA-seq. The results indicate that FD bind in the SAM and in leaves a conserved "GTCGAC" motif regulating 595 and 1754 genes respectively[37]. It was also published that FD interacts with the DNA in absence of FT and TSF, whereas these two proteins stabilize the binding of the transcription factor to the DNA[37]. Taken together these data suggest that the role of bZIP transcription factors in flowering regulation is to mediate florigenic signals at the SAM during the floral transition in Arabidopsis.

3.4.2 bZIP transcription factors controlling flowering in rice

Such as in Arabidopsis, rice FDs have a central role in flowering regulation in the SAM, but there are also some evidences that suggest that OsFD proteins work in leaves regulating florigens production in this tissue. The first bZIP transcription factor demonstrated to control flowering was OsFD1 which was initially isolated as interactor of Hd3a and part of the rice Florigen Activation Complex (FAC)[38]. *OsFD1* is expressed in both leaves and SAM and its protein has a high homology with AtFD[38,39]. Using protoplasts,

it was demonstrated that OsFD1 can activate the transcription of *OsMADS14* and *OsMADS15*, two keys activators of the floral transition in rice (see 3.6.2), but despite of that, RNA interference plants for this gene have a very mild delay in flowering time, indicating that loss of *OsFD1* does not compromise transition to flowering[38]. The FAC has a hetero-hexameric structure which is composed by two molecules of the florigen Hd3a, two molecules of OsFD1 and two molecules of Gf14c, a 14-3-3 protein which acts as molecular bridge between OsFD1 and Hd3a. The Gf14c role seems to be very relevant, in fact the direct interaction between OsFD1 and Hd3a does not occur, but both proteins can directly interact with Gf14c[38]. At the cellular level, Hd3a arriving in cells of the SAM interacts with Gf14c in the cytoplasm and is then shuttled to the nucleus where the heterodimer contacts OsFD1. Because of its central role as florigen interactor, Gf14c and homologues, are often considered florigen receptors. The crystal among Hd3a, Gf14c and the OsFD1-SAP domain was produced and the structures at the interface between Hd3a-Gf14c and Gf14c- OsFD1-SAP were resolved (Fig 3.2)[38]. The crystal shows that Hd3a and Gf14c interact forming a tetramer composed by two molecules of Hd3a and two of Gf14c in a W configuration (Hd3a-Gf14c-Gf14c-Hd3a; fig 1.2 a). Two positive charged pockets are located at the corners at the inner base of the W and allow the interaction between Gf14c and the OsFD1 phosphorylated at S192. It is relevant to note that the crystal was obtained using only 9 amino acids of the OsFD1 C-terminus which encodes for the SAP domain[38]. Thus, while the interface between Hd3a and Gf14c is well resolved, the one between Gf14c and OsFD1 might be less representative. These few amino acids are shared by most of the proteins that interact with 14-3-3 proteins because in this motif there is a Serine or a Threonine which must be phosphorylated in order to allow the interaction with 14-3-3s. The OsFD1 SAP domain contains a Serine at position 192 which must be phosphorylated to permit OsFD1 function. In fact, changing this residue to alanine abolished OsFD1 interaction with the FAC proteins. Conversely, overexpressing an OsFD1 version harboring the phosphomimic substitution S192E causes an early flowering phenotype in rice[38].

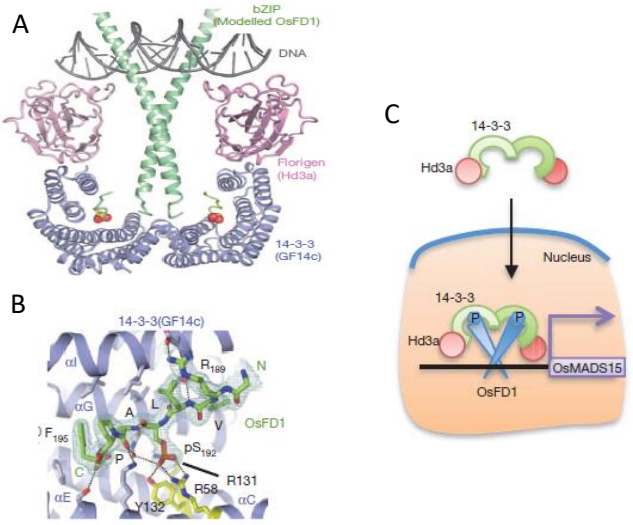


Figure 1.2: Florigen Activation complex Structure

A) Model based on the crystal among Hd3a, Gf14c and OsFD1 SAP domain. Red circles represent phosphorylation. The central part which contains the OsFD1 C-terminus leucin zipper domain and basic domain contacting DNA was modelled using the mouse CREB bZIP-C-Box DNA complex. B) Detail of important residues at the OsFD1-Gf14c interface. OsFD1:S192; Gf14c: R58, Y132, R131. C) Model of FAC activity: Hd3a binds Gf14c in the cytosol and then enters in the nucleus where Gf14c can interact with OsFD1. After its formation FAC promotes *OsMADS15* and *OsMADS14* expression.[38]

In the rice SAM the 4 *RCNs*, *RICE CENTRORADIALIS 1* (*RCN1*), *RCN2*, *RCN3* and *RCN4*, do not have a repression function in flowering time regulation instead they are promoters of secondary branches production (see 3.3.2). The interaction between these proteins and OsFD1 was tested and it was demonstrated that OsFD1 can interact with them via 14-3-3 proteins. For this reason, and in order to distinguish these complexes from FACs, it was decided to call them FLORIGEN REPRESSOR COMPLEXES (FRCs).

FACs could have different roles in the rice life cycle than regulating floral transition. An example is provided by the *OsFD2*-FAC which has an important role in leaves and panicle development[40]. Overexpression of *OsFD2* under the *Ubiquitin* promoter (*pUBQ::OsFD2*) causes the production of many smaller leaves respect to the wild type[40]. Conversely, a higher *OsFD2* expression changes also the panicles morphology producing more seeds than the wild type, and suggesting that *OsFD2* repressed the transition from branch meristem to spikelet meristem[40]. OsFD2 can regulate these processes interacting with Hd3a via Gf14b and forming an OsFD2-FAC[40]. Taken together these data show that FACs could be versatile in their function thanks to the florigen and the bZIP transcription factor which form the complex.

3.4.3 FDs controlling flowering in other species

FD-like proteins are conserved also in other species, such as maize (*Zea mays*), pea (*Pisum sativum*), tomato (*Solanum lycopersicum*) and wheat (*Triticum aestivum*).

DELAYED FLOWERING 1 (*DLF1*) is the maize FD-like bZIP transcription factor. *dlf1* mutants have flowering delay, develop more tassel branches and more ears for each node compared to the wild type[41]. In situ hybridization demonstrated that *DLF1* is expressed in the SAM[41] and interacts with ZCN8, the FT homologue of maize, in Yeast Two Hybrid assays (Y2H)[42]. All these data suggest that *DFL1* has a role to promote the floral transition, similarly to *AtFD*.

Pisum sativum *VEGETATIVE2* (*VEG2*) encodes for a bZIP transcription factor which has an FD-like function in pea. *veg2* mutants delay flowering time respect to the wild type and show defects in inflorescence determinacy. It was demonstrate that VEG2 can form florigen activation complexes with all the five pea FT-like proteins and it is supposed that in this way it is able to promote the floral transition[43].

In tomato (*SELF PRUNING G-BOX PROTEIN*) *SPGB* is the putative FD-like protein. *SPGB* is a bZIP transcription factor expressed in leaves and SAM of tomato and can interact with SELF PRUNING (SP),

the tomato ortholog of CEN and TFL1 and SINGLE FLOWER TRUSS (SFT) the ortholog of FT. *spgb* mutants show less leaves per sympodial shoot compared to the wild type, are late flowering and interact with SP and SFT via 14-3-3 protein. Using in situ hybridization, the *SPGB* expression could be detected in the vegetative meristem but decrease progressively during the floral transition of the sympodial meristem[44,45]. These data suggest conservation of the FAC system and mode of assembly in tomato.

In wheat at least three different FD-like bZIP transcription factors were found, *TaFDL2*, *TaFDL6* and *TaFDL13*[46]. Unfortunately, no mutants for these genes are available, but interaction assays with these proteins show different behavior among them. In fact, using Y2H, Li et al demonstrated that TaFDL2 and TaFDL6 can directly interact with TaFT, a wheat florigen, whereas only TaFDL13 binds TaFT2, the other florigen. Moreover, they demonstrated that the promoter of *VERNILAZATION 1* (*VRN1*), an *AP1* homolog, can be bound only by TaFDL2 in EMSA assays. Interestingly, *TaFDL2* and *TaFDL6* are expressed at high levels in both leaves and SAM, instead *TaFDL13* mRNA is present in a higher level in SAM than in leaves[46]. In another paper the same authors demonstrated that TaFDL2 and TaFDL6 can interact with six different wheat 14-3-3 proteins, from A to H, and that TaFDL15 can interact with 4 Gf14s, A, B, C and E[47]. Interestingly in the same paper they show that TaFDL13 is a splicing variant of TaFDL15 in which an intron retention causes a premature stop codon that generates a truncated protein lacking the SAP domain and is not able to interact with 14-3-3 proteins[47]. It is important to remember that in any case TaFDL13 can interact with TaFT2, instead TaFDL15 fails in this interaction[46]. These observations generate some questions about the importance of 14-3-3 proteins in the interaction between FD-like and FT-like proteins. All these results indicate that probably in wheat not only more FD-like proteins possibly regulate the floral transition (genetic evidence still lacking), but they could have different functions interacting with different partners in different tissues, such as *OsFD2* does in rice (see 3.4.2). This possible model could help to explain how wheat can regulate in a fine way a complex process.

3.5 Inflorescence Meristem (IM) formation

During floral transition the SAM that normally produces leaves undergoes a complete and deep change which allows the production of inflorescences. Rice develops a panicle, characterized by the formation of primary branch meristems that differentiate secondary branch meristems, in turn producing spikelets, which are compressed reproductive branches forming protective bracts enclosing a single bisexual flower. This process if formed by a series of SAM transformations which are divided into different morphological stages (Fig. 1.3). Because of the complexity of this process, space, time and level of gene expression are

very important during these events. Several regulatory proteins take part to it. A lot of positive and negative feedback loops seem to be involved in floral transition and the disruption of these fine regulated mechanisms could avoid the correct progress of the process itself. During transition, the first step is the formation of the inflorescence meristem (IM) which is then followed by the formation of the branches or floral meristems, depending upon the species considered.

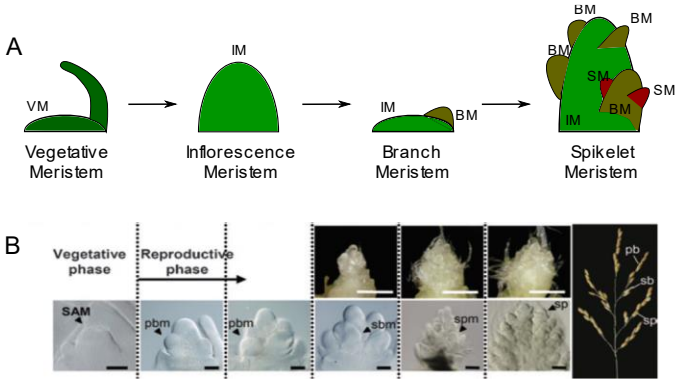


Figure 3.3: Floral transition meristem transformations

A) *Oryza sativa* SAM transition: the SAM changes its identity from VM to IM and then at IM sides the developmental program shifts from IM to BM. After that BRs incur in another transformation which changes BMs in SMs.

B) Image of SAM changes during the transition[48]

In rice the transition from Vegetative Meristem to Inflorescence Meristem is promoted by *OsMADS50* which is the *AtSOC1* homolog and shares the 50.6% of amino acid identity with this gene. Such as in Arabidopsis, *OsMADS50* is expressed in both leaves and SAM and its level increases during rice floral transition[49]. Plants which overexpressed *OsMADS50* flower in the regeneration medium during the plant transformation, instead *osmads50* mutants show late flowering phenotype compared to the wild type[49]. It was demonstrated that in leaves *OsMADS50* regulates the expression of *Hd3a* and rice *AP1*-like genes, *OsMADS14*, *OsMADS15* and *OsMADS18* which are also FAC targets[38]. All these data taken together show that *OsMADS50* activity likely controls the first stages of the rice floral transition[49].

Despite of the importance of the FAC during transition from VM to IM, few genes are known to be regulated by this complex. For this reason, using RNA-sequencing, an extensive expression analysis was done comparing wild type SAM with *hd3a/rft1* double mutant SAM[39]. From this analysis 159 genes were up-regulated and 743 genes were downregulated in the wild type respect to the mutant, meaning that lack of florigens causes a deregulation in the expression of at least 902 genes[39]. Among the florigens positively regulated genes particularly enriched was the class of MADS box transcription factors, such as *OsMADS14*, *OsMADS15*, *OsMADS18* and *OsMADS34*, a result that validates an earlier observation[50]. Genes involved in cell cycle progression or auxin signaling perception, such as *OsAUR1*, *PCNA*, *CycB2;2*, a proline oxidase and *OsIAA19*[39] were also upregulated. Interestingly a high enrichment of Transposable Elements (TEs) was found in genes that are downregulated by florigens which reach the 70% of total downregulated genes. The reason why of this downregulation is poorly understood for the moment but

some hypothesis was produced. It seems plausible that TEs must be blocked to allow a major DNA stability during floral transition because this process is very complicated and TEs perturbations could damage the delicate balance proper to meristem transition[39]. However, it is also to be noted that since *hd3a/rft1* double mutants are never-flowering, plants were propagated vegetatively, and meristems sampled from them were likely much older than the wild type ones, indicating that some remobilization of TEs might have occurred during aging.

3.6 Inflorescence Morphology: Spike and Panicle

In grass species there are two different types of inflorescences: spikes or panicles. The main difference between these kinds of structures is that in spikes, flowers are directly attached to the inflorescence stem while in panicles there is the presence of some secondary structures, called branches, that support spikelets (fig 3.4). Nevertheless, the differences between these two structures, the developmental processes that allow their formation, show some common check points regulated by homolog proteins.

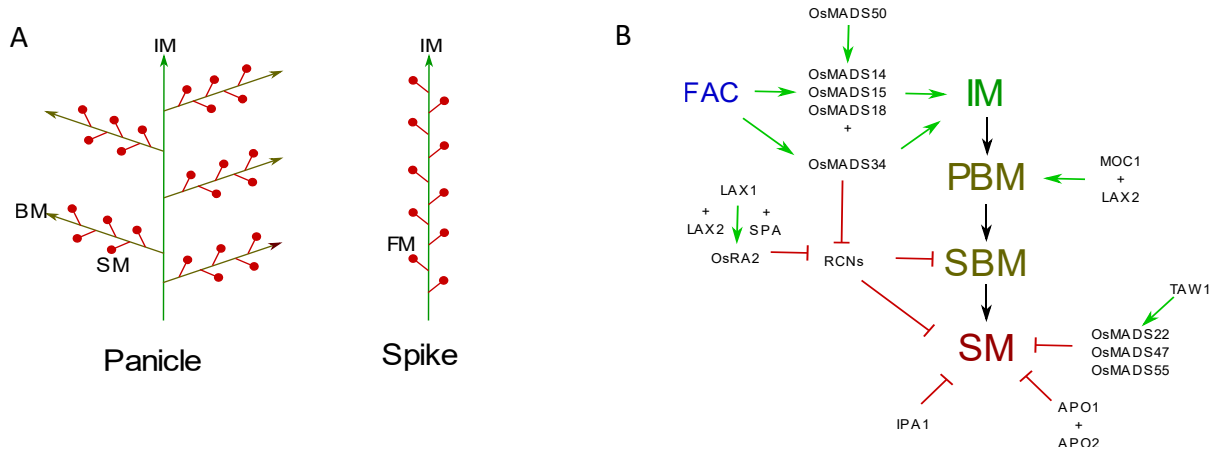


Figure 3.4: Spike and Panicle formation

A) Structure of panicle and spike: in green IM, in brownish BM and in red SM or FM. B) Pathway of rice floral transition from Inflorescence Meristem (IM) to Primary Branch Meristem (PBM) to Secondary Branch Meristem (SBM) to Spikelet Meristem (SM)

In rice the inflorescence developed in a panicle structure in which SAM transformation requires more steps to move from the IM to the spikelet meristem (SM). These steps require the formation of Branch Meristems (BMs) which are meristems from which branches are formed and that bear the spikelet meristem (SM). In

rice there are two types of BM, primary branch meristems (PBMs) formed by the IM, and secondary branch meristems (SBMs), that are formed by PBMs. Unfortunately, the mechanisms that allow the transition from IM to BM and then to SM are still poorly understood, whereas is clear that the time from one step to another is crucial to determine the number of branches and spikelets.

Axillary meristems play an important role in the transition from IM to BM because they indicate the position where BM will be formed. In rice *LAX PANICLE1* (*LAX1*) and *SMALL PANICLE* (*SPA*) are basic helix-loop-helix (bHLH) transcription factors which are expressed where axillary meristems will be formed. *lax1* mutants show a reduction of the number SBMs and the double mutant *lax1/spa* causes a most severe phenotype which lacks branches completely[51]. Because bHLH transcription factors usually interact each other, it was supposed that LAX1 and SPA directly interact to promote BMs formation[51]. Another important cofactor of *LAX1* is *LAX PANICLE2* (*LAX2*) which encodes for a nuclear protein with an unknown function[52]. Differently from *LAX1*, *LAX2* is expressed in all the BMs and its expression profile is broader even if it overlaps with the *LAX1* in axillary meristems[52]. *lax2* shows a reduced number of total lateral branches and spikelets, whereas the double *lax1/lax2* mutant fails completely to form SBMs and produces only a terminal spikelet per branch[52]. Because LAX2 can interact with LAX1 but has no DNA-binding domain, it is supposed that LAX2 is a coregulator of LAX1 in SBM determinacy[52]. Recently *OsRAMOSA2* (*OsRA2*) was shown to be a target of *LAX1* pathway[53]. *OsRA2* is expressed in branches meristems and *osra2* mutants show a reduced number of secondary branches[53]. Moreover, *lax1* mutants downregulates the level of expression of *OsRA2* suggesting that this gene acts downstream to *LAX1* in BM determinacy, whereas *OsRA2* function is upstream *RCNs* regulation. The genetical prove of this regulation is done by the *rcn2* mutant which shows a higher number of secondary branches respect to the wild type, a phenotype that could be intensified overexpressing *OsRA2* under the *UBIQUITIN* (*UBI*) promoter in the *rcn2* mutant background[53].

MONOCULM1 (*MOC1*) is another important gene in this process which encodes for a GRAS family transcription factor[54]. *MOC1* is expressed in the axillary meristems and *moc1* mutants show a lower production of tillers and panicle branches meaning that this gene plays a critical role in axillary meristems formation[54]. Double *lax2/moc1* mutants show extreme defects in primary and secondary branches formation and produce a single branch with only a terminal spikelet[52]. Despite no evidences were produced, it could be that LAX2 interact with MOC1, such as with LAX1, and in this way regulates primary branches formation in rice[52].

In Arabidopsis thaliana AP1 is a master gene that allow the transition from IM to FM. In rice there are four different AP1-like genes, *OsMADS14*, *OsMADS15*, *OsMADS18* and *OsMADS20*, and all of them seems to have a role during rice panicle formation[55].

The most studied AP1-like genes of rice are *OsMADS14* and *OsMADS15*. In *Hd3a* or *RFT1 RNAi* mutants the expression of these two genes is strongly downregulated at the SAM compared to the wild type[26]. *OsMADS14* and *OsMADS15* are direct targets of the FAC and their upregulation during floral transition is correlated with the transition itself[38]. Recently it was shown that *OsMADS15* expression increases in the SAM during IM, PBM and SBM transition and that the protein localizes in nuclei of cells[39]. Single mutants of *osmads14* and *osmads15* show only minor problems during flower development, whereas the double *osmads14/osmads15* mutant shows strongly aberrant flowers with duplications or transformation of floral organs[56]. *OsMADS18* is expressed in all rice tissues with a high level in roots and flower meristems. In flowers it seems to be expressed preferentially in secondary branch meristems. Even though *OsMADS18* is involved in different processes such as tillers production, flower development and also seed germination, RNAi mutant lines of *OsMADS18* show no flowering phenotype compared to the wild type, suggesting that its function is redundant with other MADS-box transcription factors[55]. The overexpression of *OsMADS18* shows early flowering and reduced internode elongation compared to the wild type suggesting that an increased level of this gene promotes floral transition in rice[55]. *OsMADS20* is the strangest AP1-like transcription factor of rice. It is expressed in the vegetative meristem in high level but then, after the floral transition, it is downregulated very fast and its expression disappears in the IM. Single mutants for *OsMADS20* show no flower development phenotype making this gene a true enigma for the researchers[56]. *OsMADS20* behavior is completely different from the one of other *AP1*-like genes, which could suggest a different role of this MADS box during floral transition or a more complicated involvement of MADS boxes in rice floral development. Redundancy between members of the AP1-like clade of MADS-box has been addressed by construction of triple *osmads14/osmads15/osmads18* RNA interference plants and quadruple simultaneously silence all four of the AP1/FUL-like genes (ssa). Such multiple mutants show defects in floral organs, in particular glumes and lemmas are transformed in leaf-like organs, paleas became carpel-like structure, whereas lodicules appear to be papery or change in stamen-like organs[50].

OsMADS34, or *PAP2*, is a SEP-like gene and plays a key role during the floral transition in rice. It starts to be expressed in the IM and its level increases drastically when the SM will be formed from the BM. Single *osmads34* mutants shows no phenotype compared to the wild type. Such as AtSEP proteins, OsMADS34 can directly interact with AP1-like genes[50], for this reason, the quadruple *osmads14*/*15*/*18*/*34* mutant was produced. The lacking of these four genes stops the transition from VM to IM and causes

continuous leaves production instead of panicles development[50]. Few is known about the targets of *OsMADS34* , but recently it was published that it can repress rice *RCN* genes expression and in this way delay the transition from BM to SM formation[57].

All these data indicated that AP1-like genes in rice have a double role in the transition. Probably with the help of *OsMADS34* they regulate the initial steps of the transition from VM to IM and then in a second step they could act during flower development promoting the transition from BM to SP.

The transition from and indeterminate meristem (IM or BM) to a determinate one (SM) is finely regulated in rice. The mutant *aberrant panicle organization 2* (*apo2*) is allelic to the *LFY* rice orthologue, also called *RICE LFY/FLO* (*RLF*)[58]. *APO2/RLF* is expressed in IM, BM and SM too and its mutants show different defects in rice plants such as reduced panicle height, a delay in flowering time and small panicles. This last phenotype could be explained by the fact that in *apo2* mutants the size of IM and BM is reduced and there is a precocious transition from these stages to the SM phase[58,59]. All these data suggest that *RLF* in rice has an opposite function compared to *AtLFY*. In fact, in rice *RLF* is necessary to maintain the SAM indeterminate, whereas in Arabidopsis *LFY* promotes the transition from indeterminate (IM) to determinate (FM) SAM[58,59]. Another important gene involved in this pathway is *APO1* which expression overlaps with the one of *APO2*. *APO1* is the orthologue of the Arabidopsis thaliana *UNUSUAL FLORAL ORGANS* (*UFO*) which encodes for an F-box protein[59]. In rice the function of *APO2* and *APO1* is strongly related to one another and the *apo1* phenotype is like the *apo2* one, with small panicles and flowering defects suggesting that these genes act in the same pathway[58]. Evidences show that APO1 and APO2 can directly interact and in this way slow down the transition from BM to SM to allow the correct timing of this transformation[58].

SQUAMOSA PROMOTER BINDING PROTEINS (*SPLs*) are transcription factors which regulate the transition processes in plants. Such as *APO1* and *APO2*, the rice *SPL14*, also called *Ideal Plant Architecture1* (*IPA1*), represses the transition from BM to SM. *IPA1* is expressed only in the epidermal cells of IM, BMs and SMs during the transition[60]. An increased level of *IPA1* causes panicles phenotype with more branches and spikelets, suggesting that this gene maintains in some way the BM indeterminate and regulates the timing of the transition to SM[61,60].

Another important gene which acts in the transition between BM and SM is *TAWAWA1* (*TAW1*). *TAW1* has a role such as repressor of SM identity in rice and it is expressed in BMs[56]. Dominant mutants of this gene have spikelets with more branching respect to the wild type and contrary, loss-of-function mutants have spikelets with less branching respect to the wild type[62]. *TAW1* is a member of the ALOG family

whose members contain a DNA binding domain and act such as transcriptional cofactors. Some evidences from Arabidopsis and maize indicate that *TAW1* homologs maintain SAM stem cells identity and in some cases define the boundaries between the SAM and the new organs[62]. Thus, it is possible to suppose a similar role for *TAW1* also in rice, even if more experiments must be done in order to confirm this hypothesis. It was also demonstrated that TAW1 regulates the expression of *OsMADS22*, *OsMADS47* and *OsMADS55* which are three different SVP genes in rice[62]. Overexpression of these genes promotes the vegetative development in both rice and Arabidopsis suggesting an analog SVP role in rice and Arabidopsis.

4 RESULTS

4.1 The OsFD1- like bZIP transcription factors clade

The FAC has a central role in flowering time, and bZIP transcription factors are central to its function. However, no flowering phenotype is detectable in RNAi OsFD1 plants. Therefore, we decided to further investigate the role of other bZIP transcription factors possibly involved in the rice floral transition, to test the possibility that some genes are redundant with or act in parallel to *OsFD1*. In the rice genome there are 89 bZIP transcription factors divided in 11 classes, according to their DNA-binding specificity and amino acid sequences in basic and hinge regions[63,64].

We selected some bZIPs based on aminoacid similarity with AtFD and AtFDP, such as OsFD1 and other FD-like proteins from maize and wheat (fig 4.1 and Brambilla et al[65]: suppl fig 3A). All the proteins in the phylogenetic tree show high similarity with AtFD and OsFD1, but the lower part of this tree contains a clade including bZIP transcription factors of known function that have been implicated in regulation of ABA signalling. Even though a link between ABA signalling and flowering time control has been established[66], we decided to focus our attention on proteins belonging to the OsFD1-like clade.

The OsFD1-clade includes *OsFD1* (*OsbZIP77*), *OsFD2* (*OsbZIP55*), *OsFD5* (*OsbZIP56*), *OsFD6* (*OsbZIP54*), *OsFD3* (*OsbZIP24*), *OsbZIP27*, *OsFD4* (*OsbZIP69*), *Hd3a Binding Factor 1* (*HBF1/OsbZIP42*), *Hd3a Binding Factor 2* (*HBF2/OsbZIP9*), *OsbZIP62* and *OsbZIP29*. Within this clade *OsFD2*, *OsFD5* and *OsFD6* are involved in leaves development[40], for this reason I decided to focus my attention on the other members. More systematic sequence comparisons showed that *OsbZIP27*, similarly to *OsFD6*[40], is a pseudogene. Among the remaining ones, *OsbZIP27* and *OsbZIP29* could not be amplified from cDNA prepared from leaves nor from SAMs. We discovered from available microarray data that *OsbZIP29* is expressed only in roots. Given these evidences, we chose to further analyse the role of *OsFD3*, *OsFD4*, *HBF1*, *HBF2* and *OsbZIP62*.

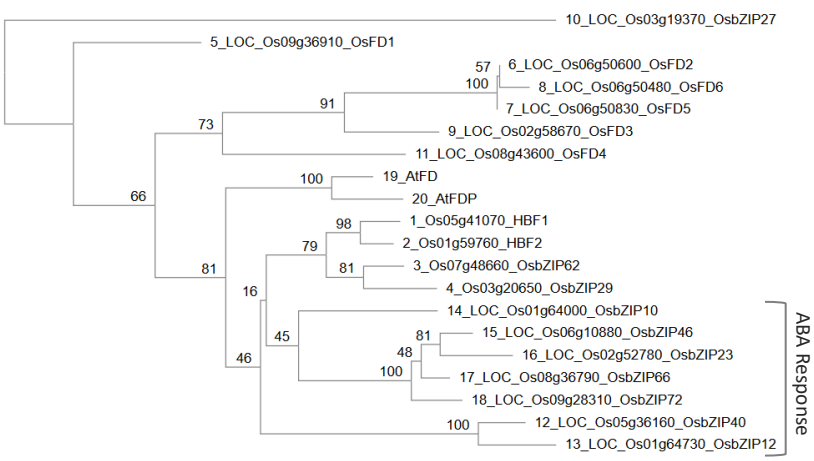


Figure 4.1: Phylogenetic tree of FD-like rice protein.

Alignment of protein sequences was done using "MAFFT" online tool and then visualized using "Phylo.io". Gap open penality=1.53; Offset value=0. Bootstrap of 100.

4.2 *Hd3a Binding Factor 1* (*HBF1*) and *Hd3a Binding Factor 2* (*HBF2*) downregulate florigens expression in leaves

In order to better understand the role of *RFT1* and *Hd3a* during the floral transition, we generated transgenic lines to conditionally overexpress each florigen after induction with Dexamethasone (Brambilla et al[65]: fig 2a)[67]. After the induction of *DEX::RFT1* lines and *DEX::Hd3a* lines, expression of the transgenic clones of the florigens increased in leaves (Brambilla et al[65]: fig 3a and 3c), but we discovered that after dex induction, transcription of the endogenous *RFT1*, *Hd3a* and their activator *Ehd1* decreased significantly in leaves (Brambilla et al[65]: fig 3b and 3d). This indicated the existence of a negative feedback loop promoted by the florigens which auto represses their own expression directly or via *Ehd1* downregulation.

Because the florigens cannot bind DNA alone but require bZIP transcription factors to do it[38], we decided to verify if some OsbZIPs in our list (fig 4.1) could participate in this feedback loop and delayed the floral transition by repressing florigens production in leaves.

Analysing the *oshbf1-1* T-DNA mutant line, we discovered that mutations in *OsHBF1* causes early heading. This result suggests that *OsHBF1* has a repressing function in rice flowering (Brambilla et al[65]: fig 5d and suppl fig 4a, 4b).

Because OsHBF1 shares high homology with OsHBF2, we decided to use the CRISPR-Cas9 technology to generate new mutant alleles for both genes. Design of the single guide RNA (sgRNA) was done in order to target at the same time both *OsHBF1* and *OsHBF2*. We obtained different combinations of double mutant alleles which presented at the same time mutations in *OsHBF1* and *OsHBF2*. Double *oshbf1/oshbf2* mutants showed an early flowering phenotype. Yet, no additive effect could be detected in the double mutant compared to the *oshbf1* single and single *oshbf2* mutants showed no phenotype compared to the wild type (Brambilla et al[65]: fig 5h, 5i, 5j, 5k and suppl fig 5a, 5b, 5c). Therefore, we could not exclude completely the role of OsHBF2 during floral transition. For this reason, we decided to overexpress *OsHBF1* and *OsHBF2* under the control of the *ACTIN* promoter (*pACTIN*). In both transgenic lines we obtained a late flowering phenotype (Brambilla et al[65]: fig 5c). Taken together, these data indicate that OsHBF1 has a role in flowering repression in rice, whereas OsHBF2 acts on this process only when overexpressed.

From these data we could conclude that inside the *OsFD1-like* clade, the HBF-like subclade contains two bZIPs, *OsHBF1* and *OsHBF2*, which are repressors of rice floral transition. Moreover, we knew from our results that florigens generated a negative feedback loop on their own production and from literature that

Hd3a and RFT1 need bZIP transcription factors to bind the DNA[38]. For these reasons we decided to verify if OsHBF1 and OsHBF2 could interact with the florigens and in this way regulate florigens production.

Because florigens expression occurs in leaves, we decided to analyse if *OsHBF1* and *OsHBF2* are expressed in that tissue and could delay flowering via the florigens. We discovered that both genes are expressed in both SAM and leaves (Brambilla et al[65]: suppl fig 3b and 3c) indicating that they could repress florigen production in leaves. Using real-time PCR, we quantified the level of florigens expression in *oshbf1*, *pACT::HBF1* and *pACT::HBF2*. We found that *Hd3a* , *RFT1* and also *Ehd1* expression was upregulated in *oshbf1* respect to the wild type, instead they are downregulated in HBF overexpressors (Brambilla et al[65]: fig 5a, 5b and 5e). These data explained *HBFs* phenotypes and indicated that these bZIPs could interact with the florigens to regulate their expression in leaves.

In order to regulate flowering in rice, the florigens and OsbZIPs interact in the FAC[38], so we decided to verify if OsHBF1 and OsHBF2 could interact with FAC proteins. Using Yeast 2 Hybrid (Y2H) experiments we found that both HBFs can interact directly with Gf14c, the 14-3-3 protein that bridges the interaction between the florigens and OsFD1[38] (Brambilla et al[65]: fig 4a). Also, direct interactions between the florigens and OsHFB1 and OsHBF2 was tested. We discovered that Hd3a directly interacts with both OsbZIPs, but no interaction was found when RFT1 interaction was tested (Brambilla et al[65]: fig 4a). Because in rice a direct interaction between the florigens and OsbZIPs was never observed, we decided to confirm the data using Bimolecular Florescence Complementation (BiFC), FRET-Flim and GST-Pull down assays (Brambilla et al[65]: fig 4b, 4c, 4d and 4e). All the data confirm that OsHBF1 and OsHBF2 can directly interact with Hd3a, instead the interaction between HBFs and RFT1 could occur only via Gf14c.

In rice, florigens expression is promoted by Ehd1[11], so we next tried to understand if OsHBF1 is able to bind directly the promoter of *Ehd1*, *Hd3a* or *RFT1* to repress their expression. Plant bZIP transcription factors usually bind the DNA region that contains sequences with a conserved motif "ACGT" in the core. Three types of motives are present in plants and could be potentially bound by bZIPs, the A-box "TACGTA", the C-box "GACGTC" and the G-box "CACGTG"[31,33]. Scanning the 1500 bps before the ATG of *Hd3a* and *RFT1* no conserved binding sites were found. However, in the *Ehd1* promoter three "ACGT" core elements were found (Brambilla et al[65]: suppl fig 5d). In order to verify if OsHBF1 could directly bind these motifs, we performed Elettro Mobility Shift Assays (EMSA) designing an oligonucleotide containing the first core motif found upstream of *Ehd1* start codon. As shown in figure 6d of Brambilla et al[65], OsHBF1 is able to bind the oligonucleotide labelled with Cy5 and the band disappears after adding the cold oligonucleotide. From those data we could conclude that OsHBF1 can bind the *Ehd1*

promoter, and that *Ehd1*, *Hd3a* and *RFT1* mRNA expression could be repressed either directly or indirectly by this binding. Recently, I performed DNA Affinity Purification Sequencing (DAP-seq) using the OsHBF1 transcription factor and I confirmed the results of EMSA. An enrichment in bound DNA was identified around the -482 bp region but I further found that a significant enrichment could be detected also at +292 bp from the ATG, falling within the first intron which has a length of 1260 bps (fig 4.2). At 184 bp from the beginning of the intron I could find a canonical G-box "CACGTG" underlying the peak. This result confirms the role of OsHBF1 in *Ehd1* regulation and opens the possibility that multiple OsHBF1 binding sites have a regulatory role in *Ehd1* expression.

To conclude we demonstrated for the first time that FD-like proteins have a role in flowering repression in leaves. *OsHBF1* and *OsHBF2* generate a negative feedback loop on the *Ehd1* promoter to directly repress its expression and indirectly that of the florigens in leaves (fig 4.3).

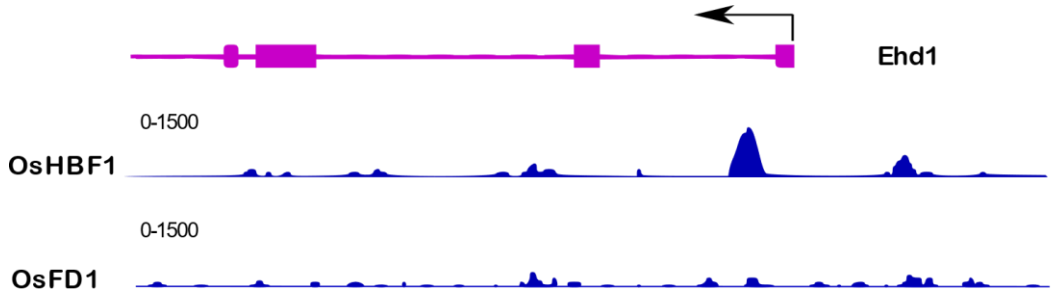


Figure 4.2: DAP-seq Ehd1 promoter analysis.

OsHBF1 bind *Ehd1* in two different positions: -482 bps and +292 bps from the start codon. Instead no *Ehd1* sequences enrichment could be found in OsFD1 DAP-seq experiment.

4.3 *OsFD1* can activate the floral transition

4.3.1 *OsFD1* promotes florigens expression in leaves

Because *OsHBF1* and *OsHBF2* repress flowering in the leaves, we decided to verify if other bZIPs could have a promotive function in that tissue. Using Real Time PCR we discovered that also *OsFD1* and *OsFD3* are expressed in leaves (Brambilla et al[65]: suppl fig 1c; fig 4.4). Conversely, *OsFD4* and *OsbZIP62* are expressed only in the shoot apical meristem (Brambilla et al[65]: suppl fig 3d; fig 4.4). The role of *OsFD1* in the SAM was already known[38], but we asked if it could have a functional role in leaves. First, we overexpressed *OsFD1* under the promoter of *ACTIN* (*pACT::OsFD1*). Transgenic lines showed mild early flowering respect to the wild type (Brambilla et al[65] 5c). Because the *pACTIN* promoter works in both

leaves and SAM, we could not conclude that the early flowering phenotype occurred because of increased expression of *OsFD1* in leaves. Yet, in an independent paper recently published, Jang et al[68] generated some lines that overexpress *OsFD1* under the *Hd3a* promoter (*pHd3a::OsFD1*). *Hd3a* is expressed only in the phloem and xylem parenchyma cells[27], so that in *pHd3a::OsFD1* lines, *OsFD1* expression is increased exclusively in those tissues. Transgenic lines showed a very early flowering phenotype respect to the wild type [68], indicating that OsFD1 promotes flowering also when overexpressed in leaves.

Looking at the expression of *Hd3a*, *RFT1* and *Ehd1* in *pACT::OsFD1* leaves we could detect an higher level of their transcripts (Brambilla et al[65]: fig 3e) which could explain the early flowering phenotype and further suggested a functional role of *OsFD1* as active flowering promotor also in leaves. Recently, we used DAP-seq in order to verify if OsFD1 could directly bind *Ehd1* similarly to OsHBF1 (see 4.4.6). However, no enrichment of OsFD1 binding sites was found in the *Ehd1* promoter or in the *Hd3a* or *RFT1* promoter (fig. 4.2), indicating that *OsFD1* promotes flowering in leaves independently of *Ehd1* regulation.

Thus, *OsFD1* has a role in flowering regulation in leaves to promote reproductive development. It regulates transcription of *Ehd1* and of the florigens indirectly, but in which pathway OsFD1 works in leaves is still unknown (fig 4.3).

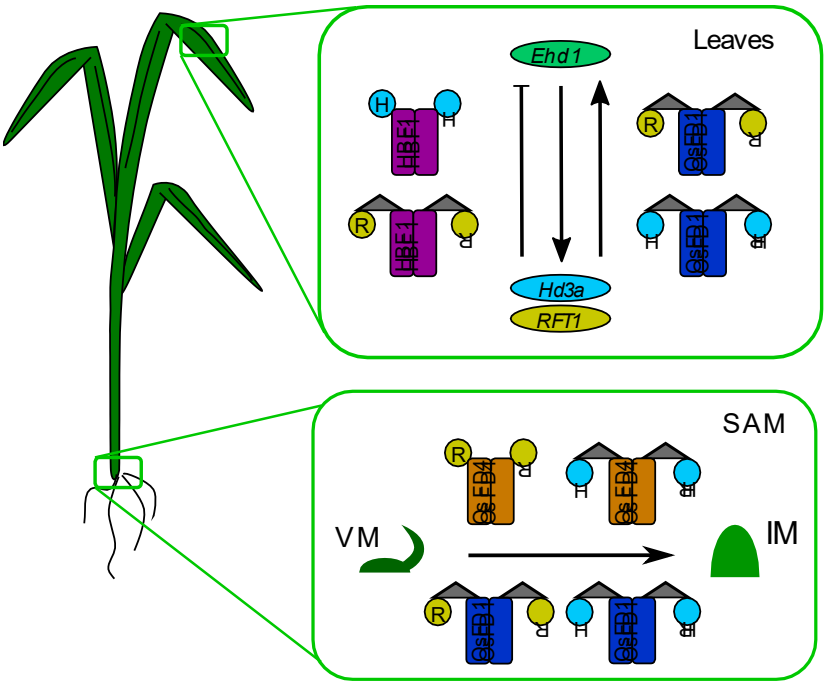


Figure 4.3: Model of FACs regulation in Leaves and SAM

As shown in the picture two level of floral transition regulations exist in rice. The first occurs in leaves where Leaves Florigen Activation Complexes (LFACs) control florigens production. The second works in the SAM by SAM Florigen Activation Complexes (SFACs) which regulate the transition from VM to IM.

4.3.2 *OsFD1* CRISPR mutants delay flowering under inductive condition

The rice floral transition model proposes that OsFD1 promotes the expression of *OsMADS15* and *OsMADS14* in the SAM and in this way allows the floral transition. Despite of the importance of OsFD1, RNA interference plants for this gene showed only a mild late flowering phenotype respect to the wild type, with poor statistical support[38]. For this reason, we decided to use the CRISPR technology to generate *osfd1* mutants (Manuscript supl 1c). Contrary to *osfd1 RNAi*, *osfd1-1* and *osfd1-2* showed a very strong late flowering phenotype compared to the wild type under inductive short-day conditions (Manuscript: fig 1b, 1c, 1d). *osfd1-1* time course experiments showed that in leaves, *Ehd1* was downregulated continuously from 0 to 18 DAS (Days After Shift from non-inductive to inductive condition) respect to the wild type (fig 4.5), whereas florigens expression showed a peculiar behaviour. In fact, *Hd3a* and *RFT1* were significantly reduced at 0 and 6 DAS in *osfd1-1* compared to the wild type, but at 12 and 18 DAS they showed similar expression levels in the mutant and in the wild type (fig 4.5). This suggests that *osfd1* mutants delay the onset of *Hd3a* and *RFT1* photoperiodic induction in leaves, but after several days of SD exposure, their expression level returns normal. Moreover, the level of *OsMADS14* and *OsMADS15* in *osfd1-1* leaves was reduced compared to the wild type, except for *OsMADS15* expression at day 12 after the shift to SD (fig 4.5). Since OsFD1 is active in the SAM, we decided to analyse also the expression of *OsMADS* genes in this tissue to quantify expression differences between *osfd1-1* and the wild type. Using qRT-PCR, we demonstrated that also in the SAM *OsMADS14* and *OsMADS15* were downregulated respect to the wild type at every time point analysed (fig 4.5). This effect is likely caused by both the lack of OsFD1 activity at the SAM, as well as reduced amounts of *Hd3a* and *RFT1* proteins reaching the SAM. Taken together these data confirm the double role of *OsFD1* during the floral transition which activates in leaves a positive feedback loop on florigens production via *Ehd1* expression and in the SAM regulates *OsMADS14* and *OsMADS15* to promote the transition from Vegetative Meristem (VM) to Inflorescence Meristem (IM) (fig 4.3).

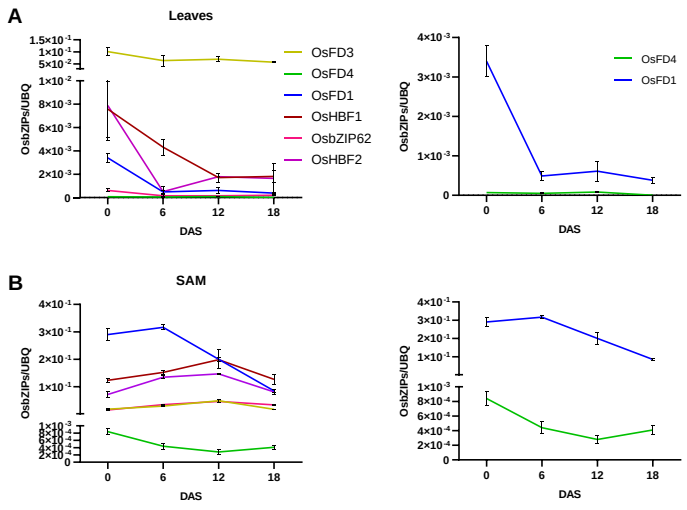


Figure 4.4: OsbZIPs Expression in SAM and leaves

A) Expression of OsFD1-like bZIPs in leaves. Left: all the bZIPs, right: detail of OsFD1 and OsFD4 expression only.

B) Expression of OsFD1-like bZIPs in the SAM. Left: all the bZIPs, right: detail of OsFD1 and OsFD4 expression only.

Time course experiments were performed on NB wild type for leaves and Dj wild type for the SAM.

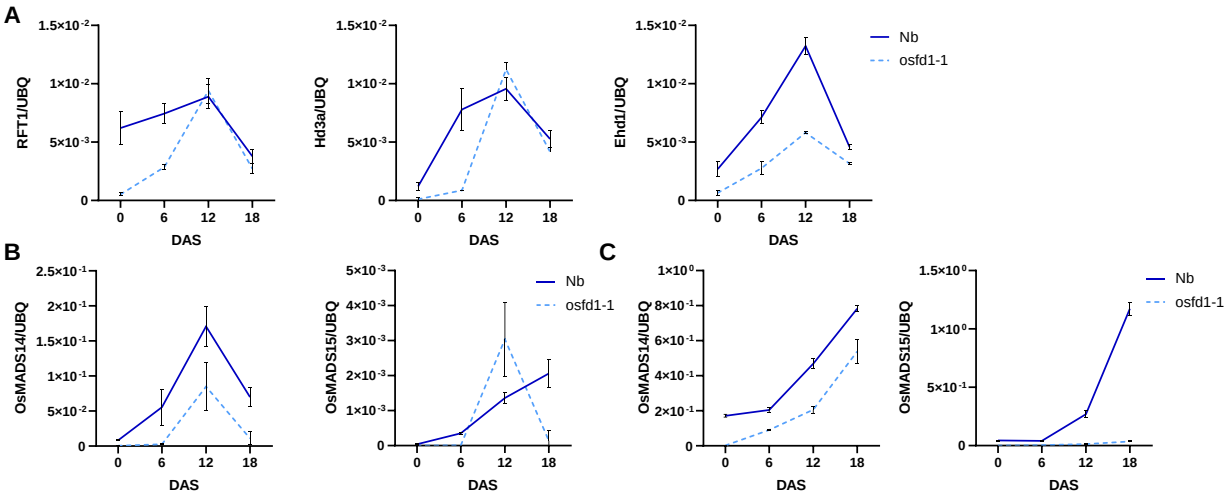


Figure 4.5: osfd1-1 time course.

A) *RFT1*, *Hd3a* and *Ehd1* expression in leaves; B) *OsMADS14* and *OsMADS15* expression in leaves; C) *OsMADS14* and *OsMADS15* expression in SAM. NB wild type (dark blue) and *osfd1-1* (light blue); DAS (Days After Shift)

4.4 OsFD4 promotes flowering in rice in the Shoot Apical Meristem via FAC formation

4.4.1 OsFD4 promotes flowering in rice

OsFD1 has a central role in flowering regulation in both leaves and SAM. When I started my PhD, we were interested in understanding the role of bZIPs during the transition of the SAM from VM to IM. For these reasons, we decided to look for *OsFD1-like* bZIP transcription factors which act preferentially in the SAM. As mentioned above (see 4.3, fig 4.4), only *OsFD4* and *OsbZIP62* are expressed only in the SAM and not in leaves. We isolated *osfd4-1* knock out T-DNA insertion lines obtained from a collection in the cultivar Dongjin, which contain the T-DNA insertion in the 3'-UTR of the gene. Despite of the position of the insertion, using RT-PCR we discovered that in *osfd4-1* plants no *OsFD4* expression could be found (Manuscript: suppl fig 1a, 1b). We performed experiments to verify the effect of the mutation on heading date. We observed that *osfd4-1* mutants delay flowering time under both inductive short-day conditions and not inductive long-day conditions (Manuscript: fig 1a, 1d). To be sure about the phenotype, we generated new *osfd4* mutant alleles using CRISPR-Cas9 technology (Manuscript: supl fig 1c). *osfd4-3* has an insertion of 16 bps in the 5' region of the *OsFD4* CDS which causes a frame shift. Quantification of

heading date using this allele confirmed that OsFD1 mutants delay the floral transition under short-day conditions (Manuscript: fig 1b). Because both *OsFD1* and *OsFD4* are expressed in the SAM, we decided to generate double *osfd1-2 osfd4-1* mutants to verify if there is redundancy between these genes. As shown in figure 1d of the Manuscript *osfd1-2 osfd4-1* showed no additive effect compared to *osfd1-2* single mutants, suggesting that under SD, only *OsFD1* is required to promote flowering. At the moment we are performing the same experiments other times to have a statistical relevant number of plants. Our current hypothesis is that OsFD1 promotes the floral transition in rice leaves under SD, generating a positive feedback loop on florigen production. Subsequently, when enough florigenic proteins are produced, they move through the phloem arriving in the SAM where they promote conversion of the SAM through *OsFD1* and *OsFD4* (fig 4.3).

4.4.2 OsFD4 interacts with the florigens

In the Florigen Activation Complex (FAC), *Hd3a* and *RFT1* interact with bZIP transcription factors to promote and regulate rice floral transition. The interaction between the florigens and the bZIPs can be direct, such as in the Hd3a-OsHBF1 FAC, or indirect via 14-3-3 proteins, such as in the Hd3a-Gf14c-OsFD1 FAC. For these reasons, we decided to test the interaction among FAC proteins and OsFD4 to verify if this bZIP can form FACs with the florigens.

The C-terminus portion of OsFD1 contains the SAP domain which allows the interaction between OsFD1 and Gf14c. Comparing OsFD4 and OsFD1 protein sequences, we found that also OsFD4 has the SAP domain at the C-terminus (Manuscript suppl fig 1d). Using Yeast Two Hybrid (Y2H), we tested the interaction between OsFD4 and all the *Gf14s* expressed in the SAM. In rice there are eight 14-3-3 proteins (from *Gf14a* to *Gf14h*) but only six of them are expressed in the SAM (from *Gf14a* to *Gf14f*)[69]. The results showed that all the Gf14s expressed in the SAM could interact with OsFD4 (Manuscript fig 3a) and, also, with Hd3a and RFT1 (Manuscript fig 3b) suggesting that all these 14-3-3 proteins could act as a molecular bridge between bZIPs and the florigens. Then, we tested the direct interaction between OsFD4 and the florigens and we found that OsFD4 could directly interact with RFT1 but not with Hd3a in Y2H (Manuscript suppl fig 3a). We decided to use Bimolecular Fluorescence Complementation (BiFC) in the heterologous system *Nicotiana benthamiana* to verify this interaction (Manuscript fig 3c). The results confirmed that RFT1 and OsFD4 directly interact in nuclei of cells of tabacum leaves.

These data suggest that OsFD4 can form FACs interacting indirectly via Gf14s or directly with florigens. Since RFT1 promotes the floral transition under non-inductive long-day conditions and *osfd4-1* mutants

show a stronger flowering delay under long-day than under short-day conditions, these results could indicate that the RFT1-OsFD4 FAC has a major role under non-inductive LD conditions.

4.4.3 OsFD4 can homodimerize and heterodimerize with OsFD3, but not with OsFD1

bZIP transcription factors contain two conserved domains, the basic leucin zipper domain and the dimerization domain. The first domain allows protein-DNA binding, whereas the second one allows the interaction between two bZIP proteins. This dimerization is crucial because bZIPs can bind the DNA only as homo- or hetero- dimers[31]. For these reasons, we decided to test the interaction between OsFD4 and others bZIPs. Using Y2H, we found that OsFD4 could homodimerize or form heterodimers with OsFD3, instead no yeast growth was detected when we tested the interaction between OsFD4 and OsFD1, OsHBF1, OsHBF2 or OsbZIP62 (Brambilla et al[65]: tab 1; Manuscript fig 4). Interestingly, we discovered that OsFD1 does not interact with itself in yeast, whereas OsFD3 could form both heterodimers with OsFD4 as well as homodimers (Manuscript fig 4a, 4b). These data suggest that only few combinations of heterodimerizations are possible between these bZIPs. We verified homodimerization of OsFD4, and heterodimerization of OsFD1 and OsFD3 also using BiFC assays in *Nicotiana benthamiana*, obtaining the same results (Manuscript fig 4c). These results suggest that OsFD1 and OsFD4 which are both expressed in the SAM, can form different FACs and do not interact with each other. Moreover, they open the possibility that OsFD3 and OsFD4 could be part of the same FAC via heterodimerization in the SAM.

4.4.4 OsFD4 regulates expression of AP1-like genes in rice

In Arabidopsis thaliana one of the most important targets induced by the FAC is *AP1* which promotes the transition from Inflorescence Meristem to Floral Meristem[70]. In rice there are 4 different *AP1-like* genes, called *OsMADS14*, *OsMADS15*, *OsMADS18* and *OsMADS20* which are expressed in the SAM and which change their expression during the floral transition[50]. OsFD1 can directly regulate the expression of *OsMADS14* and *OsMADS15* via FAC formation[38]. For this reason, we decided to quantify the expression level of *OsMADS* genes in the SAM of *osfd4-1* respect to the wild type. To do this analysis we performed time course experiments shifting plants after two months of growth under non-inductive condition to inductive conditions, and then sampling SAMs at different time points (0, 6, 12 and 18 Days After Shift-DAS). As shown in Manuscript fig 5, *OsMADS14* and *OsMADS15* were downregulated in *osfd4-1* respect to the wild type, whereas no significant changes could be detected in *OsMADS18* and *OsMADS20* expression in *osfd4-1*. In rice AP1-like genes regulate the floral transition thanks to another SEPALLATA-

like MADS-box transcription factor induced during photoperiodic induction, called *OsMADS34*[50]. We quantified *OsMADS34* expression in *osfd4-1* and we could observe that this gene was also downregulated respect to the wild type (Manuscript fig 5). Taken together these data demonstrate that *OsFD4* has a role during the first stages of floral transition, when the SAM changes from Vegetative to Inflorescence Meristem.

4.4.5 Use of DNA Affinity Purification Sequencing to find OsHBF1, OsFD1 and OsFD4 binding sites

To better understand the role of bZIP transcription factors at the shoot apical meristem and identify a set of potential target genes, we decided to perform DNA Affinity Purification sequencing (DAP-seq) of OsFD4, OsFD1 and OsHBF1. DAP-seq is a new method that allows to find in vitro DNA regions bound by transcription factors of interest[71,72]. The procedure is composed by two major parts. During the first one a library of fragmented rice DNA is created, and the transcription factor of interest is produced in vitro. In the second part, the DNA and the protein are put together to make them interact. During this step DNA fragments containing consensus motives recognized by the transcription factor are bound. In this way, it is possible to purify the protein together with its bound DNA and at the end, release the DNA and sequence it.

After the sequencing, alignment of reads and peaks calling were performed by our collaborators in the USA, according to the methods published by Galli et al 2018. Instead, motif enrichment was analyzed using MEME by Jérémy Lucas and François Parcy.

After bioinformatic analysis, enriched fragments harboured the same consensus motif for OsFD1, OsFD4 and OsHBF1 which shared a core motif of "CACGT" (Manuscript fig 6a). At the flanks of this motif two bases "GC-" had different probabilities to be found depending upon the bZIP considered. In the DNA bound by OsFD4, the entire motif "GCCACGT" was highly conserved across most binding sites (Manuscript fig 6a).

Because OsFD1, OsFD4 and OsHBF1 have different roles during the floral transition, these data suggest that the consensus motif bound by bZIP transcription factors involved in this process is conserved, and that the motif alone cannot explain binding to different target genes of these bZIPs.

Similarly, to ChIP assays, when sequences of DNA fragments are aligned with the genome, they form peaks of enrichment which contain the consensus motif inside. To assign a locus to every peak we used

the online tool PAVIS which called genes that contained one or more peaks 5000 bps upstream of the ATG or 3000 bps downstream of the STOP codon including exons and introns in the analysis. We observed that OsFD4, OsFD1 and OsHBF1 had an enrichment in 925, 1717 and 15937 genes respectively. Most of the binding sites were in the upstream region of the gene (OsHBF1 35.9%, OsFD1 47.4%, OsFD4 42.8%, Manuscript fig 6b). When the upstream sequence was considered, we observed that binding occurred most frequently in the 500 bps before the Transcription Start Site (TSS), with a peak frequency observed between -100 and -50 bp from the TSS (Manuscript fig 6c). Differences in the number of genes bound by OsHBF1 and OsFD1 or OsFD4 could be explained because of their divergent role during the floral transition, and by their different expression pattern. In fact, *OsHBF1* is a repressor of this process broadly expressed, whereas *OsFD1* and *OsFD4* are two flowering activators, mostly expressed in the SAM.

To verify the level of redundant targets among OsFD1, OsFD4 and OsHBF1, we compared target genes in Venn Diagrams. This analysis showed that 698 genes were commonly bound by all the tested bZIPs, 220 genes were shared between OsFD4 and OsHBF1, 991 between OsFD1 and OsHBF1, while no common genes were present in the intersection between OsFD1 and OsFD4 (Manuscript fig 6d). The remaining genes were bound by single bZIPs, 7 genes by OsFD4, 28 genes by OsFD1 and 14028 genes by OsHBF1.

The DAP-seq method uses the entire naked genome to find transcription factor targets which implies that chromatin structure is not considered when collecting the data. To be sure that candidate genes were expressed in the tissue of interest, we asked which genes bound by OsFD1 and OsFD4 were also expressed in the SAM during the transition to reproductive growth. The expression data were obtained by published RNA-seq[73] in which SAMs were sampled at 0 DAS and 12 DAS (DAS means Days After Shifting from non-inductive condition to inductive condition), then we crossed all the gene expressed in these time points with DAP-seq datasets. We also compared the lists of genes bound by OsHBF1 and OsFD1 with genes expressed in leaves. Also in this case, expression data were obtained by published RNA-seq[74] in which leaves were sampled at 0 DAS and 12 DAS (Manuscript fig 6d). Only a minor part of genes found using DAP-seq intersected with gene lists from RNA-seq data. In the SAM, OsFD4 and OsFD1 bound 240 common genes expressed in the SAM, whereas unique targets of OsFD4 and OsFD1 were 58 and 320, respectively. In leaves, OsHBF1 and OsFD1 unique targets were 3691 and 7 respectively, whereas common targets expressed in this tissue were 566. These data confirmed the importance of tissue specificity, which is not captured by DAP-Seq, and identify a set of candidate genes bound by the bZIPs and expressed at the SAM.

4.4.6 OsFD4, OsFD1 and OsHBF1 have different Binding Syntax

OsFD1, OsFD4 and OsHBF1 have different roles in rice floral transition regulation, but despite of that they bind the same consensus motif. Thus, the sequence of the consensus motif alone cannot explain binding differences. To understand if some differences could be found among these bZIPs, we decided to verify the spacing between two consensus motives bound in the DAP-seq based on the transcription factor tested. We performed this analysis in collaboration with François Parcy laboratory which recently published a Position Weight Matrix (PWM) analysis method, further elaborating on available DAP-seq data[75]. This analysis allows to find distances between two consensus motives bound by the same transcription factor and to calculate their configuration on the DNA strands which could be arranged as Everted Repeats (ERs), Direct Repeats (DR) or Inverted Repeats (IR) (Manuscript fig 7a). We first decided to verify if the method could be applied to rice DAP-seq analysis testing if our data well fitted in the model via ROC curve statistical analyses (Manuscript supl fig 3). This method uses as a negative control a ROC curve obtained from random consensus motives found in the entire genome and which has a value of 0,5. Then the ROC curve of the sample of interest was calculated using only consensus motives experimentally identified in DAP-seq experiments and bound by a specific transcription factor. The more the ROC curve diverges getting closer to 1, the most predictable the sample binding sites distance becomes respect to the random control. Our results showed that OsFD4, OsFD1 and OsHBF1 syntaxes were highly predictable with ROC values of 0,985, 0,971 and 0,9 respectively. We performed PWMs for each bZIP and we found that unique profiles were generated. OsFD4 had an enrichment of 4-fold change or more above the negative control in the DR0, ER1-5, ER18-20, ER44, IR34 and IR41 configurations (Manuscript fig 7b). OsFD1 showed an enrichment of 3-fold change or more to the negative control in the DR30, ER4, ER16, ER28, ER32-34, ER41-46 and IR38 configurations (Manuscript fig 7c). OsHBF1 configuration enrichments were found in DR10, DR18, DR29, DR40, ER3, ER16-18, ER29, ER42 and IR10 with a 2-fold change or more respect to the negative control (Manuscript fig 7d). These different spacing configurations between consensus motives could explain the different activities of OsFD4, OsFD1 and OsHBF1 during floral transition. bZIP transcription factors bind single consensus motifs as dimers, in this scenario the conserved space between two consensus motives could suggest that more than one bZIP dimer could bind the DNA to allow gene regulation. Despite OsFD1 in yeast and *Nicotiana benthamiana* cannot form homodimers, it binds the DNA in DAP-seq, maybe the dimerization of OsFD1 is destabilized in cells of heterologous systems but become more stable in vitro when there are no more molecules that OsFD1alone.

4.4.7 OsFD4 and OsFD1 DAP-seq targets show different regulation in the mutant respect to the wild type

Thanks to DAP-seq analysis, we found putative target genes of *OsFD4* and *OsFD1*. We performed qRT-PCR between mutants and the respective wild type to verify if *OsFD1* and *OsFD4* targets regulation was influenced. We chose some transcription factors based on the intersection between RNA sequencing data of the SAM and the DAP-seq dataset. We analyzed the expression of two genes regulated both by *OsFD1* and *OsFD4*, *LOC_Os08g38590* (*OsMADS62*) and *LOC_Os06g48950* (*OsARF19*), three genes regulated only by *OsFD1*, *LOC_Os01g14440* (*WRKY transcription factor*), *LOC_Os01g64360* (*MYB transcription factor*), and *LOC_Os04g51000* (*RICE FLORICAULA/LEAFY*), and two *OsFD4* target genes, *LOC_Os04g31730* (*B3 transcription factor*) and *LOC_Os07g41580* (*NF-YB transcription factor*) (Manuscript fig 8 and suppl fig 4). Both *OsMADS62* and *OsARF19* showed a downregulation in *osfd4-1* and *osfd1-1* respect to the wild type, confirming the role of these two bZIPs in their regulation. *LOC_Os04g51000* and *LOC_Os07g41580* had a different expression only in *osfd1-1* and *osfd4-1* respectively, whereas the other genes tested were less expressed in both mutants. These data suggest that OsFD4 and OsFD1 similarly regulate some genes, but their action is not completely redundant, at least based on these few examples.

Thanks to the DAP-sequencing analysis we found new targets of different FACs and we showed that genes regulation can change based on the bZIP transcription factor that is incorporated in the complex. Moreover, using the PWM analysis we discovered that the binding architecture of bZIP dimers at target genes is complex, and can distinguish between bZIPs. Also, our analysis considers only dimers formed by the same bZIP. However, since formation of heterodimers is possible among different bZIPs, binding configurations could be much more variable. Another level of complexity could be represented by chromatin configuration that could support or prevent bZIP binding to the DNA.

4.5 *osfd3* single mutants have no effect on flowering time

OsFD3 is an OsFD-like bZIP transcription factor which could have a role in rice floral transition, based on its protein homology with OsFD1 which reach the 55%. Using qRT-PCR we discovered that this gene was expressed in both leaves and SAM (fig 2.4). Moreover, Y2H experiments demonstrated that OsFD3 could interact with both florigens, Hd3a and RFT1, both directly and via all the 14-3-3 proteins expressed in the SAM, suggesting the possibility that this gene could form FACs and in this way regulate the floral transition (Manuscript supl fig 2). As stated above (see 4.4.4), we tested also the interaction between

OsFD3 and OsFD4 and found that the two proteins strongly interact. Thus, these data could indicate that OsFD3 and OsFD4 could work together as heterodimers to regulate the rice floral transition. Using CRISPR-Cas9, we generated *osfd3* mutants to perform phenotypical analyses. After rice transformation, we identified different alleles, whereas only *osfd3-1* which had a deletion of 2 bases produced sufficient seeds to be analyzed (Manuscript suppl 1c). Under short-day conditions, *osfd3-1* showed no flowering phenotype compared to the wild type (Manuscript fig 1b). Since it could act redundantly with *OsFD1* or *OsFD4*, more analysis must be done on *osfd4/osfd3* double mutants to evaluate its role during the floral transition and panicle development in rice.

4.6 Florigen expression under the control of DEX inducible system

Hd3a and *RFT1* are the rice florigens and they have a central role in the floral transition[26]. Plants lacking both genes never shifted from vegetative to the reproductive phase[26]. Despite the high homology of these proteins, some differences occur between them. Both florigens are expressed and trigger the floral transition under inductive short-day conditions, whereas only RFT1 regulates this process under not-inductive long-day conditions[27,25]. For these reasons we decided to generate Dexamethasone-inducible overexpressing lines both for *Hd3a* and for *RFT1* under the rice constitutive *GOS2* promoter (Fig 4.6a)[67]. These lines were described also in paragraph 4.2, as they have been used to study HBF1 and HBF2. However, in that study, only the effect of Hd3a and RFT1 induction in leaves was determined. During my PhD, I also contributed to a study aimed at determining the effect of Hd3a or RFT1 induction on gene expression at the SAM.

We induced plants after two months under non-inductive long-day conditions spraying the leaves with 50ml of 10uM Dexamethasone solution and then, after 15 hours, we sampled RNA from leaves and SAM to verify the efficiency of the induction. *Hd3a* and *RFT1* expression increased in leaves and only mildly in the SAM (Fig 4.6b). To verify the effect of induction at the SAM we measured the level of *OsMADS14*, *OsMADS15* and *OsMADS34*, which are known targets of florigens via FACs and we found that expression of all MADS genes increased in the SAM of treated plants respect to non-treated ones (Fig 4.6c).

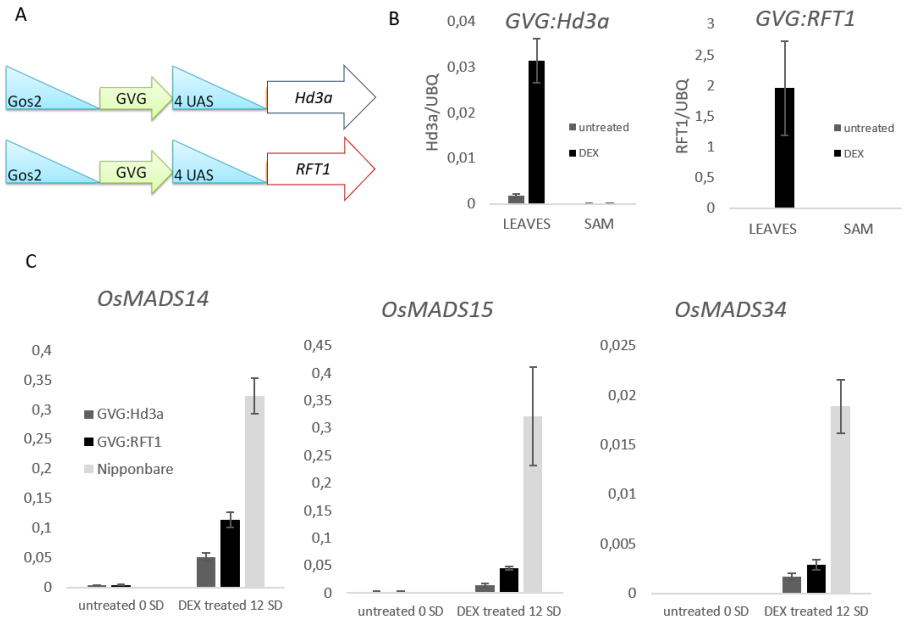
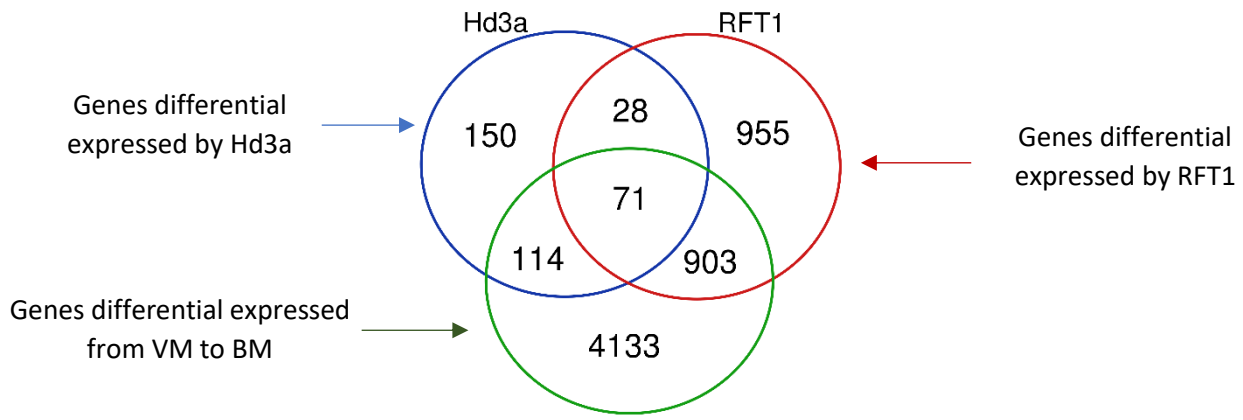


Figure 4.6: Dexamethasone inducible system setting
a) Dexamethasone system scheme; b) transgenic plants could overexpress *Hd3a* or *RFT1* upon DEX treatment in leaves; no *Hd3a* or *RFT1* overexpression was observed in the SAM. Plants were grown under long days (16 hours light) for 8 weeks before DEX treatment; c) *OsMADS14*, *OsMADS15* and *OsMADS34* expression at the SAM in *GVG:Hd3a/RFT1* inducible expressors compared to Nipponbare induced by exposure to 12 short days. Error bar: standard deviation between 3 technical replicates.

These data suggested that in our inducible system, florigenic proteins were produced in leaves of treated plants and then moved via the phloem to arrive in the SAM where *RFT1* and *Hd3a* promoted the expression of their targets, such as *OsMADS14* and *OsMADS15*.

Next, to understand the role of florigens during the floral transition and to study their differences in this process, we performed RNA-seq experiments on SAMs in which we compared gene expression of non-treated plants with RFT1-induced or Hd3a-induced ones. In this way we obtained a list of genes that are regulated only by *RFT1* or *Hd3a* at the SAM, and we could compare them to find differences between the two florigens. We found 1858, 265 and 99 genes regulated only by RFT1, by Hd3a or by both florigens, respectively (Fig 4.7). As expected, analysing both datasets, we found *OsMADS14*, *OsMADS15* and *OsMADS18*, which are known targets of florigens, in the list of the most expressed genes induced by *Hd3a* and *RFT1* (Data not shown). We choose some genes among the new targets to perform qRT-PCRs and validate their behaviour in Dex-induced samples and not induced ones. Finally, we generated *hd3a* and



rft1 single CRISPR mutants to confirm the role of the florigens in the regulation of our candidate genes. At the moment these experiments are still in progress.

Figure 4.7: Venn Diagram of genes differential expressed after Hd3a induction (blue), after RFT1 induction (red) and after natural induction from Vegetative Meristem (VM) to Branches Meristem (BM) (green)

4.7 *PREMATURE INTERNODE ELONGATION 1* (*OsPINE1*) a new target of Hd3a and RFT1

In rice the SAM is enclosed by leaves and positioned at the base of the plant, for this reason during the floral transition the stem elongates to ensure the emergence of the panicle from the protecting leaves. Thanks to RNA-seq performed from SAM tissues, comparing non-inductive long day conditions versus inductive short-day conditions, we found *PREMATURE INTERNODE ELONGATION 1* (*OsPINE1*) which is a Zinc-finger transcription factor that regulates internodes elongation in rice. *OsPINE1* is expressed in the lower internodes during the vegetative phase of rice plants and when the floral transition occurs it is repressed thanks to florigens production (Gómez-Ariza et al[73]: fig 1). *ospine1* CRISPR mutants had no effect on flowering time compared to the wild type, but it showed a prostrate phenotype in which the internodes started to elongate during the vegetative phase (Gómez-Ariza et al[73]: fig 2b). The reason of this precocious growth is a hypersensitivity of the *ospine1* mutant to Gibberellins (GA), which in plant is involved in growth and cells elongation. In fact, *ospine1* plants treated with Paclobutrazol (PAC), a GA biosynthesis inhibitor, showed a rescue of the phenotype, whereas when treated with exogenous bioactive GA showed a stronger response to the hormone treatment respect to the wild type (Gómez-Ariza et al[73]: fig 2c). Taken together these results indicate that *OsPINE1* represses stem elongation during the vegetative growth via GA perception inhibition, then during the floral transition, reduction of *OsPINE1* expression causes an increase in GA sensitivity that contributes to stem elongation.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

During my PhD I focused my work on rice flowering time and floral transition regulation, and particularly on the role of Florigen Activation Complexes (FACs) during this process. When I started my thesis, there were no data about the role of FD-like bZIP transcription factors in leaves. We then demonstrated that *OsHBF1* and *OsFD1* have a role during flowering time regulation in this tissue via FACs formation. My main project was the study of bZIP transcription factors which regulate the floral transition in the SAM. First, because *osfd1* RNA interference plants have very mild flowering phenotype, we generated CRISPR *osfd1* mutants and I observed that under inductive short-day conditions *osfd1-1* and *osfd1-2* strongly delay flowering respect to the wild type, meaning that *OsFD1* has a role in floral transition under this condition. Because *OsFD1* has a double role in leaves and in SAM and because it promotes floral transition in leaves via the short-day specific promoter *Ehd1*, we are performing *osfd1* flowering phenotyping under not-inductive long-day conditions to verify if a floral transition delay is detectable also in this case. Additionally, to the OsFD1 analysis, we found *OsFD4* which promotes the rice floral transition. The reason why we decided to focus our attention on this transcription factor was its SAM specific expression, whereas also *OsFD1* is expressed in the SAM, we are performing in situ hybridizations to compare the localization of *OsFD4* and *OsFD1* transcripts during the floral transition from Vegetative Meristem (VM) to Inflorescence Meristem (IM). Using protein-protein interaction assays, we demonstrated that OsFD4 forms FACs interacting indirectly with Hd3a and directly with RFT1[25]. The stronger phenotype of *osfd4* under non-inductive conditions and OsFD4-RFT1 direct interaction, could suggest that *OsFD4* has a photoperiod dependent function, for this reason now we are analyzing double *osfd4 rft1* mutants to verify its phenotype under long-day conditions. We used the DAP-seq method to find targets of *OsFD1, OsFD4* and *OsHBF1* and in this way tried to understand the cause of their different roles during the floral transition. DAP data showed that these bZIPs share a common "CACGT" binding motif, which does not allow to explain their different functions. Because of that we looked at the distance between these binding motives in each sample and we found that OsFD4, OsFD1 and OsHBF1 have a proper and unique behavior in binding the DNA. Interestingly, bZIP transcription factors bind a single consensus motif such as dimers[31], the conserved space between two consensus motives could suggest that FD-like bZIP transcription factors could form more complex structures than dimers to bind the DNA and finally regulate the floral transition. In any case more experiments must be done to support this hypothesis. The number of targets that we found using DAP-seq is different for each transcription factors, even if *OsFD1* and *OsFD4* are more comparable than *OsHBF1* (1717, 925, and 15937, respectively). It is possible to speculate that this huge difference could be explained thanks to the difference in these bZIPs function, in fact *OsFD1* and *OsFD4* are two activators

of floral transition, whereas *OsHBF1* is a repressor. The use of DAP-seq for these three genes give a big help in the knowledge of rice FD-like transcription factors, in fact now is it possible to analyse a broader range of genes regulated by different FACs. The use of RNA-seq on now available *osfd4*, *osfd1* and *hbfs* mutants might help to further refine these data. In parallel to these DAP-seq experiments, we performed RNA-seq on Hd3a and RFT1 DEX-inducible lines. Because in FACs bZIPs and florigens work together all these data could be used to understand if the FACs specificity for each bZIP could change based on the florigen bound in the complex or not.

Taken together all these data demonstrate the predominant role of FACs during the floral transition. FACs are formed by florigens Hd3a or RFT1 and different bZIPs transcription factors, which sometimes need 14-3-3 proteins to bridge the interaction between them. FACs plasticity allows to increase the level of complexity and to produce a fine regulation of genes expression during rice development. In this scenario, bZIP transcription factors become the key that regulate the ample range of actions which are also florigen-dependent. The available high-throughput datasets that we generated can be now used to find new genes with a role during the floral transition or any other bZIP/florigen-dependent process.

6 MATERIA AND METHODS

Material and Methods here described are the ones that I performed during my PhD. Some analyses which are not reported were done by other colleagues and methods are available in the attached papers.

Plant growing conditions, RNA sampling and qRT

Nipponbare and Donjing plants were grown under long-day conditions (14,5 light/9,5 dark) or short-day conditions (10 h light/ 14 h dark). For gene expression experiments, SAM or leaves samples were collected starting at ZT 0 in time courses of several following days. SAMs were manually dissected under a stereomicroscope. RNA was extracted with *TRIzol*® (Termofisher Scientific). c-DNA was transcribed with Im-Prom-II RT (Promega) and qRT was performed with the primers listed in supplementary table 1. Maxima SYBR qPCR master mix (Termofisher Scientific) was used in qRT PCR experiments.

Rice Transformation

Rice transformation was performed using Nipponbare seeds to induce callus formation, and the EHA105 *Agrobacterium tumefaciens* strain was used to infiltrate calli. Transgenic calli were isolated from non-transgenic calli by growing them on selection media supplemented with hygromycin in 2 rounds of selection (50 mg l−1and 100 mg l−1 hygromycin, respectively). Plants regenerated from transgenic calli were grown in rooting medium for 2 weeks before transferring to soil in the greenhouse.

CRISPR Cloning and *osfd4-1* T-DNA insertion mutants

osfd4-1 mutant corresponds to the Salk line PFG_2D_41663.R, homozygous T-DNA insertional mutants were selected. CRISPR-Cas9 was performed using the system described by Miao et al[76]. Targeting oligo for OsFD1, OsFD4, OsFD3 and simultaneously OsHBF1-OsHBF2 were designed thanks to CRISPR P online tool and about 100-200 bps from the start site.

Protein-protein interaction assays

Yeast two hybrid tests were performed cloning the cds into the vectors pGADT7 and pGBKT7 (Clontech) and transformed into AH109 and Y187 yeast strains respectively. Interactions were tested by mating and growth on selective media -L-W-H -A.

BiFC experiments were performed in tobacco epidermal cells with the vectors pBAT TL-B sYFP-N and pBAT TL-B sYFP-C.

DAP seq method

For DAP-seq library, gDNA was extract from rice leaves, then 5 micrograms of genomic DNA were diluted in EB (10 mM Tris-HCl, pH 8.5) and sonicated to 200 bp fragments in a covaris S2 sonicator. DNA was purified using AmpureXP beads at a 2:1 bead:DNA ratio. Samples were then end repaired using the End-It kit (Lucigen) and cleaned using Qiaquick PCR purification (Qiagen) according to the manufacturer's recommendations. Purified samples were A-tailed using Klenow 3–5′exo- for 30 min at RT and then purified using Qiaquick PCR purification as described above. Purified samples were then ligated overnight with a truncated Illumina Y-adapter as described in Bartlett et al. Libraries were purified by bead cleaning using a 1:1 bead:DNA ratio, eluted from the beads in 30 µl of EB, and quantified with the Qubit HS fluorometric assay. Separately, HALO-tagged TFs were expressed in an in vitro rabbit reticulocyte TNT expression system and they were immobilized on Magnet HALO-Tag beads, washed, and incubated with the DNA library according to Galli et al 2018. Then read mapping, filtering, and peak calling was done according to Galli et al 2018.

APPENDICES

Appendices 1:
Antagonistic Transcription Factor Complexes Modulate the Floral Transition in Rice

PhD contribution:

In this work we analyzed the role of FD-like bZIP transcription factors and Florigen Activation Complexes (FACs) in leaves. We demonstrated that *OsFD1* promotes flowering via positive feedback loop on florigen expression in leaves and we characterized *OsHBF1* and *OsHBF2* which generate a negative feedback loop on florigen production via downregulation of *Ehd1* in this tissue.

For this paper, we generated Dexamethasone inducible lines of *Hd3a* and *RFT1* and I selected and set the induction conditions to make the expression of florigens int the transgenic lines comparable to the natural production of *Hd3a* and *RFT1*. I generated and selected double *oshbf1* and *oshbf2* CRISPR mutants and I performed quantification of gene expressions using qRT-PCR for CRISPR and T-DNA mutants, overexpressor lines and Dexamethasone inducible lines.

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Antagonistic Transcription Factor Complexes Modulate the Floral Transition in Rice

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Plants measure day or night lengths to coordinate specific developmental changes with a favorable season. In rice (*Oryza sativa*), the reproductive phase is initiated by exposure to short days when expression of *HEADING DATE 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) is induced in leaves. The cognate proteins are components of the florigenic signal and move systemically through the phloem to reach the shoot apical meristem (SAM). In the SAM, they form a transcriptional activation complex with the bZIP transcription factor OsFD1 to start panicle development. Here, we show that Hd3a and RFT1 can form transcriptional activation or repression complexes also in leaves and feed back to regulate their own transcription. Activation complexes depend on OsFD1 to promote flowering. However, additional bZIPs, including Hd3a BINDING REPRESSOR FACTOR1 (HBF1) and HBF2, form repressor complexes that reduce *Hd3a* and *RFT1* expression to delay flowering. We propose that Hd3a and RFT1 are also active locally in leaves to fine-tune photoperiodic flowering responses.

INTRODUCTION

The floral transition sets the beginning of the reproductive phase and is completed upon switching of the shoot apical meristem (SAM) from indeterminate vegetative to determinate reproductive growth. In many plant species, these changes are triggered by daylength (or photoperiod), which is measured in leaves to synchronize inflorescence development with the most favorable seasons. This signaling mechanism requires systemic communication signals that integrate environmental inputs and connect distant tissues of the plant.

Rice (*Oryza sativa*) preferentially flowers under short days (SDs). When daylength falls under a critical threshold, proteins encoded by the *HEADING DATE 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) loci are produced in leaves and delivered through the phloem to the SAM, where they induce developmental reprogramming (Tamaki et al., 2007, 2015; Komiya et al., 2009). Both proteins share homology with FLOWERING LOCUS T (FT) of *Arabidopsis thaliana* and belong to the phosphatidylethanolamine binding protein (PEBP) family of regulators, which includes also TERMINAL FLOWER1 (TFL1) homologs (Kojima et al., 2002; Ho and Weigel, 2014). However, whereas FT-like proteins are strong activators of flowering, TFL1-like proteins are flowering inhibitors (Wickland and Hanzawa, 2015).

Under inductive photoperiods, both *Hd3a* and *RFT1* are transcribed, and their protein products are essential for flowering to the extent that artificial reduction of their mRNA expression results in never-flowering plants (Komiya et al., 2008; Tamaki et al., 2015). However, transcription of *RFT1* can be induced also under long days (LDs), and its floral promotive activity under these conditions contributes to the facultative nature of the photoperiodic flowering response of rice (Gómez-Ariza et al., 2015; Komiya et al., 2009).

Induction of *Hd3a* and *RFT1* expression in leaves results from the integration of photoperiodic information with diurnal timing set by the circadian clock. Environmental signals ultimately converge on the transcriptional activation of *Early heading date 1* (*Ehd1*), encoding a B-type response regulator unique to rice (Brambilla and Fornara, 2013; Doi et al., 2004; Cho et al., 2016). Transcription of *Ehd1*, *Hd3a*, and *RFT1* thus correlates under SD in leaves, showing a transient induction that persists only for the time required to irreversibly commit flowering at the SAM (Galbiati et al., 2016; Doi et al., 2004; Cho et al., 2016; Komiya et al., 2008). Once a sufficient amount of Hd3a and/or RFT1 proteins reaches the SAM, expression of target genes that promote inflorescence formation is induced (Taoka et al., 2011; Tamaki et al., 2015).

FT-like proteins have no DNA binding property. Therefore, upon reaching the cytoplasm of cells at the SAM, they bind to transcription factors of the bZIP family, including FD in Arabidopsis and OsFD1 in rice (Wigge et al., 2005; Taoka et al., 2011). The

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complex, originally found to be dimeric based on studies in Arabidopsis, was later demonstrated to contain also a 14-3-3 protein of the Gf14 family (G-box factor 14-3-3) that bridges the interaction between OsFD1 and Hd3a. The resulting ternary complex, named florigen activation complex (FAC), is targeted to the nucleus where it further dimerizes, forming a heterohexameric complex tethered by OsFD1 on target DNA sequences (Zhao et al., 2015; Taoka et al., 2011). Similar interactions take place in many plant species, including tomato (*Solanum lycopersicum*; Park et al., 2014), potato (*Solanum tuberosum*; Teo et al., 2017), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*; Li et al., 2015), maize (*Zea mays*; Danilevskaya et al., 2008), and hybrid aspen (*Populus tremula* 3 *tremuloides*; Tylewicz et al., 2015), suggesting that this molecular module is widely conserved among angiosperms. This conservation is further corroborated by interspecific interactions demonstrated to occur between Hd3a/RFT1 and FD (Jang et al., 2017). In many such examples, FD-like genes can provide DNA binding specificity by recognizing ACGT-containing consensus sequences on the DNA of target promoters (Izawa et al., 1993; Li and Dubcovsky, 2008; Taoka et al., 2011; Wigge et al., 2005). Competition between FT-like and TFL1-like proteins for interaction with FD and 14-3-3 proteins partly explains their opposite function on flowering and shoot architecture. Again, such competitive behavior is widespread among angiosperms (Pnueli et al., 2001; Randoux et al., 2014; Hanano and Goto, 2011; Park et al., 2014).

The rice genome encodes seven Gf14 proteins, four of which (the b, c, d, and e) can assemble into a FAC (Taoka et al., 2011). The Gf14c protein was the first to be functionally characterized as an Hd3a interactor (Purwestri et al., 2009; Taoka et al., 2011). Because of their redundancy and pleiotropic effects, it has not been possible to study *gf14* mutants, but transgenic rice overexpressing *Gf14c* had delayed flowering (Purwestri et al., 2009). Despite the apparent contrast with the nature of a FAC, this result might indicate that a tightly regulated balance between FAC components needs to be achieved at the SAM to promote flowering. Alternatively, floral repressor complexes containing Gf14c might exist and become predominant upon overexpression of this specific 14-3-3 protein.

Besides FD-like transcription factors and 14-3-3 proteins, FT-like genes can interact with members of the TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) transcription factor family. The ability to bind distinct members of this group of regulators partly discriminates between FT- and TFL1-like proteins and indicates that TCPs are preferential interactors of FT-like proteins (Mimida et al., 2011; Niwa et al., 2013; Ho and Weigel, 2014). Finally, apple (*Malus domestica*) Vascular Plant One Zinc finger (MdVOZ1a) was isolated as an interactor of apple FT and shown to alter inflorescence architecture when expressed in Arabidopsis (Mimida et al., 2011). Whether interactions between FT-like and VOZ-like proteins are conserved among flowering plants is yet to be assessed.

Downstream targets of the FAC at the SAM include members of the MADS box transcription factor family that are necessary to switch the meristem to reproductive growth. In Arabidopsis, induction of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1, FRUITFULL* (*FUL*), and *APETALA1* takes place shortly after arrival of FT at the SAM (Andrés and Coupland, 2012). Similarly, *OsMADS14*, *OsMADS15*, and *OsMADS18*, genes belonging the *FUL* clade, and *OSMADS34/PAP2*, a *SEPALLATA* (*SEP*)-like gene, are progressively activated upon floral transition in rice (Kobayashi et al., 2012; Litt and Irish, 2003). Mutants in which all four genes are silenced develop inflorescence stems where flowers are replaced by vegetative shoots (Kobayashi et al., 2012). This general mode of action of the florigens at the SAM has been observed in several plant species (Jang et al., 2015; Jaudal et al., 2015; Li and Dubcovsky, 2008). However, FACs can be deployed also in tissues different from the SAM to control a broad spectrum of developmental processes different from inflorescence formation. For example, components of FACs governing leaf development have been reported in both Arabidopsis and rice (Teper-Bamnolker and Samach, 2005; Tsuji et al., 2013). Potato tuber formation depends on FACs forming at the stolon meristem in response to FT export from the leaves (Navarro et al., 2011; Teo et al., 2017). Seasonal growth cessation in trees is induced by FACs assembled in vegetative apical meristems that stop elongation and leaf production before the onset of winter (Tylewicz et al., 2015). These findings illustrate the plasticity and robustness of FACs as integrators of photoperiodic signals into distinct developmental networks.

Given the high number of OsbZIP-coding genes in rice, the combinatorial interactions possibly leading to different florigen-containing complexes are very high (Tylewicz et al., 2015; Park et al., 2014; Tsuji et al., 2013; Li et al., 2015). Additionally, the floral transition in rice is associated with both induction and repression of gene expression at the SAM, and different complexes could operate by promoting or repressing expression of specific targets (Tamaki et al., 2015). Here, we demonstrate that canonical FACs can also form in leaves where Hd3a and RFT1 interact through Gf14c with OsFD1. These complexes are required to activate a positive feedback loop on *Ehd1*, *Hd3a*, and *RFT1* expression. This function is counterbalanced by two OsbZIP transcription factors closely related to OsFD1 that directly bind Hd3a and function as negative regulators of the *Ehd1* florigens module in leaves. Finally, we provide evidence for a meristematic function of one such OsbZIP to repress the floral transition by reducing the expression of inflorescence identity genes. We propose that dynamic formation of distinct complexes fine tunes flowering in leaves and at the SAM of rice.

RESULTS

An Active Florigen Activation Complex Can Form in Leaves

The rice FAC is a transcriptional activation complex assembled in cells of the SAM by Hd3a or RFT1, a Gf14 protein and OsFD1, and its primary targets include members of the *OsMADS* transcription factor family (Kojima et al., 2002; Taoka et al., 2011; Tsuji et al., 2013; Tamaki et al., 2015; Kobayashi et al., 2012). It has been proposed that FAC complexes control a wide range of developmental processes in distinct tissues of several plant species, but to which extent a FAC might function outside of the SAM and in rice leaf tissues is unclear. The diurnal mRNA expression of components of the FAC was quantified under inductive and noninductive photoperiods, including SD (10 h light) and LD (16 h

light) in the leaves (Supplemental Figures 1A to 1D). The expression of *Gf14c* did not depend upon the photoperiod and showed a peak at Zeitgeber (ZT) 15 (Supplemental Figure 1B). Expression of *OsFD1* was detected under both photoperiods; however, its expression under LD was constant during the time course, whereas it oscillated under SD with a peak in the middle of the night (Supplemental Figure 1C). Similarly, expression of *Hd3a* and *RFT1* was induced during the night and peaked toward the end of it (Supplemental Figure 1A).

Since all FAC components were coexpressed in leaves under SD, the expression of *OsMADS14* was used as readout for the activity of the FAC. *OsMADS14* mRNA showed a peak during the night only in leaves of plants grown under SDs, similarly to *OsFD1* (Supplemental Figure 1D). Additionally, expression of both *OsMADS14* and *OsMADS15* was induced in leaves upon shifting plants from LD (16 h light) to SD (10 h light), as more Hd3a and RFT1 became available for FAC formation (Supplemental Figures 1E and 1F). Expression of *OsMADS* TFs is therefore sensitive to expression of FAC components in both leaves and meristem (Taoka et al., 2011; Kobayashi et al., 2012).

Based on relative transcript quantifications, *OsFD1* maximum expression was 5 times lower relative to *Hd3a* or *RFT1* and ;50 times lower than *Gf14c* (compared with *y* axis scales in Supplemental Figures 1A to 1C). Although relative mRNA amounts cannot be accurately compared between genes, these data suggested that OsFD1 might be a limiting factor to FAC formation in leaves. To test this hypothesis, the coding sequence of *OsFD1* was expressed under the constitutive rice *ACTIN2* promoter (*proACT:OsFD1*), and expression of *OsMADS14* and *OsMADS15* was quantified at 6 and 13 d after shifting plants from LD to SD (Figures 1A to 1C). In *proACT:OsFD1* plants, *OsMADS14* and *OsMADS15* expression was strongly upregulated in leaves at the indicated time points, compared with wild-type plants grown under the same conditions, indicating that increasing OsFD1 abundance results in higher induction of FAC target genes (Figures 1B and 1C).

Following the same rationale, we conditionally overexpressed *Hd3a* or *RFT1* in leaves under LD, when *Gf14c* and *OsFD1*, but not *Hd3a* or *RFT1*, are expressed. To control overexpression, dexamethasone-inducible (DEX) *Hd3a*- or *RFT1*-overexpressing plants were produced (*proGOS2:GVG 4xUAS:Hd3a* and *proGOS2:GVG 4xUAS:RFT1*; hereafter referred to as *GVG:Hd3a* and *GVG:RFT1*; Figure 2A). We used a previously validated system for inducible gene expression, composed of a DEX-inducible component that drives expression of the genes of interest (Ouwerkerk et al., 2001). Using this system, we avoided the need for a chimeric florigen-glucocorticoid receptor protein, whose size might impinge on Hd3a or RFT1 protein movement or activity.

Transgenic plants containing *GVG:Hd3a* or *GVG:RFT1* could overexpress transgenic *Hd3a* or *RFT1* only upon DEX treatments (Figures 2B and 2C). While a negligible basal expression of *OsMADS14* and *OsMADS15* was observed in leaves of untreated plants under LD, expression of *OsMADS14* and *OsMADS15* was strongly activated 16 h after DEX treatment, concomitantly to *Hd3a* or *RFT1* induction (Figures 2D and 2E).

Taken together, these experiments indicate that *OsMADS14* and *OsMADS15* transcription in leaves is activated upon

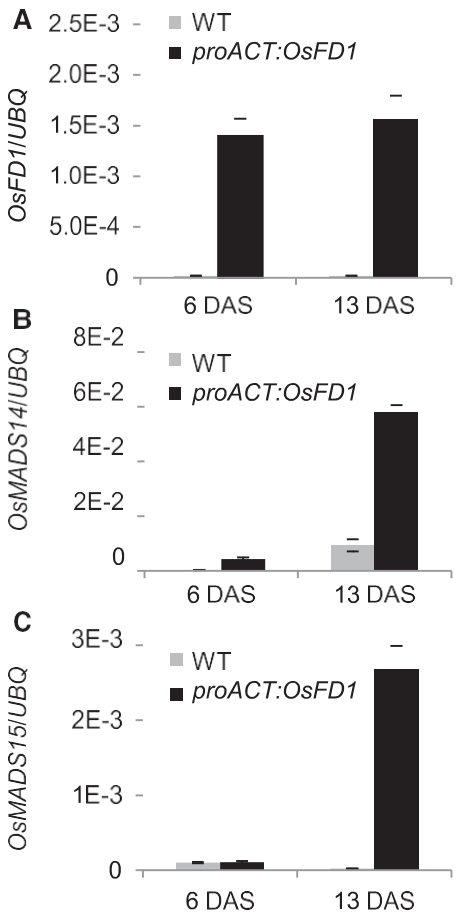

Figure 1. Overexpression of *OsFD1* in Leaves Induces Transcription of Targets of the FAC.

Expression of *OsFD1* (A), *OsMADS14* (B), and *OsMADS15* (C) in leaves of transgenic *proACT:OsFD1* plants. Plants were grown under LD (14.5 h light) for 6 weeks and then shifted to SD (10 h light). Leaves were collected at ZT0 at 6 and 13 d after shift to SD (DAS). *UBIQUITIN* (*UBQ*) was used as standard for quantification of gene expression. Data are represented as mean 6 SD. E-*n* = 3 10^{2n}. ANOVA tests for graphs in (A) to (C) are shown in Supplemental File 1.

coexpression of all FAC components that are likely to form an active complex, as in the SAM.

A Negative Feedback Loop Independent of OsFD1 Limits Florigen Expression in Leaves

The expression of *Hd3a* and *RFT1* is transiently activated in leaves of plants grown under natural field or artificial conditions. This observation suggests the existence of a mechanism that down-regulates their expression upon commitment to flowering and that could possibly depend on *Ehd1*, encoding a common upstream promoter of *Hd3a* and *RFT1* expression (Goretti et al., 2017; Ogiso-Tanaka et al., 2013; Gómez-Ariza et al., 2015). Under our growing conditions, expression of the florigens reached a peak ;12 to 15 d after shifting plants from LD to SD (Galbiati et al., 2016). We tested whether *Hd3a* and *RFT1* are causal to their

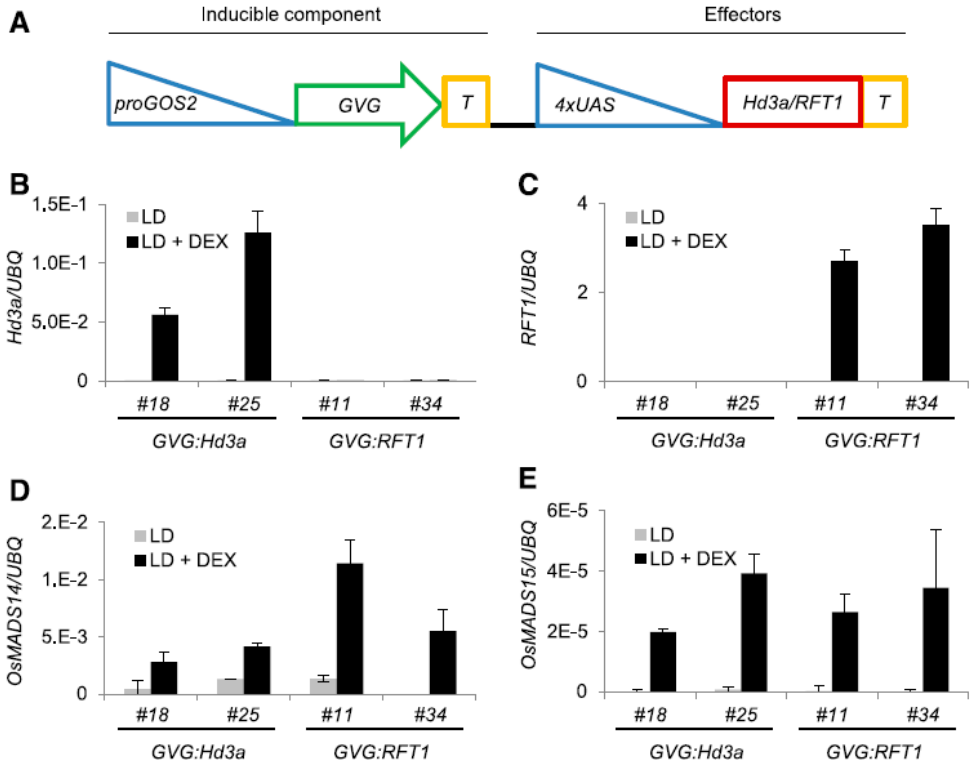

Figure 2. Expression of *OsMADS14* and *OsMADS15* in Leaves Is Dependent on Expression of *Hd3a* and *RFT1*.

(A) Schematics of the inducible system used in this study. The GVG chimeric protein is expressed under the *GOS2* promoter to produce the inducible part of the vector. The *Hd3a* or *RFT1* coding sequences are cloned under the control of the *4x UPSTREAM ACTIVATION SEQUENCE* (*UAS*) to produce the effector component of the vector. T indicates the terminator.

(B) to (E) Expression of *Hd3a* (B), *RFT1* (C), *OsMADS14* (D), and *OsMADS15* (E) in leaves of DEX-inducible transgenic plants grown under LD. Leaves were harvested at ZT0. *GVG:Hd3a* and *GVG:RFT1* indicate DEX-inducible *Hd3a*- and *RFT1*-overexpressing lines, respectively. Two independent transgenic lines are shown for each construct. Plants were either DEX- or mock-treated, and transcripts were quantified using primers designed on the coding sequences. *UBQ* was used as standard for quantification of gene expression. Data are represented as mean ± SD. xE-*n* = 3×10^{2n}. ANOVA tests for graphs in (B) to (E) are shown in Supplemental File 1.

own downregulation in leaves after the floral transition. The *GVG:Hd3a* or *GVG:RFT1* transgenic plants were grown under LD (16 h light) and then shifted to SD (10 h light) to induce expression of the endogenous *Hd3a* and *RFT1* transcripts in leaves. After 13 SD, half of the plants were DEX treated to overexpress transgenic *Hd3a* or *RFT1* (Figures 3A and 3C). Leaf samples were harvested 16 h after DEX treatment at ZT0, when endogenous *Ehd1*, *Hd3a*, and *RFT1* were highly expressed. Quantification of transcripts indicated that the endogenous *Ehd1*, *Hd3a*, and *RFT1* transcripts were strongly downregulated in DEX-treated plants compared with mock-treated controls (Figures 3B and 3D). A similar reduction of transcripts abundance was observed when either of the two florigens was induced (Figures 3A to 3D). We tested several independent lines of both *GVG:Hd3a* and *GVG:RFT1* for DEX-dependent control of *Ehd1*, *Hd3a*, and *RFT1* transcripts. Despite a varying degree of inducibility among independent transgenic lines, as quantified by the increase in *Hd3a* and *RFT1* expression in response to DEX, we consistently observed reduction of endogenous *Ehd1*, *Hd3a*, and *RFT1* transcripts (Supplemental Figures 2A and 2B). Therefore, both Hd3a and RFT1 can mediate a negative feedback loop on *Ehd1* and, indirectly, on their own expression. The negative loop is activated also at low levels of expression of transgenic *Hd3a* or *RFT1*, suggesting that it finely adjusts expression of the florigens during floral induction.

A canonical OsFD-containing FAC could be required for negative regulation of *Hd3a* and *RFT1* expression. Since OsFD1 is limiting to FAC formation in leaves at 12 d after shift (DAS), expression of the florigens was analyzed in *proACT:OsFD1* plants at this time point. Compared with wild-type plants, constitutive expression of *OsFD1* induced the upregulation of *Hd3a*, *RFT1*, and *Ehd1* expression (Figures 3E and 3F). These data suggest that OsFD1 can promote expression of *Ehd1*, *Hd3a*, and *RFT1* in leaves and is not part of the mechanism that self-limits expression of the florigens.

Identification of FAC Components Expressed in Leaves

In rice and other plant species, many bZIP TFs have been already described that form alternative FACs with the florigens and control different developmental processes (Tylewicz et al., 2015; Tsuji et al., 2013; Li et al., 2015). We evaluated whether other TFs abundant in leaves might form alternative FACs with a flowering repressive function. We performed untargeted and targeted yeast two-hybrid screens using Hd3a and RFT1 as baits. Only the results of targeted screens will be presented in this study. We selected members of the bZIP family of transcription factors based on sequence similarity with OsFD1, wheat TaFDL2 (Li et al., 2015; Li and Dubcovsky, 2008) and maize DLF1 (Muszynski et al., 2006)

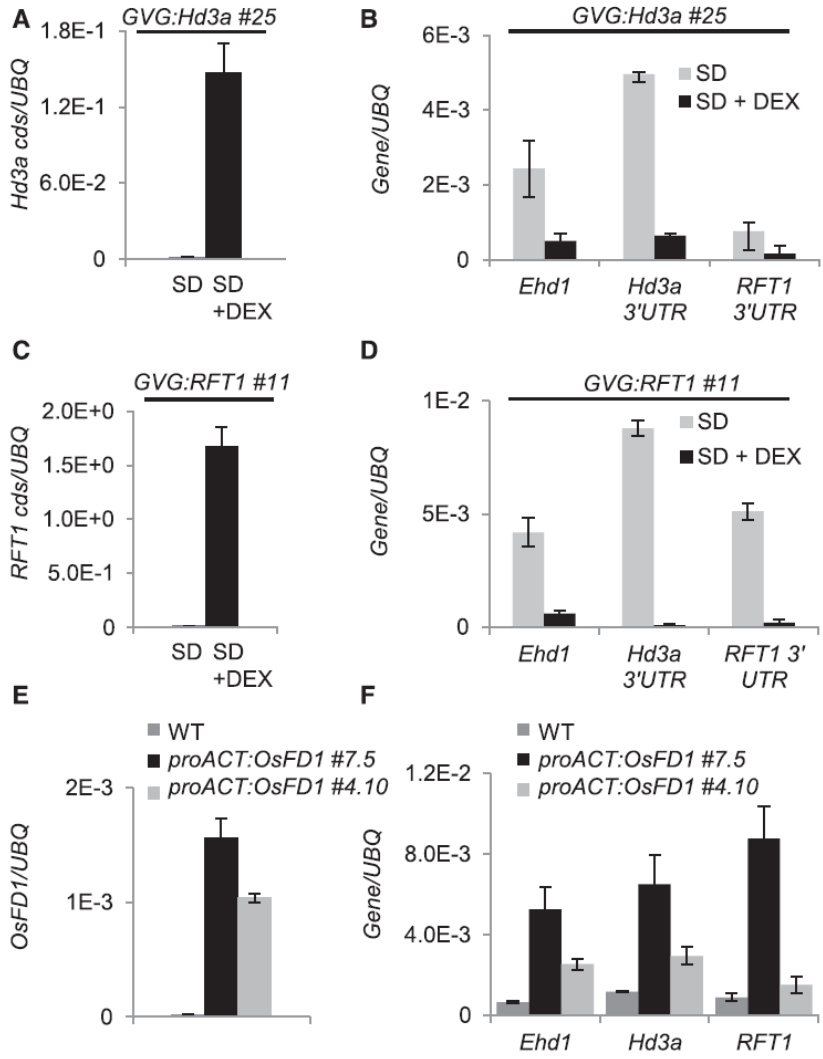

Figure 3. A Negative Feedback Loop Independent of *OsFD1* Reduces *Ehd1*, *Hd3a*, and *RFT1* Expression during Floral Induction in Leaves.

(A) to (D) DEX-induced overexpression of *Hd3a* ([A] and [B]) or *RFT1* ([C] and [D]) causes strong increase of *Hd3a* (A) or *RFT1* (C) transcript accumulation from transgenic sequences, but downregulation of *Ehd1*, *Hd3a*, and *RFT1* endogenous transcripts, compared with mock-treated controls ([B] and [D]).

(E) and (F) Two independent transgenic *proACT:OsFD1* lines show increased expression of *OsFD1* (E) and of *Ehd1*, *Hd3a*, and *RFT1* in leaves compared with the wild type (F). DEX was applied at 13 DAS, and leaf samples were collected at ZT0, 16 h later. *proACT:OsFD1* plants were collected at ZT0 and 12 DAS. Leaves from 10 plants per treatment were sampled. *UBQ* was used as standard for quantification of gene expression. Data are represented as mean 6 SD. Primers on *Hd3a* or *RFT1* coding sequences or on the 39 untranslated regions were used to distinguish transgenic+endogenous ([A] and [C]) from en- dogenous transcripts, respectively ([B] and [D]). ANOVA tests for graphs in (A) to (F) are shown in Supplemental File 1.

(Supplemental Figure 3A and Supplemental Data Set 1), and we tested their interaction with Hd3a and RFT1. Since it has been shown that bZIP TFs bind DNA by forming homo- and hetero-dimers, we also tested their ability to homo- and heterodimerize. OsFD1 interaction with Gf14c was used as positive control (Taoka et al., 2011). A summary of all interactions is reported in Table 1. We excluded from this analysis *OsbZIP29* as we could not amplify it from cDNA of LD- or SD-grown plants, *bZIP54/OsFD6* as it is inferred to be a pseudogene (Tsuji et al., 2013), and finally genes whose interaction patterns have already been determined (Tsuji et al., 2013). The OsbZIP24/OsFD3 and OsbZIP69/OsFD4 proteins could not interact in our yeast assay with Hd3a or RFT1, although a recent report indicates weak interaction with RFT1 (Jang et al., 2017). OsbZIP24/OsFD3 could interact with Gf14c, while OsbZIP69/OsFD4 could not. Conversely, OsbZIP62, OsbZIP42, and OsbZIP9 could interact with Hd3a but not with RFT1, indicating some binding preference for one of the florigens. However, they also interacted with Gf14c, which could possibly bridge the interaction with both florigens.

Among the bZIP TFs tested, we identified OsbZIP62, OsbZIP42, and OsbZIP9 as interactors of Hd3a and Gf14c (Table 1, Figure 4A). Based on their functional characterization, we renamed

OsbZIP42 and OsbZIP9 as Hd3a BINDING REPRESSOR FACTOR1 (HBF1) and HBF2, respectively. The HBF1 and HBF2 proteins share 19.13% and 20.75% amino acid identity with OsFD1 and cluster in the same branch of the bZIP phylogenetic tree (Supplemental Figure 3A). They share 68% identity with each other when the full-length proteins are considered.

To further validate the direct interactions of HBF1, HBF2, and OsbZIP62 with Hd3a, bimolecular fluorescent complementation (BiFC) experiments were performed. The YFP N terminus was fused to each bZIP transcription factor creating HBF1-YFP N, HBF2-YFP N, and bZIP62-YFP N chimeric proteins, whereas the YFP C terminus was fused to Hd3a (Hd3a-YFP C) (Figure 4B). Leaves of *Nicotiana benthamiana* were infiltrated with Hd3a-YFP C and each of the bZIP chimeric fusions, and nuclei of the epidermis showed strong YFP fluorescence, indicating physical interactions between Hd3a and HBF1, HBF2, or bZIP62 as well as nuclear localization of the heterodimers. No fluorescence was observed in nuclei coexpressing OsFD1-YFP N and Hd3a-YFP C, confirming the indirect interaction between OsFD1 and Hd3a (Taoka et al., 2011).

Interactions were also assessed by Förster resonance energy transfer (FRET) fluorescence lifetime imaging microscopy (FLIM) (Berezin and Achilefu, 2010). In FRET-FLIM measurements, the readout for FRET is a reduced lifetime of the donor molecule in the FRET sample, compared with the donor-only sample. FRET occurs when two molecules interact directly. A decrease in the Hd3a-GFP donor lifetime was observed in the presence of HBF1-mCherry, HBF2-mCherry, and OsbZIP62-mCherry, confirming direct interactions in *N. benthamiana* epidermal nuclei (Figures 4C and 4D). No significant reduction of donor lifetime was observed when coexpressing Hd3a-GFP and OsFD1-mCherry (Figures 4C and 4D).

Direct interactions between HBF1, HBF2, and Hd3a were conclusively assessed in vitro by GST pull-down assays. We fused HBF1 and HBF2 to the maltose binding protein (MBP) and incubated them with either Gf14c-GST or Hd3a-GST immobilized on a glutathione resin. Both bZIPs bound Gf14c-GST and Hd3a-GST, but not GST alone (Figure 4E; Supplemental Figure 3E). These data confirm that interactions between HBF1, HBF2, and Hd3a occur in nuclei and do not require an intermediate 14-3-3 protein.

Finally, since bZIP TFs bind the DNA as dimers (Schütze et al., 2008; Reinke et al., 2013), we also tested the possibility that HBF1 and HBF2 could heterodimerize with each other or with OsFD1. We did not observe heterodimerization between these proteins in yeast (Table 1) or using the FRET-FLIM system (data not shown), indicating that HBF1, HBF2, and OsFD1 are likely part of distinct transcriptional complexes.

Diurnal time courses were used to determine the spatiotemporal expression of *OsbZIP62*, *HBF1*, and *HBF2* (Supplemental Figures 3B to 3D). The mRNA expression of *OsbZIP62* was most abundant in the SAM under SD and showed no strong oscillation during the 24-h cycle, despite a slight decline during the night. Transcript abundance was negligible in leaves, indicating that OsbZIP62 is likely not part of a complex limiting *Hd3a* expression in leaves but is possibly part of an Hd3a-containing complex in cells of the SAM (Supplemental Figure 3D). Transcripts of *HBF1* and *HBF2* were highly expressed in the SAM and showed expression also in leaves. *HBF1* transcription in leaves reached a peak during the night, when *Hd3a* transcripts are also abundant (Supplemental Figures 3B and 3C). Taken together, these data indicate that HBFs can potentially form distinct complexes both in the SAM and leaves.

HBF1 and *HBF2* Encode Floral Repressors That Reduce *Ehd1*, *Hd3a*, and *RFT1* Expression in Leaves

Whether *HBF1* and *HBF2* could influence flowering or expression of the florigens in leaves was assessed by overexpressing them under the constitutive *ACT* promoter (Supplemental Figures 3F and 3G). Expression of *Ehd1*, *Hd3a*, and *RFT1* was monitored

Table 1. Targeted Yeast Two-Hybrid Analysis between Hd3a, RFT1, Gf14c, and Selected OsbZIPs

		AD Clones									
		Hd3a	RFT1	Gf14c	OsFD1	OsbZIP69/ OsFD4	OsbZIP24/ OsFD3	OsbZIP62	OsbZIP9/ HBF2	OsbZIP42/ HBF1	Empty AD
BD Clones	Hd3a	–	–	20	-	–	–	15	20	20	–
	RFT1	–	–	20	-	–	–	–	–	–	–
	Gf14c	–	–	20	20	–	15	10	20	20	–
	OsFD1	–	–	10	–	–	–	–	–	n.t.	–
	OsbZIP69/ OsFD4	–	–	–	–	20	20	–	–	–	–
	OsbZIP24/ OsFD3	–	–	15	–	–	20	–	–	–	–
	OsbZIP62	–	–	20	–	–	–	–	n.t.	–	–
	OsbZIP9/ HBF2	–	–	10	–	–	–	–	n.t.	–	–
	OsbZIP42/ HBF1	10	–	15	–	–	–	–	–	n.t.	–
	Empty BD	–	–	–	–	–	–	–	–	–	–

Interaction strength is shown as the highest 3-aminotriazole concentration on which diploid colonies could grow when plated on selective medium. A dash indicates no interaction. n.t., not tested. BD fusions were expressed in yeast strain Y187 (matα), and AD fusions were expressed in yeast AH109 (matA). Diploid yeast was produced by mating. Growth was observed after 6 d at 30°C.

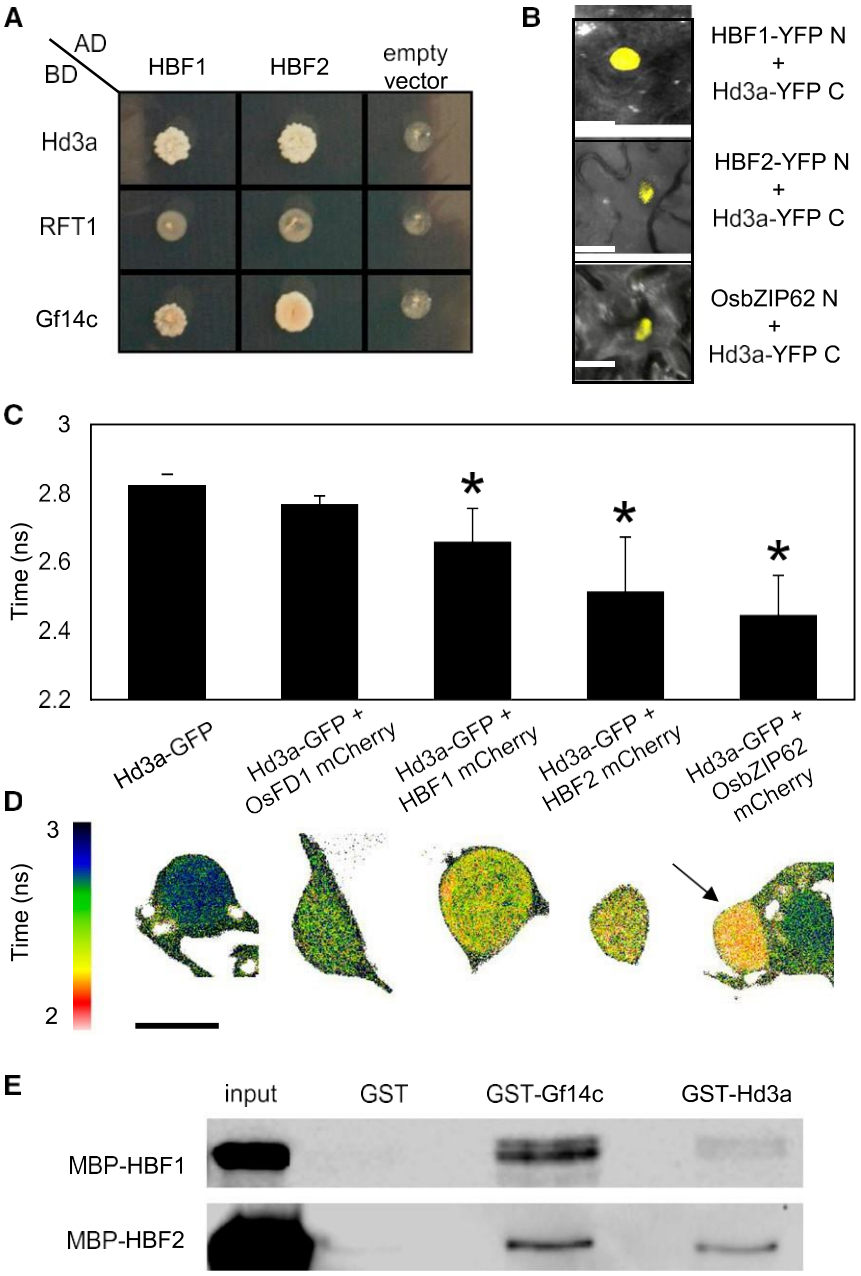

Figure 4. HBF1 and HBF2 Interact with GF14c and Directly with Hd3a.

(A) Yeast two-hybrid assays between Hd3a, RFT1, and Gf14c fused to the binding domain (BD) and HBF1 or HBF2 fused to the activation domain (AD) of Gal4. Colonies were grown on selective -L-W-H medium supplemented with 10 mM 3-aminotriazole.

(B) BiFCassaysshowingrestoredYFPfluorescenceinnucleiuponcoexpressionofHd3a-YFPCwithHBF1-YFPN,HBF2-YFPN,orOsbZIP62-YFPN.Bar=10*m*m.

(C) FRET-FLIM measurements of the Hd3a-GFP donor lifetime in the presence of the acceptors OsFD1-mCherry (no FRET), HBF1-mCherry, HBF2- mCherry,or OsbZIP62-mCherry.Theaveragelifetimeof10transformednucleipermeasurementisshown6 SD.Anasteriskindicatessignificanceforp<0.0003 (Student's *t* test). ANOVA test for the graph is shown in Supplemental File 1.

(D) Colorcodeindicatingthelifetimeof GFPateachpixelinonerepresentativenucleusfortheinteractionsshownin(C).Fortheinteractionbetween Hd3a andOsbZIP62two adjacentcellsareshown,whereonlytheleftnucleus(arrow)coexpressesbothconstructs,whiletherightoneexpressesonlyHd3a-GFP. Accordingly, shortened lifetime is observed only in the left nucleus.

(E) GST pull-down assay showing interactions between MBP-HBF1 and MBP-HBF2 with GST-Gf14c and GST-Hd3a, but not with GST alone. An im- munoblot using an anti-MBP antibody is shown. Protein sizes are MBP-HBF1, 79.5 kD, and MBP-HBF2, 79.5 kD. Resin loading control is shown in Supplemental Figure 3E.

during photoperiodic induction of plants shifted from LD (16 h light) to SD (10 h light). Leaves of the *proACT:HBF1* and *proACT:HBF2* plants showed a marked downregulation of *Ehd1*, *Hd3a*, and *RFT1* expression compared with the wild type, unlike what observed in *proACT:OsFD1* transgenic plants (Figures 5A and 5B). In agreement with the overall downregulation of the *Ehd1*-florigen module, *proACT:HBF1* and *proACT:HBF2* plants flowered late when grown for 2 months under LD and then shifted to SD (Figure 5C).

We obtained the *hbf1-1* mutant from the PFG T-DNA collection in the cultivar Dongjin (Jeon et al., 2000). Quantification of transcripts in the mutant showed that expression of *HBF1* was strongly reduced because of insertion of the T-DNA in the promoter (Supplemental Figures 4A and 4B). We analyzed the flowering behavior of the *hbf1-1* mutant and observed that it headed earlier by ;5 d compared with segregating wild-type siblings under continuous LD (14.5 h light) and by ;9 d under SD (10 h light) (Figure 5D). To link the mutant phenotype with photoperiodic regulation of the *Ehd1*-florigen module, transcript abundance of *Ehd1*, *Hd3a*, and *RFT1* was determined at two time points after shifting plants from LD to SD (10 and 17 DAS). The mRNA accumulation of all genes was higher in the *hbf1-1* mutant compared with the wild type at both time points, indicating derepression of the module (Figures 5E to 5G). To exclude an indirect effect of HBF1 on *Ehd1* expression, the expression of six genes upstream of *Ehd1* was also measured (Supplemental Figures 4C and 4D). None of them showed a difference in gene expression between the wild type and the *hbf1-1* mutant. The only exception was *Ghd7*, which was slightly downregulated in the mutant compared with the wild type (Supplemental Figure 4D).

To confirm that loss of *HBF1* function promotes flowering and also to assess a possible functional redundancy between *HBF1* and *HBF2*, we generated a series of double *hbf1 hbf2* mutants in the cultivar Nipponbare, using the CRISPR/Cas9 technology (Miao et al., 2013). We designed a single-guide RNA (sgRNA) on a region highly conserved between *HBF1* and *HBF2* on their first exon, to simultaneously target both loci (Supplemental Figure 5A). Upon regeneration of transgenic plants, we obtained six independent lines harboring different combinations of biallelic or homozygous indels (Supplemental Figure 5B). We selected five T2 lines (#1.2, #2.1, #4.1, #4.2, and #6.1) from four independent T1s (#1, #2, #4, and #6), all of which were homozygous for *hbf1* mutations and homozygous or biallelic for *hbf2* (Supplemental Figure 5C). All lines were double *hbf1 hbf2* loss-of-function mutants, except line #4.1, which contained a homozygous 227 bp in-frame deletion at the *HBF1* locus, likely not causing loss of gene function (Supplemental Figure 5C). We measured their flowering time under LD (14.5 h light) and after growth for 8 weeks under LD followed by SD (10 h light). Under both conditions, all *hbf1 hbf2* double loss-of-function mutants flowered earlier compared with the wild type (Figures 5H to 5K), but flowering was not accelerated in line #4.1. These data indicate that loss of nine amino acids (EDFLVKAGV before the bZIP domain) in the HBF1 protein likely does not affect its function. They further indicate that the *hbf2* mutation does not additively contribute to the phenotype caused by single *hbf1* mutations. As opposed to the effect of the *hbf1-1* allele in Dongjin, the Nipponbare *hbf1 hbf2* CRISPR mutants showed predominantly accelerated flowering under LD (;13 d was the largest difference observed between line #1.2 and the wild type), rather than under SD (the same line #1.2 flowered ;5 d earlier than the wild type). We attribute these differences to the different sensitivity of Dongjin and Nipponbare to loss of HBF1 function.

HBF1 Can Bind the *Ehd1* Promoter

Expression of *Ehd1* is dependent upon HBF1 activity. The *Ehd1* promoter region was scanned in search of conserved motifs recognized by bZIP TFs, and we found three CACGTC motifs that are characteristic of abscisic acid response elements (ABREs) and G-boxes (Li and Dubcovsky, 2008) (Supplemental Figure 5D). As expected by the central position of *Ehd1* in flowering regulatory networks, many other motifs were identified in its promoter region spanning 1.5 kb upstream of the ATG (Supplemental Figure 5D). The possibility of a direct interaction between HBF1 and the *Ehd1* promoter was assessed using electrophoretic mobility shift assay. The HBF1 protein was purified and incubated with a Cy5-labeled oligonucleotide identical to the region of the *Ehd1* promoter containing the ABRE, located at 2482 bp (Supplemental Figure 5D). HBF1 binding to this oligonucleotide resulted in a band shift (Figure 6D). Addition of an excess of unlabeled oligonucleotide reversed the shift of the fluorescent probe. However, no band shift could be detected when HBF1 was incubated with a promoter fragment containing a CArG-box, demonstrating that HBF1 binding to the ABRE-containing region was specific (Figure 6D). No ABREs or G-boxes were identified by scanning the *Hd3a* or *RFT1* promoters, although indirect binding of HBF1 to these genes cannot be completely excluded.

HBF1 Represses Transcription of *OsMADS14* and *OsMADS15* in the Shoot Apical Meristem

The *HBF1* and *HBF2* transcripts could be identified in both leaves and SAMs, suggesting that they are expressed in both florigen-producing and -receiving tissues. Their overexpression delayed flowering, and in leaves it reduced mRNA expression of *Hd3a* and *RFT1*. Whether these proteins also had a role in the SAM to control flowering or gene expression was tested by misexpression studies. To this end, the promoter of *ORYZA SATIVA HOMEOBOX1* (*proOSH1*) was cloned and used to drive expression of *HBF1*. *OSH1* is expressed in undifferentiated cells of the SAM but not in organ primordia arising from it (Itoh et al., 2000; Sentoku et al., 1999). Transgenic *proOSH1:HBF1* rice plants that overexpressed *HBF1* were produced. Transcriptional analysis of leaves and SAMs of T2 lines indicated that expression driven by the *OSH1* promoter was effective at increasing expression of *HBF1* at the SAM but not in leaves (Figure 6A). The same plants had delayed flowering by a few days compared with non-transgenic segregating controls (Figure 6B). Our dissection of SAMs included also some of the youngest leaf primordia arising from the meristem; however, the *OSH1* promoter is not active in this tissue (Tsuda et al., 2011). Thus, we conclude that the flowering delay is caused by increased expression of *HBF1* in meristematic cells. Transcripts of *Hd3a* and *RFT1* were not expressed at the meristem; therefore, although we cannot fully exclude the expression of other *FT*-like genes, feedback regulation of these florigens is likely not occurring at the apex.

Finally, the expression of *OsMADS14* and *OsMADS15* was found to be significantly reduced in SAMs (Figure 6C). These data indicate that *HBF1* can repress flowering and expression of

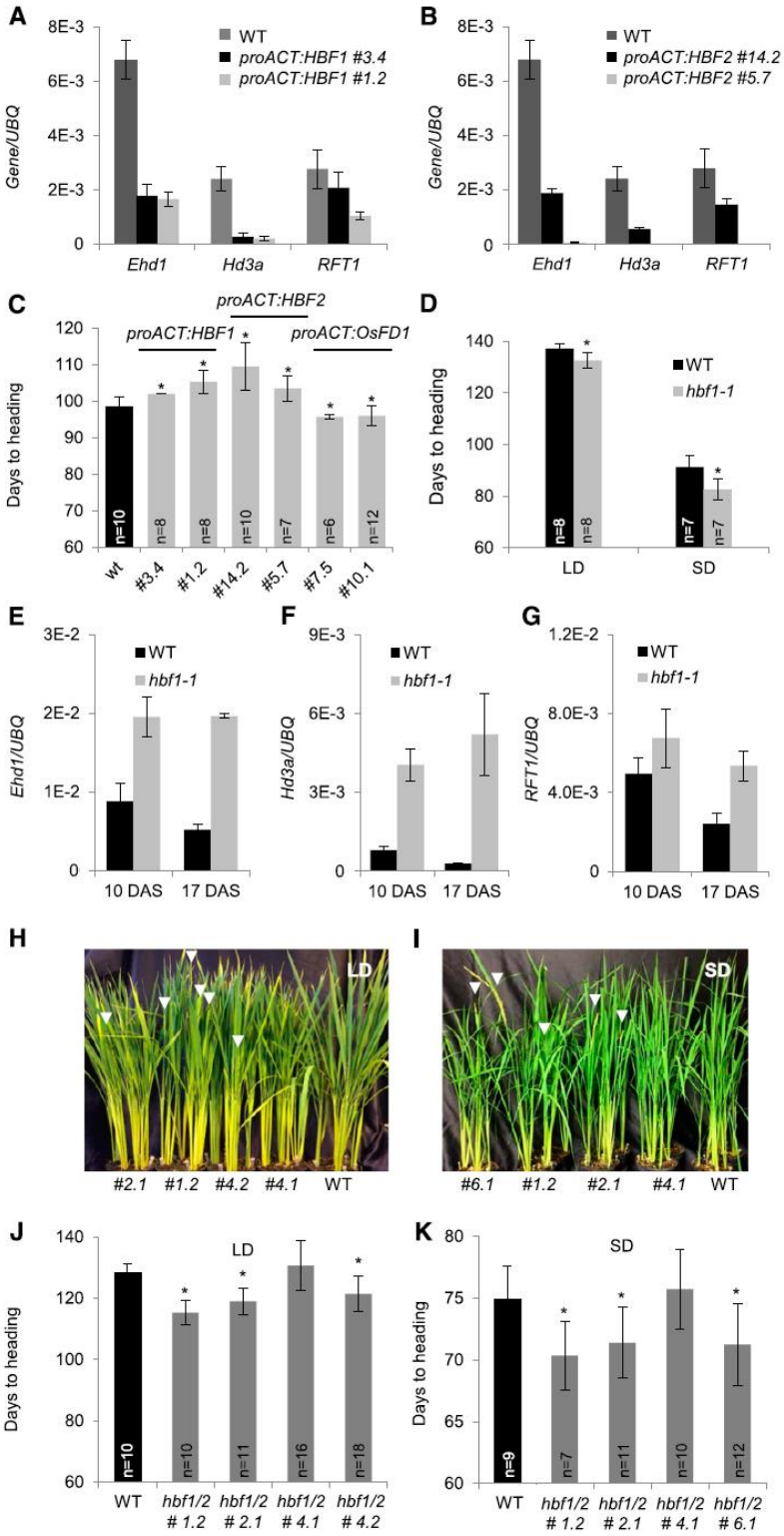

Figure 5. *HBF1* and *HBF2* Encode Floral Repressors Reducing *Ehd1* Expression.

(A) and (B) Quantification of mRNA levels of *Ehd1*, *Hd3a*, and *RFT1* in leaves of *proACT:HBF1* (A) and *proACT:HBF2* (B) overexpression plants grown for 8 weeks under LD (16 h light) and then shifted to SD (10 h light). *UBQ* was used as standard for quantification of gene expression. Data are represented by mean 6 SD.

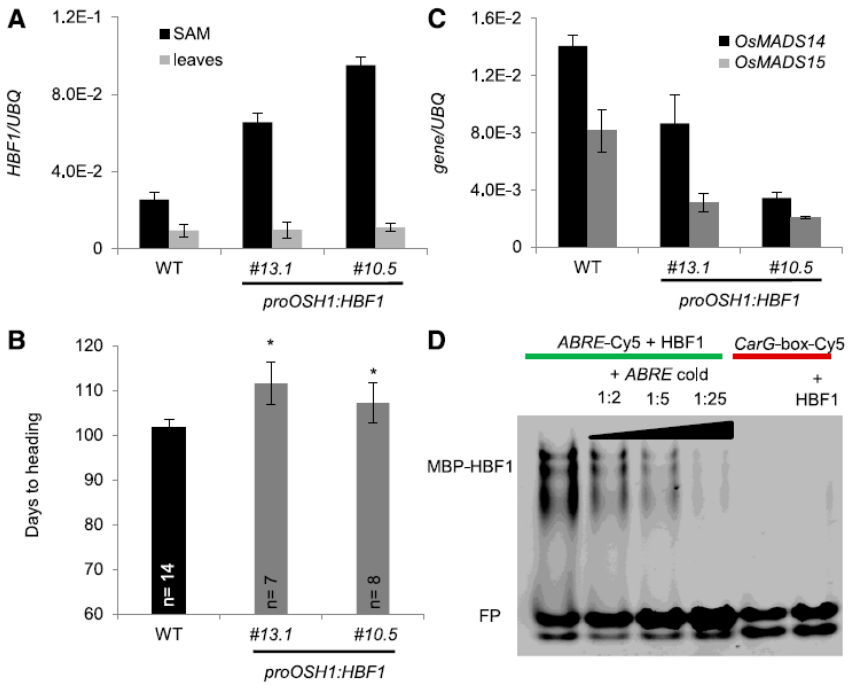

Figure 6. HBF1 Represses Flowering at the SAM.

(A) Quantification of *HBF1* expression in SAMs and leaves of plants misexpressing *HBF1* from the *OSH1* promoter. Two independent transgenic lines are shown.
(B) Heading dates of *proOSH1:HBF1* transgenic plants grown for 8 weeks under LD (16 h light) and then shifted to SD (10 h light) (*n* indicates the number of plants scored). Asterisks indicate $P < 0.05$ in an unpaired two-tailed Student's *t* test.
(C) Quantification of *OsMADS14* and *OsMADS15* expression in SAMs of transgenic *proOSH1:HBF1* plants. Samples in (A) and (C) were collected from apical meristems grown under LD and then exposed to 12 inductive SD. *UBQ* was used as standard for quantification of gene expression. All data are represented by mean ± SD. E-*n* = 3×10^{-2n}.
(D) Electrophoretic mobility shift assay between MBP-HBF1 and *ABRE*-Cy5 (lanes 1–4) and HBF1 and *CArG*-box-Cy5 (lane 6). The specificity of interaction between HBF1 and *ABRE*-Cy5 was tested by incubation with increasing amounts of unlabeled oligonucleotides (labeled/unlabeled oligonucleotide ratios 1:2, 1:5, and 1:25). HBF1 was incubated with an oligonucleotide containing a *CArG*-box-Cy5 (lanes 5 and 6) as a negative control. FP, free probe. ANOVA tests for graphs in (A) to (C) are shown in Supplemental File 1.

inflorescence identity genes at the SAM and therefore has a dual transcriptional repressive function in distinct plant compartments.

DISCUSSION

Dexamethasone treatment of plants expressing inducible versions of *Hd3a* and *RFT1* indicated the existence of transcriptional repression of the florigens mediated by a feedback negative loop.

Thus, we propose a modification of the rice floral induction model to include an autoregulatory loop centered on *Hd3a* and *RFT1*. The florigens regulate their own expression in leaves by forming distinct FACs with several OsbZIP proteins (Figure 7). These complexes can either promote or repress *Ehd1*, *Hd3a*, and

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Figure 5. (continued).

(C) Days to heading of wild type, *proACT:HBF1*, *proACT:HBF2*, and *proACT:OsFD1* overexpressors grown for 8 weeks under LD (16 h light) and then shifted to SD (10 h light).
(D) Heading dates of wild type (Dongjin) and *hbf1-1* mutants grown under continuous LD (14.5 h light) or continuous SD (10 h light).
(E) to (G) Expression of *Ehd1* (E), *Hd3a* (F), and *RFT1* (G) in *hbf1-1* mutant plants compared with the wild type.
(H) to (K) mRNA levels are shown at 10 and 17 d after shifting plants from LD to SD.

(H) and (I) Nipponbare wild type and T2 *hbf1 hbf2* CRISPR mutants grown under continuous LD (14.5 h light) (H) or shifted from LD (16 h light) to SD (10 h light) 8 weeks after sowing (I). Arrowheads indicate the emerging panicles.

(J) and (K) Quantification of heading dates in the same plants as in (H) and (I), respectively (*n* indicates the number of plants scored). Asterisks indicate $P < 0.05$ in an unpaired two tailed Student's *t* test. E-*n* = 3×10^{-2n}. The detailed genotypes of the mutants are reported in Supplemental Figure 5C.

ANOVA tests for graphs in (A) to (G), (J), and (K) are shown in Supplemental File 1.

RFT1 depending on the interacting bZIP. In particular, OsFD1 acts as transcriptional activator in leaves, whereas the closely related HBFs repress expression of the florigens in the same tissue. Thus, Hd3a and RFT1 proteins can engage in both florigen activation and repression complexes. Binding of HBF1 to the promoter of *Ehd1* further provides molecular evidence for feedback regulation of the florigens. The preference of RFT1 and Hd3a to interact with OsFD1 or the HBFs can be driven by relative expression patterns or modifications of OsFD1 and the HBFs under different growing conditions. Both the *HBF1* and *HBF2* transcripts are expressed in the SAM as well, and tissue-specific overexpression of *HBF1* at least, could reduce the expression of targets of the FAC at the apex.

These data identify a previously unknown function for the rice florigens in leaves and suggest the existence of a regulatory layer limiting Hd3a and RFT1 signaling to fine-tune production of the florigens in leaves and their effect on gene regulatory networks at the apical meristem.

The Rice Florigens Act in Leaves to Regulate Their Own Expression

A growing number of studies demonstrate that FT-like proteins are involved in a wide range of developmental processes, including tuberization (Navarro et al., 2011), bulbing (Lee et al., 2013), stomatal opening (Kinoshita et al., 2011), leaf curling (Teper-Bamnolker and Samach, 2005), vegetative growth in trees (Hsu et al., 2011), plant architecture in tomato (Park et al., 2014), and tillering in rice (Tsuji et al., 2015). In many such instances, they function in tissues different from the SAM. However, FT-like proteins have been most prominently described in the context of flowering time control in response to environmental cues. During this process, they act as long distance flowering promoters produced in leaves and translocated to the SAM, inducing developmental switches upon the formation of a FAC (Lifschitz et al., 2006; Corbesier et al., 2007; Mathieu et al., 2007; Tamaki et al., 2007). The data presented in this study suggest that a FAC can form also in rice leaves to activate expression of the same targets normally transcribed in the SAM. That a FAC is active also in leaves was initially suggested by experiments in Arabidopsis (Teper-Bamnolker and Samach, 2005). Expression of *FT* or *Tomato FT* (*TFT*) in transgenic Arabidopsis plants from the viral 35S promoter caused leaf curling that could be suppressed by mutating *FD*, *SEP3*, or *FUL*. These data indicated that a FAC formed in leaves under specific conditions could perturb leaf development by promoting transcription of targets usually expressed at the SAM (Teper-Bamnolker and Samach, 2005).

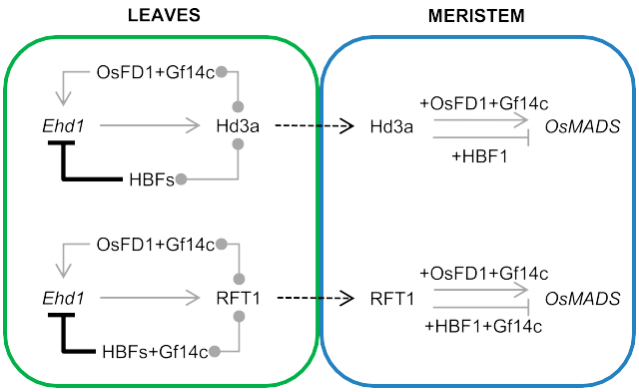

Figure 7. Combinatorial Circuitry Controlling Production of and Response to Florigenic Proteins in Rice.

In leaves, Hd3a and RFT1 can promote expression of *Ehd1* by forming a canonical FAC with OsFD1 and Gf14c, and they can repress it by in- teracting with HBFs. Hd3a can interact directly with HBFs, whereas RFT1 might interact indirectly with HBFs through GF14c. Binding of HBF1 to the *Ehd1* promoter is direct. Upon translocation to the meristem, Hd3a and RFT1 proteins can promote transcription of *OsMADS* target genes by forming a canonical FAC. HBF1 at least can repress transcription of the same targets by forming a repressive FAC. Gray arrows and flat-end arrows indicate transcriptional activation and repression, respectively. Con- nectors indicate protein-protein interactions. Thick, black flat-end arrows indicate direct repression by protein-DNA binding. Dashed arrows indicate protein movement.

Whether a FAC has any biologically relevant function in leaves of Arabidopsis remains to be clarified. However, the identification of *Ehd1*, *Hd3a*, and *RFT1* as targets of florigen-containing complexes in leaves of rice suggests that one function of these complexes is feedback tuning of the expression of some of its own components. In particular, by reducing transcription of *Ehd1*, florigen repressor complexes can indirectly limit expression of *Hd3a* and *RFT1*, downstream targets of *Ehd1* (Doi et al., 2004; Zhao et al., 2015). Since seasonal expression of the rice florigens is transient and is strongly reduced upon completion of the floral transition, a plausible biological role for this autoregulatory loop could be to switch off transcription of the florigens upon floral commitment. Alternatively (or in parallel), it could fine-tune the production of Hd3a and RFT1 during photoperiodic induction (Gómez-Ariza et al., 2015; Ogiso-Tanaka et al., 2013). More data will be required to distinguish between these possibilities and validate them, but it is clear that reproductive commitment requires a tight balance between flowering promoting and repressive complexes, whose equilibrium could be controlled by modulating the expression levels of distinct bZIPs by developmental or environmental factors (Tang et al., 2016; Wu et al., 2014; Zhang et al., 2016) or by controlling their activity through phosphorylation (Kagaya et al., 2002; Choi et al., 2005; Furihata et al., 2006). Indeed, phosphorylation of OsFD transcription factors is required for binding to 14-3-3 proteins and is limiting to FAC function (Taoka et al., 2011).

Autoregulatory motifs are likely very common in gene regulatory networks but can be identified and studied only by quantifying endogenous transcripts in plants expressing transgenic copies of the same gene or its closely related homologs. Such approach has led to the identification of a loop regulating *StSP6A* expression, encoding a tuberigen, the mobile protein causing tuber formation at the apical meristem of potato stolons, and sharing high sequence similarity with *Hd3a* (Navarro et al., 2011). A similar autoregulatory loop in the expression of an endogenous florigen has been recently reported in chrysanthemum (*Chrysanthemum seticuspe*), where transcriptional induction of *CsFTL3* required a complex formed by CsFTL3 and CsFDL1 proteins (Higuchi et al., 2013). It is noteworthy that regulatory loops involving two FT-like proteins are also very common among Angiosperms. The FT-like SP5G proteins of potato and tomato inhibit expression of the *SINGLE FLOWER TRUSS* (*SFT*) florigen and of *StSP6A*,

respectively (Abelenda et al., 2016; Soyk et al., 2017). Similar modules in which an FT-like protein inhibits developmental transitions by repressing a second FT-like gene have been reported also for flowering in sugar beet (*Beta vulgaris*; Pin et al., 2010; Higuchi et al., 2013) and bulbing in onion (*Allium cepa*; Lee et al., 2013). In rice, both autoregulatory and relay mechanisms between Hd3a and RFT1 are possible under inductive conditions, when both proteins are expressed. Their differential ability to directly bind to HBFs might underlie differences in their capacity to take part in positive or negative relay mechanisms, but this type of cross-regulation is difficult to dissect genetically because of the redundancy between these factors. However, in general, autoregulatory and relay mechanisms among florigen-like proteins are emerging as very common modules controlling developmental switches.

Florigen-Containing Complexes Exhibit Combinatorial Properties

Florigen activation complexes from several species have a modular structure where distinct bZIP proteins can interact with different FT-like proteins in a combinatorial fashion (Sussmilch et al., 2015; Tsuji et al., 2013). Temporal and spatial dynamics of complex formation highly expand the regulatory possibilities of such complexes to control plant development. In rice leaves, Hd3a and RFT1 can form complexes displaying transcriptional promoting or repressive activity depending on the interacting bZIP. Since HBF1, HBF2, and OsFD1 do not heterodimerize, they cannot be part of the same complex, in agreement with their opposite functions. Additionally, since HBF1 and HBF2 do not interact with each other, they are possibly part of independent complexes.

Different examples in plants suggest that the functional specificity of these regulatory complexes can be provided by the bZIP as well as the FT-like protein. In rice, branching of shoots and altered panicle architecture are induced upon overexpression of OsFD2 (Tsuji et al., 2013). This bZIP can interact with Hd3a, and the interaction is bridged by the Gf14b protein. Given that OsFD2 controls patterns of vegetative growth, it could be speculated that FACs are active during distinct phases of the plant life cycle and not only during reproduction. Additionally, it raises the interesting possibility that complexes dynamically changing the Gf14 protein component might take on different roles. However, functional studies with Gf14 mutants are complicated by their pleiotropy and essential nature (Purwestri et al., 2009).

In hybrid aspen, overexpression of FDL1 but not FDL2 delays bud set and growth cessation, indicating FDL1 specificity for these developmental processes. However, both FDLs could interact with FT1 and FT2 to activate downstream targets in transient heterologous systems (Tylewicz et al., 2015). In these examples, specificity is likely contributed by the FD-like transcription factor.

Conversely, distinct PEBP components binding to the same bZIP protein can switch its function. Arabidopsis FD can interact with FT but also with TFL1, to form a flowering repressive complex (Hanano and Goto, 2011; Ho and Weigel, 2014). Similar interaction patterns are also possible in tomato between SP3G/SPP, an FD homolog, and the TFL1-like protein SELF PRUNING or the SFT florigen, where the balance between complexes regulates shoot architecture and, ultimately, yield (Pnueli et al., 2001; Park et al., 2014). Finally, the floral transition in Arabidopsis axillary meristems is controlled by the TCP transcription factor BRANCHED1, directly interacting with the PEBPs FT and TWIN SISTER OF FT but not with TFL1 (Niwa et al., 2013). Overall, these patterns indicate that a basal conserved module can be repurposed in distantly related species to control several developmental programs and that plasticity in complex assembly determines the balance between developmental programs.

METHODS

Plant Materials

The *hbf1-1* mutant corresponds to the Salk line PFG_2D-00885 in the cultivar Donjing. Homozygous T-DNA insertional mutants were selected using primers listed in Supplemental Table 1. The cultivar Nipponbare was used in all other experiments.

Growth Conditions, Sampling, and Quantification of Gene Expression

Plants (rice [*Oryza sativa*]) were grown under LD (14.5 h light/9.5 h dark or 16 h light/8 h dark) or SD conditions (10 h light/14 h dark) in Conviron PGR15 growth chambers. Light was provided by T8 fluorescent and halogen in- candescent lamps. Light intensity was adjusted to level 3 for both sets of lamps, resulting in ; 450 ㎜ol/m^2/s. Plant material was collected from the distal part of mature leaves, from at least three plants/time point, at ZT0. Only for the experiments described in Figures 5E to 5G and in Supplemental Figures 4C and 4D, plants were sampled at ZT20 under SD, as this time point corresponds to peak expression of *Ehd1*. Only for the data described in Figures 5A and 5B, all samples were quantified in the same experiments and then split into separate graphs for clarity of presentation. For SAM sampling, at least five apices/sample were manually dissected under a stereomicroscope using scalpels. Sample included the meristem, the two younger leaf primordia arising from it, as well as part of the rib meristem. RNA was extracted from leaves using the TRIzol reagent (Thermo Fisher Scientific) and from SAMs using the NucleoSpin RNA Plant kit (Macherey- Nagel). To prepare and quantify cDNAs, the RNA was retro-transcribed using the ImProm-II reverse transcriptase (Promega), and the Maxima SYBR qPCR master mix (Thermo Fisher Scientific) was used to measure gene expression in a Mastercycler Real Plex^2 (Eppendorf). All primers used in RT-qPCR experiments have an annealing temperature of 60°C. For quantification of transcripts of *Hd3a* and *RFT1* endogenous mRNAs, *Ehd1*, *OsMADS14*, *OsMADS15*, and *UBQ*, we used primers described by Galbiati et al. (2016) and Gómez-Ariza et al. (2015). All other primers used in this study are listed in Supplemental Table 1.

Construction of Transgenic Plants and DEX Treatments

The *OsbZIP* coding sequences were amplified from leaf or SAM cDNAs using primers listed in Supplemental Table 1 and subsequently cloned in pDONR207 (Invitrogen). Plant expression vectors were obtained by Gateway cloning, recombining the coding sequence after the *ACTIN* promoter in the pH2GW7 plasmid. The *Hd3a* and *RFT1* coding sequences were amplified from leaves of Nipponbare with primers Os1-Os2 and Os3- Os2, respectively. The *pINDEX2* vector was used for DEX-inducible ex- pression of *Hd3a* and *RFT1* (Ouwerkerk et al., 2001), but it was first turned into a Gateway-compatible (Invitrogen) destination vector by blunt cutting with *Pml*I and insertion of an *Eco*RV-digested Gateway RFC cassette. A *proOSH1:Gateway* destination construct was generated cloning a 1.5-kb promoter fragment using primers Os_6 and Os_7 (Supplemental Table 1). The *pINDEX4* vector and *proOSH1* were then cut using *Mun*I and *Mlu*I and

ligated to create *pINDEX4 proOSH1*. The RFA Gateway cassette was inserted into the *proOSH1 pINDEX4* vector after bluntcutting using *Eco*RV and *Stu*I. Subsequently, the DEX-inducible cassette was removed by blunt cutting using *Swa*I and *Bbr*PI and self-ligation of the vector. The *proOSH1:HBF1* vector was generated by LR recombination (Invitrogen). For rice transformation, embryogenic calli were produced from Nip- ponbare seeds, prepared and transformed according to the protocol of Sahoo et al. (2011) using the EHA105 strain of *Agrobacterium tumefaciens*. Transgenic plants were selected on 50 mg/L and 100 mg/L hygromycin during selections I and II, respectively. Gene expression of *Hd3a* and *RFT1* was induced by leaf spray with 10 *m*M DEX solution + 0.2% Tween, in transgenic homozygous T3 plants. DEX treatments were performed at ZT8 and sampling was done 16 h later at ZT0. Induction efficiency was assessed by RT-qPCR on leaves using primers specific for the *Hd3a* or *RFT1* coding sequences.

Protein-Protein Interaction Studies

For yeast two-hybrid studies, the coding sequences were cloned into the vectors pGADT7 and pGBKT7 (Clontech) Gateway (Invitrogen) and transformed into AH109 and Y187 yeast strains, respectively. Interactions were tested by mating and growth of diploid yeast on selective -L-W-H medium supplemented with 3-aminotriazole. BiFC experiments were performed in *Nicotiana benthamiana* epidermal cells with the vectors pBAT TL-B sYFP-N and pBAT TL-B sYFP-C. FRET-FLIM experiments were performed in *N. benthamiana* epidermal cells transformed with the *b*-estradiol-inducible vectors pABIND-GFP and pABIND-mCherry (Bleckmann et al., 2010; Somssich et al., 2015). *b*-Estradiol induction of the transgenes was performed with 20 mM *b*-estradiol and 0.1% Tween 20 4 to 6 h before measurements. FRET-FLIM measurements were performed on 10 cotransformed nuclei at least, and mean, $_{SD}$, and P value (Student's *t* test) of the donor lifetime for the various sets of experiments were cal- culated, as described by Stahl et al. (2013).

GST Pull-Down

The GST-Hd3a and GTS-GF14c fusion proteins were obtained by recombining the coding sequence into pDEST15 (Invitrogen), expressing them using BL21 (DE3) cells (Invitrogen) and purifying them with Gluta- thione Sepharose 4b (Sigma-Aldrich). The concentration of each fusion protein was determined using Bradford assays. Equal amounts of GST-fusion proteins and GST were incubated in TIF buffer (150 mM NaCl,

20 mM Tris, pH 8.0, 1 mM $MgCl_2$, 0.1% Nonidet P-40, and 10% glycerol) and added to 2 mL of clarified bacterial lysate of BL21 (DE3) cells ex- pressing HBF1 and HBF2 proteins fused to MBP (pMAL vector adapted to

Gateway system). The bacterial lysate was obtained by sonication of a bacterial pellet resuspended in TIF buffer supplemented with cOmplete Protease Inhibitor Cocktail (Roche). The reaction mixture was incubated for 2 h at 4°C under gentle rotation. After three washes with TIF buffer and two washes with PBS buffer, the resins were resuspended with SDS-PAGE loading buffer and eluted at 99°C for 5 min. The eluted proteins were re- solved in 10% SDS-PAGE, and immunoblot analysis was performed using a monoclonal anti-MBP HRP-conjugated antibody (BioLabs).

Phylogenetic Analysis

Sequences of bZIP proteins were retrieved from public databases and aligned using the CLC Genomics Workbench program with the following parameters: gap open cost = 20.0; gap extension cost = 10.0; end gap cost = as any other; alignment mode = very accurate. An unrooted phy- logenetic tree was created on the alignment using the neighbor-joining algorithm. Distances were measured using the Jukes-Cantor model. Bootstrap values are indicated at each node based on 1000 replicates. Sequence alignments are reported in Supplemental Data Set 1.

CRISPR-Cas9 Editing

The CRISPR-Cas9 vector was previously described (Miao et al., 2013). The single-guide RNA oligo (Os_934) targeting both *HBF1* and *HBF2* was designed based on the first exon of both genes, upstream of the region encoding the bZIP domain and expressed in transgenic Nipponbare. Transformation was performed as described above. The *HBF1* and *HBF2* loci in the regenerating plants were amplified and sequenced using primers Os_551-Os_338 and Os_976-Os_553, respectively, to identify the muta- tions introduced by nonhomologous end joining. The same primers were used to genotype the subsequent plant generations.

Electrophoretic Mobility Shift Assays

Consensus sequences in the *Ehd1* promoter (1.5 kb upstream of the ATG) were identified using the Nsite software (Shahmuradov and Solovyev, 2015). The sequences of the ABRE and CArG-box containing primers are shown in Supplemental Table 1. The HBF1 protein fused to MBP was expressed in the *Escherichia coli* Rosetta strain and purified to homoge- neity by passing it through a maltose column followed by an ion exchange step (MonoQ). Binding of HBF1 to the *Ehd1* promoter was tested using

25 pmol of Cy5-labeled DNA duplexes (either ABRE or CArG-box sequences; Supplemental Table 1) mixed with 150 pmol of the purified protein in 20 mM Tris-HCl, pH 8.0, and 200 mM NaCl. In the competition studies, the mixture was supplemented with increasing amounts (1:2 to 1:25 molar ratio) of unlabeled DNA. Precast Novex TBE gels (Thermo Fisher Scientific) were used for the electrophoretic run.

Accession Numbers

Sequence data from this article can be found in the Rice MSU Ge- nome Annotation Release 7 under the following accession numbers: LOC_Os06g06320.1 (Hd3a), LOC_Os06g06300 (RFT1), LOC_Os08g33370 (Gf14c), LOC_Os09g36910 (OsFD1), LOC_05g41070 (HBF1), LOC_Os01g59760 (HBF2), LOC_07g48660 (bZIP62), LOC_Os06g16370.1 (Hd1), LOC_Os10g3 2600.1 (Ehd1), LOC_Os07g15770.1 (Ghd7), LOC_Os07g49460.1 (PRR37), LOC_Os03g54160.1 (OsMADS14), and LOC_Os07g01820.1 (OsMADS15).

Supplemental Data

Supplemental Figure 1. Expression of FAC components and FAC targets in leaves.

Supplemental Figure 2. Independent Hd3a or RFT1 DEX-inducible transgenic lines show a range of *Hd3a* or *RFT1* DEX-dependent induction and downregulation of *Ehd1*, *Hd3a*, and *RFT1* endogenous expression.

Supplemental Figure 3. Selection of bZIP transcription factors putatively forming a transcriptional complex with the florigens.

Supplemental Figure 4. Analysis of the *hbf1-1* mutant.

Supplemental Figure 5. Analysis of *hbf1 hbf2* CRISPR mutants and of the *HBF1* promoter.

Supplemental Table 1. Primers used in this study.

Supplemental Data Set 1. Text file of the alignment used for the phylogenetic analysis shown in Supplemental Figure 3A.

Supplemental File 1. ANOVA tables.

Appendices 2:
A transcription factor coordinating internode elongation and photoperiodic signals in rice

PhD contribution:

In this work we presented *PREMATURE INTERNODE ELONGATION 1* (*PINE1*) a Zinc-finger transcription factor which regulates internodes elongation during the floral transition. *PINE1* is expressed in the vegetative phase and must be downregulated during the flowering to allow the panicle to emerge from the last leaf. For this paper I contributed to the nuclear localization analysis of PINE1 (cloning of the constructs and confocal images). I also conducted the quantification of *PINE1* transcript via qRT-PCR in plants that overexpress *Hd3a* or *RFT1* under the Dexamethasone inducible systems. Thanks to that experiment we connected the repression of *PINE1* to the upregulation of florigens during the floral transition.

A transcription factor coordinating internode elongation and photoperiodic signals in rice

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Keywords Rice, photoperiod, heading, shoot apical meristem, internode elongation, florigens, Hd3a, RFT1, gibberellin

In several plant species inflorescence formation is accompanied by stem elongation. Both processes are accelerated in rice upon perception of shortening days. Here, we show that *PREMATURE INTERNODE ELONGATION 1* (*PINE1*), a rice zinc finger transcription factor, reduces sensitivity of the stem to gibberellin (GA). The florigens reduce *PINE1* expression to increase stem responsiveness to GA, and promote flowering. These data suggest the existence of a regulatory network coordinating flowering and GA-dependent growth.

Exposure of rice leaves to day lengths shorter than a critical threshold activates the expression of *Hd3a* and *RFT1* in the companion cells of the phloem. The cognate proteins move through the vascular system of the plant to reach the shoot apical meristem (SAM), where they commit it to reproductive development[1].

In cells of the SAM, Hd3a and RFT1 interact with proteins belonging to the 14-3-3 family, and with the bZIP transcription factor OsFD1[2,3]. The resulting heterohexameric complex binds to the promoter of *OsMADS15*, a MADS-box transcription factor, whose activity is required for proper inflorescence development redundantly with *OsMADS14*, *OsMADS34*/*PAP2* and *OsMADS18*[4,5].

Commitment to reproductive growth of the SAM needs to be coordinated with stem elongation. This ensures that emergence of the panicle from the protecting leaves (a process called heading in cereals) occurs as its development is complete. However, it is currently unclear if regulatory genes exist that integrate photoperiodic and growth-promoting signals.

Elongation depends on production and perception of gibberellins (GAs), to the extent that the metabolism of this hormone has been genetically manipulated by breeders in several species to modify plant height[6]. This led to the Green Revolution during the '60s and represented a major advancement in agriculture[7–9]. The common precursor of all GAs is converted to bioactive GAs by GA20 oxidases (GA20ox) and GA3 oxidases (GA3ox)[10]. Elongating internodes of rice express *OsGA20ox2* and *OsGA3ox2* in the meristematic and elongation zones at the base of the internodes, whereas their expression is reduced in the mature zone. This pattern corresponds to the gradient of growth and differentiation of cells in the internode[11].

We searched for genes responding to variations in day length and that could account for early regulatory functions during transition of the SAM to reproductive growth. Differently from previous studies that addressed transcriptomic changes at the SAM during floral induction[4,5,12], we sampled shoot apices at early stages, before any morphological change became visible. We developed a morphology-independent system to determine the temporal window of SAM commitment, and used this information to guide sampling of apices for transcriptional profiling. Pools of plants of the cultivar Nipponbare (NB) were grown for 6 weeks under non-inductive long days (LD) and transiently exposed to SD before returning them to LD. Expression of *Hd3a* and *RFT1* transcripts increased in leaves only under inductive photoperiods but dropped to undetectable levels as soon as plants were returned to LD (Fig. 1b). At least 12 SD were required to irreversibly promote flowering of NB, whereas plants treated with less than 12 SD did not flower before the end of the experiment and behaved as plants maintained under continuous LD (Fig. 1a). The same experiment was performed using Taichung 65 (T65), a photoperiod-insensitive cultivar harboring mutations in *Hd1* and *Ehd1*, encoding transcriptional activators of the florigens[13]. Flowering could not be observed in T65 even after transient exposure to 18 SD and expression of the florigens remained very low (Fig. S1a-b). No major morphological changes could be observed at the SAM of NB before 12 SD, but

during this time the apex was fully committed to panicle formation (Fig. S1g). Therefore, sequencing experiments were performed on RNA time courses from shoot apices (Fig. S1f) exposed to 0, 4, 8 and 12 SD, and differentially expressed genes (DEGs) were identified (Dataset S1). During commitment of the apex and after filtering for log fold change (logFC) >1.5 or <-1.5 (FDR ≤0.05), we identified 301 up-regulated and 222 down-regulated genes across all time points (Fig. 1c). Among the 10 most up-regulated genes at the end of commitment we identified *OsMADS14*, *OsMADS15* and *OsMADS34*, indicating activation of the panicle development program (Fig. S2). RNA-seq on shoot apices of T65 identified 122 and 129 genes up and downregulated, respectively (Fig. S1c and Dataset S1).

We reasoned that downregulation of genes required to maintain vegetative features under LD must be crucial to induce the reproductive switch. We then searched for regulatory factors whose expression decreased in response to SD exposure. We identified the C2H2 zinc finger *ZOS12-10* (*LOC_Os12g42250*) as the most downregulated transcription factor of the dataset (Fig. S2 and Dataset S1) and renamed it *PREMATURE INTERNODE ELONGATION 1* (*PINE1*). Transcription of *PINE1* was reduced also during commitment of T65 apices, but at a slower rate, consistent with reduced sensitivity of T65 to changes in day length (Fig. 1d). Transcript quantification in additional varieties suggested that SD-dependent reduction of expression is a general feature of *PINE1* (Fig. 1e). Transcription of *PINE1* was high in the full shoot apex, the SAM proper and the region below the SAM, almost undetectable in mature leaf blades, and very low in mature culms and developing flowers (Fig. 1g). Diurnal time courses confirmed these findings, and further indicated a biphasic rhythm at the shoot apex that dampened under SD (Fig. S3e-f). Transgenic plants expressing the GFP under control of the *PINE1* promoter showed fluorescence in the SAM and in young leaf primordia, as well as in tissues below the SAM (Fig. 1h-i). GFP expression was not detected in the upper nodes but examination of the basal part of the stem showed strong fluorescence at node 4 from the apex, and in basal dividing cells of the internode (Fig. 1h, k-l). Expression of *PINE1* was reduced at the SAM when plants were exposed to SD, indicating sensitivity of its transcription to day length (Fig. 1g, j and Fig. S3b-d). Finally, the nuclear localization of PINE1 was confirmed using a GFP-tagged protein expressed from the 35S promoter in epidermal cells of tobacco leaves, consistent with PINE1 being a transcription factor (Fig S3a). These data indicate that *PINE1* is transcribed at the SAM and surrounding meristematic tissues and in central nodes and dividing cells of the compressed stem. Further, its transcription depends upon the photoperiod, being reduced by SD. We next measured expression of *PINE1* in apices of transgenic plants harboring dexamethasone (dex)-inducible clones of *Hd3a* or *RFT1*, to assess if its transcription depends upon the florigens or other photoperiod-dependent but

florigens-independent signals[14]. Transgenic lines harboring either construct were grown under LD and treated with dex or mock treated. Expression of *PINE1* at the apex was quickly downregulated in response to dex in inducible lines but not in control lines, indicating that its transcriptional repression was dependent upon Hd3a or RFT1 (Fig. 1f).

To separate the effects of *PINE1* transcriptional repression from SD-dependent florigenic signals, we generated CRISPR mutants[15], and analyzed them under LD. Three independent alleles (hereafter referred to as type 1) harbored indels that created frame-shifts, whereas two alleles maintained the reading frame (type 2 mutants) (Fig. 2a). Primary transformants bearing type 1 mutations showed a prostrate habit (Fig. S4a-c). Removal of outer leaf sheaths indicated that prostrate growth was caused by premature elongation of the internodes that caused lodging. Excessive growth was observed also in subsequent generations in seedlings grown under LD (Fig. 2b). Elongation became visible as early as two weeks after germination and increased the distance between the SAM and the base of the plant during growth. Mutant plants elongated the central internodes first, followed by the most apical ones (Fig. 2b), similarly to the pattern of elongation shown by wild type plants during panicle emergence[16]. Thus, we conclude that *PINE1* is a repressor of stem elongation, preventing it to occur before the reproductive switch, and that the photoperiod promotes elongation by reducing its transcription through florigen signaling. Since *PINE1* mRNA expression is highest at the base of the stem, around the internodes that first elongate in the mutant, we suggest a cell-autonomous function for PINE1 to repress stem elongation. We also hypothesize that non-cell autonomous information encoded by the florigens or by a florigen-dependent signal moves from the apex or the leaves to repress *PINE1* expression.

Transcriptional markers of panicle development were induced at the meristem only after exposure to SD (Fig. S4d-e). Consistent with these observations, heading dates of *pine1-1* mutants were like the wild type (Fig S5).

Transgenic rice plants expressing *PINE1* under the control of the *ACTIN2* promoter (*pACT*) developed panicles and flowers with aberrant morphologies (Fig. S6a-b). At least three independent lines developed compact panicles bearing flowers with altered symmetry and duplications of floral organs (Fig. S6b-f). Scanning electron microscopy revealed that in outer floral organs, paleas were converted into lemmas (Fig. S7). In wild type flowers, *PINE1* is expressed at very low levels, and these phenotypes could be caused by strong ectopic expression in floral meristems. The overexpressors were sterile preventing the possibility of quantifying developmental defects in subsequent generations (Fig. S6g-j). Therefore, we expressed

PINE1 under the control of the *ORYZA SATIVA HOMEOBOX 1* (*pOSH1*) promoter, that drives gene expression in the meristem and underlying stem tissues, including nodes and internodes[17–19].*OSH1* is expressed also in floral meristems, yet panicles and flowers of *pOSH1:PINE1* developed normally and were fertile. However, emergence of the panicle wasn't complete being limited by reduced elongation of the internodes (Fig. S8). Taken together, these data indicate that *PINE1* is not affecting the floral transition *per se*, but rather the associated internode elongation that ejects the panicle out of the culm during heading. We conclude that *PINE1* coordinates growth of the internodes downstream of *Hd3a* and *RFT1*, and that internode elongation and flowering can be genetically uncoupled.

Constitutive internode elongation in *pine1* mutants was rescued by treatments with an inhibitor of gibberellin biosynthesis, suggesting that the phenotype is caused by increased GA abundance or by increased sensitivity to the hormone (Fig 2b). To test these possibilities, we first quantified bioactive GAs in stems of *pine1-1* mutants grown under LD. The abundance of GA_1 and GA_4 was reduced compared to the WT in samples collected at ZT0 (Fig. S9d-e). Quantifications performed on a segregating progeny of a *pine1-1a*/*pine1-2a* heterozygous plant corroborated the same difference of GA accumulation between elongating type 1 versus non-elongating type 2 alleles. Samples of this experiment were collected at ZT12, ruling out the possibility that the time of day could impact on the observed differences (Fig. S9f-g). Expression of GA biosynthetic enzymes showed a mild increase in *pine1* mutants compared to the WT (Fig. S9a-c), except for *OsGA20ox1* in *pine1-1a*, whose expression was similar to the wild type. Since GA biosynthesis is characterized by negative feed-back regulation, increased expression of biosynthetic enzymes is correlated to the observed reduction of GA abundance[20,21]. Thus, abundance of GA does not correlate to increased elongation.

Next, we quantified the sensitivity of the stem to GA under LD. To this end, 9-day-old NB wild type, *pine1-1a* and *pine1-1c* plants were treated *in vitro* with PAC, to block endogenous GA biosynthesis, and GA_3 was added to the growth medium at increasing concentrations. Internodes of NB do not elongate under LD in response to exogenously applied GA, regardless of PAC presence (Fig. 2c-d and[22]). Conversely, GA_3 induced strong elongation of *pine1-1* stems that was proportional to the concentration of GA_3 (Fig. 2c-d). Measurements of total stem length indicated that GA-treated *pine1-1* mutants were as long or longer than the corresponding untreated controls (Fig. 2d and S9h). We conclude that *PINE1* prevents the stem to respond to GA before the onset of the floral transition. Reduction of *PINE1* levels by *Hd3a* and *RFT1* allows elongation and coordinates stem growth with conversion of the meristem into a panicle (Fig. S10).

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AUTHOR CONTRIBUTIONS

J.G.A., V.B., G.V., M.L., M.C., R.S., F.G. performed experiments, generated and analysed transgenic plants and performed hormone treatments; R.C. analysed RNA-Seq data; E. Carrera and I.L.D. quantified bioactive GAs; E. Caporali performed SEM microscopy; F.F. and J.G.A conceived the project and wrote the manuscript.

MATERIALS AND METHODS

Plant material and growth conditions

Two *temperate japonica* cultivars of rice (*Oryza sativa*) were used in this study, Nipponbare and Taichung 65. Nipponbare bears functional alleles of *HEADING DATE 1* (*Hd1*) and *EARLY HEADING DATE 1* (*Ehd1*), whereas Taichung 65 bears non-functional alleles (*hd1*, *ehd1*)[13]. Plants were grown under controlled LD (16h light/8h dark) or SD (10h light/14h dark) conditions with day/night temperatures set at 28°C/24°C. Diurnal LD time courses were performed at 14.5h light/9.5h dark. Leaves, flowers and culms (including nodes and internodes) were sampled using scissors and surgical knives. Shoot apices and shoot apical meristems (SAMs) where sampled using a stereomicroscope using scalpels as described by[5]. In this work, the term shoot apex indicates samples as represented in Fig. S1f, whereas shoot apical meristem indicates the meristematic dome only. Plant samples for downstream nucleic acids preparations were rapidly frozen in liquid nitrogen and stored at -80°C. Heading date measurements have been performed using 8-12 plants/genotype.

RNA isolation and analysis of gene expression

RNA from SAMs was extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel), whereas RNA from shoot apices, leaves, flowers and culms was extracted using the TRIzol reagent (Invitrogen). The cDNA was synthesized using a Superscript II kit (Invitrogen) following manufacturer's instructions. Quantification of gene expression by real time PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) in twin.tec real-time PCR plates (Eppendorf), under the conditions described in [23]. All quantifications were calculated based on technical triplicates. Biological samples collected independently gave similar results. At least one biological replicate was collected for each experiment. *In situ* hybridizations were performed according to the protocol described in [24]. Primers are listed in Table S1.

RNA-sequencing and bioinformatics

Three biological replicates composed of at least 8 shoot apices of NB and T65 were collected at 0, 4, 8 and 12 days after shift from LD to SD. Total RNA was extracted using the Pure Link® RNA mini kit (Ambion). Sequencing was performed at GATC Biotech (Germany) using an Illumina HiSeq 2000. RNA-seq yielded 12 to 29 millions of cleaned 51 and 101 bp single reads (Dataset S1). The quality of raw data was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). About 94% of the cleaned reads were mapped on the Nipponbare genome version 7.0 (http://rice.plantbiology.msu.edu/) using bowtie2 and TopHat2[25,26], counted by HTSeq[27] and then used for gene-level differential expression analysis using R (version 3.1.0) and edgeR package (version 3.6.4) for statistical computing[28,29]. Only features with more than 1 read per million in at least 3 samples were retained, for a total of 20626 genes expressed across all conditions. Differentially expressed genes were called using a 5% false discovery rate (FDR) control and filtered for log2 fold change (FC) values $\leq$-1.5 or $\geq$1.5 in at least one time point of the time course compared to non-induced apices. Gene Ontology enrichment was calculated using PANTHER[30] from the GOC website (http://geneontology.org). Data have been submitted to GEO with series record number GSE90493.

Vector construction and plant transformation

The *PINE1* coding sequence (CDS) was amplified from cDNA using primers reported in Table S1, purified and recombined into a pDONR201 vector to create a *PINE1* entry clone using BP recombinase (Gateway®). The entry clone was recombined with an LR reaction into *pGWB6* to create a GFP-PINE1

N-terminal fusion protein, expressed under the control of the viral 35S promoter in transient assays in tobacco. LR recombination was used to introduce the same CDS into *pOSH1:Gateway* and *pACTIN:Gateway* destination vectors that have been previously described [14]. Mutations in *PINE1* were introduced using CRISPR/Cas9 mutagenesis. The *PINE1* protospacer (5'-GCGTCTCCTTCCATTCCTCC-3') was cloned into the *pOs-sgRNA* vector using primers PINE1-CRISPR-F and PINE1-CRISPR-F, and then recombined into the *pH-Ubi-Cas9-7* vector using an LR reaction[15]. The *PINE1* promoter was amplified using the pPINE1-F and pPINE1-R primers, bearing HindI and BamHI restriction sites, respectively, and cloned into pGEM T-Easy vector (Promega). The restriction sites were used to introduce the promoter in *pGWB6* substituting the 35S promoter.

Nipponbare seeds were used to induce callus formation and the EHA105 *Agrobacterium tumefaciens* strain was used to infiltrate calli. Transgenic calli were isolated from non-transgenic calli by growing them on selection media supplemented with hygromycin in two rounds of selection (50mg/L and 100mg/L of hygromycin, respectively) as described in [31]. Plants regenerated from transgenic calli were grown in rooting medium for two weeks before transferring on soil in the greenhouse.

DNA extraction and genotyping

DNA extraction and PCR analyses were performed as described in [23]. Primers are listed in Table S1.

Scanning Electron Microscopy

Nipponbare and *pACT2:PINE1* overexpressing lines were grown in the greenhouse under LD conditions and apical meristems were sampled after 16, 18, 20 and 22 days after shifting to SD. At least three replicates were collected for each sampling. Samples were fixed for 24h at 4°C in FAE solution (10% formaldehyde; 5% acetic acid; 50% ethanol). Fixed tissues were washed with water and post-fixed with aqueous 2% osmium tetroxide for 2h at room temperature. Tissues were rinsed several times in deionized water and dehydrated in a graded series of ethanol for 15 min per rinse. This step was followed by critical point drying with liquid CO_2 and sputter-coating with gold in a Nanotech sputter coater. Specimens were analyzed using a LEO 1430 Scanning Electron Microscope.

GA quantification and treatments

Rice seeds of wt Nipponbare and *pine1* mutant lines were sown on soil in a growth chamber, under LD conditions. Seedlings were treated with PAC after 5 days from germination. Five mL per plant of 10μM PAC were supplied directly to the soil twice/week for 6 weeks. Mock-treated plants have been used as controls. For the quantification of *pine1* internode sensitivity, Nipponbare and *pine1-1* mutant seeds were surface sterilized with bleach, rinsed and germinated on ½ MS medium in Eco2box (Duchefa). After 9 days from plating on MS, boxes containing at least 10 plants each were treated with a 10mL solution containing PAC 10μM or PAC 10μM + GA_3 10μM, 50μM or 100μM. Pictures were taken after 14 days from the beginning of treatments and internodes measured using the ImageJ software. Quantification of GA_1 and GA_4 was performed on 20-day-old stems collected at the base of the culm, according to the protocol described in[32]. GA quantifications were performed in triplicates of 8 plants each.

Imaging

Confocal images were obtained using a Nikon A1 Confocal Laser Microscope with Nikon A1 Plus Camera. Three independent *pPINE1:GFP* transgenic lines were imaged, giving similar patterns of expression. Pictures of plants and seedlings were taken using a Nikon D3200.

Materials are available upon request.

DATA AVAILABILITY

RNA-Seq data that support the findings of this study have been deposited in GEO with series record number GSE90493.

FIGURE LEGENDS

Fig. 1. Identification of *PINE1* and regulation of its expression by photoperiodic and florigenic signals. (a) Days to heading of Nipponbare plants after double shifts from LD to SD to LD. The number of SD of transient induction is indicated. LD+LD and LD+SD indicate plants maintained under LD or SD after the shift, respectively. At least 8 plants/treatment were scored. (b) Gene expression profiles of *Hd3a* and *RFT1* after the shift from LD to SD (continuous line) or after return to LD (dashed line). (c) Number of upregulated (red) and downregulated genes (blue) at 4, 8 and 12 days of SD induction compared to LD controls. Differentially expressed genes were filtered for FDR<0.05, and logFC≥1.5 or ≤-1.5 in at least one time point of the time course compared to non-induced apices. (d-e) Reduction of

PINE1 expression during commitment of apices in Nipponbare and T65 (d), and in additional *temperate japonica* varieties (e). (f) Quantification of *PINE1* expression in apices of *Hd3a* (*proGOS2:GVG 4xUAS:Hd3a*) and *RFT1* (*proGOS2:GVG 4xUAS:RFT1*) inducible plants, grown under LD and treated with dexamethasone (DEX) or mock-treated (NO DEX). Leaves of independent transgenic *proGOS2:GVG 4xUAS:Hd3a* lines that had no induction of *Hd3a* upon DEX treatments were used as controls. Samples were collected 16h after the treatment. (g) Quantification of *PINE1* expression from different tissues and photoperiods. Pictures on top of the bars represent the sampled zones. Scale bar, 1mm. All quantitative PCR data were normalized using Ubiquitin and are represented as mean±standard deviation of 3 biologically independent samples. For graphs in d, f and g, data are statistically different relative to 0 (d) or to non-induced controls (f, g) at $p<0.01$ (**) based on two-sided Student's t test. (h-l) Sections of *pPINE1:GFP* transgenic lines grown under LD. (h) Longitudinal section across the SAM. (i) Magnification of a SAM of an independent line. (j) Expression of GFP was reduced in a SAM exposed to SD. Transverse sections in (k) and (l) were taken at position 1 and 2 of (h), respectively. n indicates the nodes. Experiments shown in h-l were repeated three times. Three independent *pPINE1:GFP* lines gave similar results.

Fig. 2. Mutation of *PINE1* increases sensitivity of the stem to gibberellins and induces stem elongation. (a) Structure of the *PINE1* gene. The EAR motifs and zinc finger domain are highlighted. Asterisk indicates the stop codon. The nucleotide sequence of the wild type aligned to five mutant alleles is indicated. Type 1 alleles contain frame shift mutations, whereas type 2 alleles contain in frame deletions not producing premature stop codons. (b) Internodes length of different genotypes measured in 8.5-week-old plants grown under LD. Each bar represents a single plant, each internode is indicated using a color on the bar. Inset shows type 1 and 2 mutants. *pine1-1a* mutants treated with PAC are shown on the right. Arrowheads indicate the position of the meristem. Scale bar, 5 mm. (c) Basal culms of NB and *pine1-1c* treated with PAC and increasing concentrations of GA. Note the elongation of *pine1-1c* internodes (arrows). (d) Total stem length of NB and independent *pine1-1* mutants treated with PAC and increasing concentrations of GA_3. Concentrations are expressed as µM. Plants were measured 14 days after treatment and the number of individuals is indicated on top of each bar. Data are mean±standard deviation. Means of *pine1-1a* and *pine1-1c* are statistically different at $p<0.05$ by one-way ANOVA, WT data are not statistically different.

Appendices 3:
The importance of being on time: regulatory networks controlling photoperiodic flowering in cereals

PhD contribution:

In this review we summarized what is known about flowering time regulation in leaves in response to the photoperiod in different species. In particular, I wrote the part that describes sorghum (*Sorghum bicolor*) and maize (*Zea mays*) response to photoperiod and what is known about florigens activation in leaves of these plants.

The importance of being on time: regulatory networks controlling photoperiodic flowering in cereals

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Flowering is the result of the coordination between genetic information and environmental cues. Gene regulatory networks have evolved in plants in order to measure diurnal and seasonal variation of day length (or photoperiod), thus aligning the reproductive phase with the most favorable season of the year. The capacity of plants to discriminate distinct photoperiods classifies them into long and short day species, depending on the conditions that induce flowering. Plants of tropical origin and adapted to short day lengths include rice, maize and sorghum, whereas wheat and barley were originally domesticated in the Fertile Crescent and are considered long day species. In these and other crops, day length measurement mechanisms have been artificially modified during domestication and breeding to adapt plants to novel areas, to the extent that a wide diversity of responses exists within any given species. Notwithstanding the ample natural and artificial variation of day length responses, some of the basic molecular elements governing photoperiodic flowering are widely conserved. However, as our understanding of the underlying mechanisms improves, it becomes evident that specific regulators exist in many lineages that are not shared by others, while apparently conserved components can be recruited to novel functions during evolution.

Introduction

Several plant species measure day length to start specific developmental switches, e.g. transition to reproductive growth, during the most appropriate times of the year. Seasonal variations of day length provide a fundamental parameter to synchronize developmental changes, because they are not subject to fluctuations like other environmental cues, including temperature.

Plants can be categorized as long day (LD) or short day (SD) species, depending on the photoperiod most effective at triggering reproductive growth. When day length exceeds a specific critical threshold, flowering is promoted in LD plants, whereas SD plants flower in response to reduction of

day length below a critical threshold. Such thresholds are specific for each species and are largely determined by the region where the species originated and first adapted. Plants growing at low tropical latitudes tend to flower in response to exposure to long nights, whereas species adapted to higher latitudes promote flowering during seasons characterized by LD, indicative of the warm days of spring and summer. Plants adapted to temperate regions that germinate before winter, often need to satisfy a vernalization requirement (exposure to low non-freezing temperatures for several weeks) to become competent to respond to photoperiodic induction. Additionally, many plants can promote flowering even after long exposures to non-inductive photoperiodic conditions, indicating a facultative response to day length and the existence of floral promoting stimuli that can bypass the requirement for specific photoperiods. Therefore, plant interactions with its growth environment can be complex, and gene networks have evolved that respond to changing seasonal parameters.

In crop species, responses to day length have been extensively manipulated, creating varieties that can grow, flower and set seeds at latitudes outside of the range occupied by the wild progenitor. Artificial adaptation to broad latitudinal ranges has been a key step during domestication of several species, allowing cultivation and diversification in many regions of the globe. Natural genetic variation has offered the substrate for human selection and remarkably, many domestication loci encode orthologous genes in distantly related species providing a molecular perspective to look at conservation of pathways regulating flowering.

Here, we will summarize recent advances in understanding of photoperiodic flowering regulation in crop species, focusing on cereals. Starting with the tenets established using Arabidopsis as model system, we will discuss how conserved and unique elements have been deployed to evolve flowering networks of LD and SD plants and how they control production of a florigenic systemic signal in leaves.

Arabidopsis contributed to develop the basic tenets of photoperiodic flowering

Photoperiodic flowering has been mostly studied using the dicot *Arabidopsis*, through which core genetic and molecular mechanisms at the base of the process have been characterized (Song et al., 2015). Arabidopsis might not be representative of all plant species but it provides a conceptual framework that can be implemented in other species and also used to discuss evolution of novel mechanisms typical of distantly related plants.

Flowering of Arabidopsis is promoted under LD. The circadian clock is responsible for the rhythmic expression of several factors implicated in environmental responses. Among them, the GIGANTEA

(GI) and FLAVIN BINDING KELCH REPEAT F-BOX PROTEIN 1 (FKF1) proteins are expressed at the end of the light phase and interact in a light-dependent fashion (Sawa et al., 2007). The resulting complex targets a group of CYCLING DOF FACTORs (CDFs) for proteasome-mediated degradation (Fornara et al., 2009). The *CDFs* encode transcriptional repressors that limit expression of the *CONSTANS* (*CO*) zinc finger transcription factor, a central regulator within the photoperiodic flowering pathway (Putterill et al., 1995). Besides the major GI-FKF1-CDFs module, several additional mechanisms contribute to CO expression at the transcriptional and post-transcriptional level, including regulation by transcription factors (Ito et al., 2012), alternative splicing (Gil et al., 2016), photoreceptors (Song et al., 2014; Valverde et al., 2004), as well as ambient temperature signals (Fernández et al., 2016), hormonal signals (Wang et al., 2016) and post-translational modifications (Sarid-Krebs et al., 2015). However, central to the current model for photoperiodic flowering, the most prominent feature of CO is its light-dependent stability (Song et al., 2012; Valverde et al., 2004). During the night and the morning, CO protein is unstable and quickly degraded (Jang et al., 2008; Lazaro et al., 2015; Song et al., 2014). Consequently, its expression is shaped to be highest under LD, during the light phase. At this time of the diurnal cycle, CO protein, acting in the companion cells of the phloem, can directly promote expression of *FLOWERING LOCUS T* (*FT*), component of the systemic florigenic signal (An et al., 2004; Corbesier et al., 2007; Mathieu et al., 2007).

The effects of CO protein on the levels and rhythmicity of *FT* mRNA abundance are mediated by several classes of protein interactors that include transcription factors and transcriptional co-regulators, photoreceptors, histone-like proteins and ubiquitin ligases (see Brambilla and Fornara, 2016 and references therein). Therefore, the photoperiodic flowering pathway, despite being largely interconnected with other regulatory pathways, can be simplified into a linear molecular cascade, whose major output is the FT protein.

Rewiring photoperiodic networks in rice modifies day length responses

Rice flowering is accelerated by exposure to SD. Seasonal and diurnal time measurements are mediated by a circadian clock that shares components with that of Arabidopsis, and when mutated results in altered sensitivity to the length of the day (Izawa et al., 2011; Matsubara et al., 2012). Homologs of *GI*, *FKF1*, the *CDFs*, *CO* and *FT* exist in rice and have been partly linked in a cascade that resembles the photoperiodic pathway of Arabidopsis (Shrestha et al., 2014). The OsGI and OsFKF1 proteins can interact with each other and with a CDF protein, OsDOF12, similarly to their Arabidopsis homologs (Han et al., 2015; Li et al., 2009). However, mutations in *OsFKF1* delay

flowering under any photoperiod tested, whereas *osgi* mutants are late flowering under SD, while having only mild effects under LD (Hayama et al., 2003; Izawa et al., 2011). The phenotypic effects of the two mutations are therefore different. Overexpression of *OsDOF12* does not alter the levels of *Hd1* transcripts, but increases those of *Heading Date 3a* (*Hd3a*), a homolog of *FT*, under LD. Thus, the function of *OsDOF12* is opposite to that of Arabidopsis *CDFs*, effectively promoting flowering (Li et al., 2009). It is still unclear whether the interaction between OsGI and OsFKF1 is dependent upon the photoperiod, or if it is necessary for the degradation of OsDOF12 or other DOF proteins. These data indicate that a similar arrangement of regulators exists upstream of *Hd3a*, but that their molecular function or day length-dependency is very different from Arabidopsis. That the GI-FKF1 interaction is evolutionarily ancient is indicated by data from the liverwort *Marcantia polymorpha*, where the dimer controls growth phase transition (Kubota et al., 2014). However, evolution has likely re-shaped the function of the dimer several times, readjusting it depending on the species.

Map-based cloning of *Heading Date 1* (*Hd1*) showed it encodes a homolog of *CO*, pointing to conservation of the day length sensing system between rice and Arabidopsis (Yano et al., 2000). However, early studies highlighted critical differences between CO and Hd1 functions, the most prominent being that Hd1 protein behaves as strong floral repressor under LD, to the extent that mutations in *Hd1* result in accelerated flowering, and have been extensively introgressed in varieties cultivated at high latitudes (Gao et al., 2014; Gómez-Ariza et al., 2015; Goretti et al., 2017; Hayama et al., 2003; Izawa et al., 2002). A second important QTL, *Early Heading Date 1* (*Ehd1*) was later cloned and shown to encode a B-type response regulator (Doi et al., 2004). Ehd1 integrates circadian and light inputs and is required to promote flowering under both LD and SD (Itoh et al., 2010), and to modulate it also in response to abiotic stress, including water deficit (Galbiati et al., 2016; Zhang et al., 2016). It encodes a unique regulator not shared with dicot species that under SD induces flowering mainly by promoting *Hd3a* expression (Zhao et al., 2015). Under LD, expression of *Ehd1* is limited by several repressors that delay flowering (Gao et al., 2014; Gómez-Ariza et al., 2015). Among them, *Grain Number Plant Height and Heading Date 7* (*Ghd7*) and *Hd1* encode negative transcriptional regulators, whose protein products interact forming a repressor dimer (Nemoto et al., 2016). At least the Ghd7 protein can directly bind the promoter of Ehd1. Thus, recent genetic and molecular evidences indicate how a conserved inductive cascade has been repurposed and integrated with unique components to create a novel network topology (Fig.1).

As with all photoperiodic response networks, the major outputs of the regulatory cascade include florigenic proteins, including *Hd3a* and its paralog *RICE FLOWERING LOCUS T 1* (*RFT1*). Both proteins encode mobile leaf-borne systemic signals, but whereas Hd3a is required only under SD to

induce flowering, the RFT1 protein can promote flowering under both SD and LD (Komiya et al., 2008, 2009; Zhao et al., 2015). Thus, the facultative response of rice is based on a system comprising two florigens subject to differential regulation.

Mechanisms of photoperiodic flowering in other short day monocots including sorghum and maize

Sorghum (*Sorghum bicolor*) is a short day plant evolved in Africa, in the Sudan region. Six major QTLs controlling flowering time and termed *Maturity* loci (*Ma1-Ma6*) have been detected in sorghum. Almost all QTLs have been identified as photoperiodic flowering regulators and their study is demonstrating the strong homology occurring between the sorghum and rice pathways (Wolabu and Tadege, 2016).

Cloning of the *Ma3* locus showed that it encodes *SbPhyB*, a light receptor which can mediate light signalling and flowering repression (Childs et al., 1997). When *SbPhyB* is mutated, sorghum becomes insensitive to the photoperiod and flowers early compared to the wild type both under LD and SD (Yang et al., 2014a). One of the functions of *SbPhyB* is to promote the transcription of *SbPRR37* (*Ma1*) and *SbGhd7* (*Ma6*). These genes encode flowering repressors that limit mRNA expression of downstream targets under LD, including *Ehd1*, *SbFT* and *SbZCN8* (collinear orthologs of *Hd3a* and maize *ZCN8*, respectively) (Murphy et al., 2011). The flowering suppressor role of these sorghum genes reflects the function of rice *OsPRR37* and *Ghd7*, indicating that these components are shared among SD plants. Recent data suggested that the *Ma1* QTL does not correspond to *PRR37*, but rather to an *FT*-like gene, *SbFT12*, that could act as floral suppressor (Cuevas et al., 2016; Wolabu and Tadege, 2016). Additional data will be required to confirm the true identity of the *Ma1* gene.

The regulation of *SbCO* transcription mediated by *SbPRR37* has also been investigated. The data suggest that *SbPRR37* modulates *SbCO* expression at dawn, promoting its transcription under LD, whereas under SD *SbCO* gene expression seems not to depend upon *SbPRR37* (Murphy et al., 2011). *SbCO* can activate florigen production under both SD and LD conditions through the activation of *SbEhd1*, *SbCN8* and *SbCN12* (Yang et al., 2014b). The role of sorghum *SbCO* as constitutive floral activator is therefore different from that of rice *Hd1*, implicating a different regulatory mechanism.

Thirteen different *FT*-like genes have been identified in the sorghum genome, three of which (*SbFT1*/*SbCN15*, *SbFT8*/*SbCN12* and *SbFT10*/*SbCN8*) could promote flowering when constitutively expressed in Arabidopsis (Wolabu et al., 2016; Yang et al., 2014b). The transcripts of *SbCN8*, *SbCN12* and *SbCN15* peak at dawn but show distinct sensitivities to *SbCO* mutations. Whereas the transcripts of *SbCN8* and *SbCN12* are strongly reduced in the *Sbco* mutant background under LD,

SbCN15 shows only a phase shift, suggesting different regulation by *SbCO* (Yang et al., 2014b). The transcriptional patterns of *SbCN8*, *SbCN12* and *SbCN15* under different photoperiods and mutant backgrounds could provide in the future valuable data to understand similarities and differences with the dual florigen system of rice.

Maize (*Zea mays*) was domesticated in central Mexico from Teosinte, which is a short day plant. The first flowering gene cloned in maize was *INDETERMINATE 1* (*ID1*): plants with mutations in this gene delay the floral transition and produce aberrant inflorescences (Colasanti et al., 1998). *ID1* encodes a zinc-finger transcription factor expressed in immature leaves which can activate the floral transition and is not under the control of the circadian clock (Wong and Colasanti, 2007). Although the precise function of *ID1* in the photoperiodic pathway is still unclear, recent analyses demonstrated that *ID1* controls chromatin modifications of loci encoding maize florigens, and that it can regulate flowering through histone methylations (Mascheretti et al., 2015). A rice homolog of *ID1*, *OsEhd2*, is required for the regulation of *OsEhd1* (Matsubara et al., 2008). Although a maize *Ehd1* homolog has not been found yet, the high homology between *ID1* and *OsEhd2* could suggest an analogous regulatory mechanism, possibly indicating the existence of an Ehd1-like protein. Another indirect evidence supporting this view is that the CCT-domain transcription factor *ZmCCT* shows sequence homology with *OsGhd7*, and encodes a strong flowering repressor under LD (Fig.1). Mutations in *ZmCCT* cause early flowering and have been artificially selected to expand maize cultivation to higher latitudes (Hung et al., 2012).

Two *GI* homologs are present in maize, *GIGANTEA1* (*GI1*) and *GIGANTEA2* (*GI2*) (Miller et al., 2008). In Arabidopsis and rice, *GI* is under circadian clock control and regulates the expression of several genes important for the floral induction. In maize, *gi1* mutations cause early flowering under LD conditions. Transcriptional analysis of these mutants demonstrated that *GI1* is necessary to repress transcription of *CONZ1* (homolog of *OsHd1*) and *ZCN8* (homolog of *Hd3a*), both of which displayed increased expression in the *gi1* background (Bendix et al., 2013). These data demonstrate that *ZmGI* function is similar to *OsGI* which can repress flowering under LD conditions, a function opposite to that of *AtGI* (Hayama et al., 2003). Whether mutations in *CONZ1* influence flowering is unknown, but the data suggest it to be downstream of *GI1*, and possibly upstream of *ZCN8* as positive regulator of flowering (Miller et al., 2008).

From the analysis of 15 maize *FT*-like genes, *ZCN8* was identified as the strongest candidate for the maize florigen (Meng et al., 2011). *ZCN8* encodes a homolog of *FT* that delays flowering if silenced, and can complement *ft* mutants when expressed in *Arabidopsis* (Lazakis et al., 2011). The regulation

of *ZCN8* is similar to that of another putative maize florigen, *ZCN7*, and is under the control of chromatin modifications governed by *ID1* (Mascheretti et al., 2015). However, whether ZCN7 satisfies the criteria of a florigenic protein is still to be clarified.

Flowering mechanisms in long day temperate cereals

Differently from rice, sorghum and maize, the temperate cereals wheat (*Triticum spp.*) and barley (*Hordeum vulgare*) were domesticated in the Eastern Mediterranean region, in areas characterized by the alternation of cold and warm seasons. These cereals have evolved mechanisms to prevent flowering when temperatures are low, to protect the meristem from cold damage. Flowering is promoted after exposure to vernalizing conditions, when plants resume growth in the spring. During domestication, some cultivars of these species have lost sensitivity to vernalization and, depending on the response to cold, they could be classified as winter or spring types. Winter-types have an obligate requirement for exposure to cold to induce flowering. Such response is controlled by the *VERNALIZATION* (*VRN*) loci (Ream et al., 2012). *VRN1* is a MADS-box floral promoter homologous to *FRUITFULL* (*FUL*) and *APETALA1* (*AP1*) of Arabidopsis, whereas *VRN2* is a floral repressor sharing sequence similarity to *Ghd7* of rice. Under low temperatures, the expression of *VRN1* is induced and the protein directly binds to the promoter of *VRN2* to reduce its expression during vernalization (Deng et al., 2015; Trevaskis, 2006). Dominant mutations in *VRN1* or recessive mutations in *VRN2* confer a spring growth habit, and have been exploited by breeders to expand cultivation areas (Fu et al., 2005; Loukoianov, 2005; Yan et al., 2004).

Downregulation of *VRN2* is required to induce *VRN3* expression during the floral transition. VRN3 proteins (designated as TaFT and HvFT in wheat and barley, respectively) are homologs of the Arabidopsis and rice florigens, and move to the apical meristem to promote flowering upon exposure to warm temperatures and LD (Li and Dubcovsky, 2008; Yan et al., 2006). Thus, cold signals coordinate *VRN* expression to activate flowering and long-distance florigenic signaling only when a vernalization requirement has been satisfied.

As soon as *VRN2* levels decrease, exposure to long days is required to promote flowering. Temperate cereals flower earlier under long days, whereas exposure to short days delays flowering. The *PHOTOPERIOD 1* (*Ppd1*) gene has been described as the major factor controlling sensitivity to day length in wheat and barley (Beales et al., 2007; Turner et al., 2005). Mutations in PPD1 delay flowering under LD and reduce *VRN3*/*FT* expression. PPD1 proteins are homologous to PRR37 proteins of rice and sorghum, both of which repress flowering under LD. The functional divergence

of PRR37 proteins observed among LD temperate and SD tropical cereals deserves further attention, as it might be at the base of their distinct photoperiodic requirements.

Homologues of *CO* and *Hd1* have been identified in wheat and barley (Campoli and Von Korff, 2014). The *TaHd1-1* gene could complement a rice *hd1* mutant, suggesting functional conservation of protein function in a heterologous system (Nemoto et al., 2003). In barley, studies based on overexpression have provided important clues to the position of *Hd1* homologues in flowering regulatory networks. Overexpression of *HvCO1* and *HvCO2* promoted flowering under both LD and SD, but plants retained sensitivity to the photoperiod, because of independent control of *HvFT1* by *PPD1* (Campoli et al., 2012). Thus, barley flowering depends on two parallel pathways controlling *FT* expression (Fig.1). Interestingly, overexpression of *HvCO2* was recently shown to increase expression of *VRN2* under LD and SD in a winter variety (Mulki and von Korff, 2016). Despite such increase of the *VRN2* repressor, overexpression of *HvCO2* could still promote flowering, likely through a *VRN2*-independent pathway. The data might suggest that *HvCO2* mediates a floral repressive function through *VRN2*, to limit *FT* expression. Whether barley orthologues of *Hd1* display dual functions similarly to rice *Hd1* awaits further testing. The use of mutant resources and possibly of edited alleles might help to address this issue.

Figure legend

Figure 1. Simplified genetic modules controlling production of the florigens in leaves.

Rice. *OsFKF1* and *OsGI* promote flowering via transcriptional regulation of distinct target genes. However, their protein products can also interact. The Ehd1 and Hd1 proteins promote flowering by activating *Hd3a* and *RFT1* expression under SD. However, under LD, Hd1 switches its function to repress *Hd3a* transcription. *Hd3a* transcription is sensitive to induction mediated by *Ehd1* under both LD and SD, to the extent that *ehd1 rft1* double mutants cannot flower. Conversely, transcription of *RFT1* can be activated also under LD, by an unknown mechanism that eventually allows flowering also under unfavorable conditions.

Sorghum. Some of the *Maturity* (*Ma*) loci have been cloned. The SbPhyB protein (Ma3) represses flowering by promoting expression of *SbGhd7* (*Ma6*) and *SbPRR37* (*Ma1*) under LD. However, sorghum lines bearing *ma3* recessive mutations can flower early also under SD. The SbCO protein is

a constitutive activator of flowering, differently from rice Hd1. *SbPRR37* can promote *SbCO* transcription at dawn.

Maize. A higher degree of polygenic control of flowering has been observed in maize compared to other species. However, homologues of flowering genes have been cloned and some mutants characterized. In this diagram we speculate about the existence of an *Ehd1*-like function, possibly creating a topology similar to that of other SD species. Major discrete regulators are encoded by *ID1* and *ZmCCT*.

Temperate cereals. Exposure to cold is necessary to reduce *VRN2* levels in leaves. Vernalized plants can respond to LDs that promote expression of *VRN3*/*FT* via *CO* homologues and, most importantly, through PPD1, encoding a CCT-domain protein similar to PRR37.

Arrows indicate transcriptional activation; flat-end arrows indicate transcriptional repression. Dashed lines indicate that the protein products can interact. Question marks indicate the existence of unknown factors with specific functions on gene expression. L, long day; S, short day.

Appendices 4:
Manuscript first draft

TITLE: **OsFD4 promotes the rice floral transition via FAC formation in the shoot apical meristem**

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ABSTRACT:

The floral transition is a crucial moment during a plant life cycle, in which the Shoot Apical Meristem (SAM) stops to produce leaves and start making flowers. In rice, the florigens Heading Date 3a (Hd3a) and RICE FLOWERING LOCUS T 1 (RFT1), OsFD-like bZIP transcription factors, such as OsFD1 and OsHBF1, and Gf14 proteins, assemble into different Florigen Activation (or repressor) Complexes (FACs) which regulate this process in both leaves and SAM. Regulation of flowering time through the FACs has been characterized in different tissues of the plant, including leaves and SAM, yet little is known about the combinatorial complexity of FAC formation and the role of other bZIPs at the SAM, other than OsFD1 . Here, we characterize *OsFD4*, a bZIP which forms FACs only in the SAM, and promotes the floral transition in this tissue. *osfd4* mutants delay flowering under both inductive and non-inductive conditions, downregulating the level of *OsMADS14*, *OsMADS15* and *OsMADS34*, three activators of flower development. Protein-protein interaction assays indicate an extensive network of interactions between several bZIPs and Gf14 proteins, suggesting plasticity in FACs formation. Finally, we used DAP-seq to find genomic regions bound by *OsFD4*, *OsFD1* and *OsHBF1*. We show that they bind the same consensus motif but their DNA binding syntax is different, suggesting it could be key to discriminate between OsFD1-, OsFD4- and OsHBF1-FACs functions.

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 occurs in both leaves and shoot apical meristem (SAM) when some molecules, the florigens, are produced. In rice (*Oryza sativa*) this process occurs preferentially when the day length falls under a critical threshold. Yet, rice can flower also under non-inductive long-day conditions, after a more extensive vegetative phase.

The floral transition is initiated in rice leaves where two florigens, *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*), are produced. *Hd3a* and *RFT1* are homologs of Arabidopsis *FLOWERING LOCUS T* (*FT*) and members of the phosphatidylethanolamine-binding protein (PEBP) family[1,2,3]. Similarly to FT, they are produced in the vascular tissue of leaves and then their proteins can move through the phloem and arrive in the SAM where they promote expression of genes necessary to activate the inflorescence development program[4,2]. RNA interference lines showed that when expression of *Hd3a* or *RFT1* is reduced, plants flower later than the wild type[3]. However, the phenotypic effect depends upon the photoperiod. Both florigens can redundantly promote the floral transition under inductive short-day conditions[3], whereas only RFT1 allows flowering under non-inductive long-day conditions[2]. This difference is related to their transcription in leaves because when plants are grown under short-day conditions, the expression of both florigens is activated, whereas growth under non-inductive long-day conditions promotes *RFT1* expression only [2,3]. This differential sensitivity to day length depends on distinct promoter set ups[2,3].

Expression of the florigens in leaves depends on several regulators. *Early Heading date 1* (*Ehd1*) is a major activator of *Hd3a* and *RFT1* which acts directly on the promoter of these genes in a photoperiod dependent manner[5,3]. *Ehd1* is expressed preferentially under short-day conditions and encodes for a B-type response regulator with no homolog in *Arabidopsis thaliana*[5]. According to its role as flowering activator, *ehd1* mutants show a late flowering phenotype compared to the wild type, whereas its overexpression causes an early flowering phenotype under short-day conditions[5,6]. Florigen Activation Complexes (FACs) are higher-order complexes containing florigen molecules and FD-like bZIP transcription factors which could interact with each other either directly or indirectly via bridging protein called Gf14s, members of the 14-3-3 protein family[7]. In leaves, FACs can regulate expression of the florigens, generating positive and negative feedback loops on *Ehd1* production[8]. More specifically, FACs formed by the florigens and *Oryza sativa Hd3a Binding Factor 1* (*OsHBF1*) have a repressive role on the floral transition. OsHBF1 can bind the promoter of *Ehd1* to downregulate its expression in leaves, thus generating a negative feedback loop on *Hd3a* and *RFT1* production[8]. OsHBF1 forms FACs by interacting directly with Hd3a or indirectly, via Gf14c, with

RFT1[8]. Rice loss-of-function mutants of *OsHBF1* generated by CRISPR/Cas9 mutagenesis are earlier flowering compared to the wild type. A second bZIP transcription factor, *OsFD1*, was isolated as interactor of Hd3a and part of a flowering-promoting FAC[7]. *OsFD1* is expressed in both leaves and SAM and interacts with Hd3a and RFT1 only indirectly via Gf14c. In leaves, OsFD1 promotes the expression of *Ehd1* generating a positive feedback loop on florigen production, whereas in the SAM, OsFD1-containing FACs activate the transcription of *OsMADS14* and *OsMADS15*, two key activators of floral development in rice[7,9]. Thus, the florigens not only connect leaves and SAM to convey seasonal information to the apex, but their movement throughout the plant allows formation of distinct florigen-containing complexes in distinct tissues and with varying functions. In the SAM, OsFD1 activity can switch from activating to repressing flowering by forming FLORIGEN REPRESSOR COMPLEXES (FRCs). In FRCs, OsFD1 interacts with four different CENTRORADIALIS-like proteins, *RICE CENTRORADIALIS 1* (*RCN1*), *RCN2*, *RCN3* and *RCN4*[10]. *RCNs* are classified as PEBP and are members of the TFL1-like subclade, expressed in rice stem vascular tissue[10]. Similarly to Hd3a and RFT1, RCNs can move through the phloem and arrive in the SAM where they bind OsFD1 via Gf14c and in this way they regulate production of secondary branches of the panicle via FRCs formation[10]. Despite its crucial role in flowering progression, *osfd1* RNA interference plants show only a very mild flowering delay compared to the wild type[7].

FD-like bZIP transcription factors contain in the C-terminal portion of the protein the basic region, the leucine zipper domain and the SAP domain[11,12]. The basic region contains amino acids necessary for the binding to the DNA, instead the leucine zipper domain allows dimerization between bZIPs. In fact, these transcription factors can bind a single consensus motif only in form of homo- or hetero-dimers, otherwise no interaction with the DNA could be found[11]. The last conserved domain is the SAP domain which plays a crucial role in the interaction between FD-like bZIPs and Gf14 proteins. 14-3-3 proteins bind proteins which contain two types of conserved motives R/K-S-X-P or R/K-X-X-S-X-P and in particular the interaction could happens thanks to the phosphorylation of the Serine present in the terminal part of these motives[7]. In the C-terminal of OsFD1, a SAP motif is present and the Serine in position 192 has a predominant role in the interaction between OsFD1 and Gf14c and also in the regulation of flowering time[7]. In fact, when OsFD1 SAP domain is delated no more interaction is detectable between OsFD1 and Gf14c, whereas the phosphomimic mutant S192E causes an early flowering phenotype when expressed in rice plants[7].

Here we characterized *OsFD4* a FD-like bZIP which activates the floral transition specifically in the SAM. OsFD4 forms FACs via direct or indirect interaction with florigens and can form both

homodimers or heterodimers with OsFD3. We present DAP-seq data of OsFD1 and OsFD4 in which we observed differences in their target genes and in their syntax.

RESULTS

OsFD4 promotes flowering in rice

In leaves OsHBF1-FAC and OsFD1-FAC regulate florigens production via *Ehd1* regulation[8], whereas in SAM only the OsFD1-FAC is known to promote floral transition via *OsMADS14* and *OsMADS15* regulation. Despite of that *OsFD1* RNA interference plants showed only mid flowering phenotype respect to the wild type[7], suggesting that other bZIPs transcription factors could share redundancy with *OsFD1*. For this reason, we looked for bZIP transcription factors which regulate floral transition in rice, and we found *OsFD4* (*LOC_Os08g43600*). The mutant line *osfd4-1* was selected from the CIRAD collation of T-DNA insertion mutants in the Dongjin variety and showed an insertion in the 3'-UTR of the gene which caused the lack of OsFD4 expression (Supplemental Figures 1A and 1B). This mutant shows late flowering phenotype respect to the wild type under both short-day conditions and long-day conditions (Figure 1A). We generated *osfd4*, *osfd3* and *osfd1* mutants using CRISPR-Cas9 to confirm *osfd4-1* phenotype and to compare the effect of *OsFD4* with the one of *OsFD3* and *OsFD1* on flowering time (Supplemental Figure 1C). We grew plants for two months under not-inductive long-day conditions and then we shifted them to inductive short-day conditions. As shown in Figure 1B *osfd4-3* delayed flowering respect to the wild type, confirming *osfd4-1* phenotype (Figure 1B). Contrary to the *osfd1* RNAi phenotype[7], also *osfd1-1* flowered later than the wild type and showed a stronger delay respect *osfd4-3* too, whereas no effect on flowering time could be found in *osfd3-1* mutants (Figures 1B and 1C). To analyse the redundancy between *OsFD4* and *OsFD1*, we crossed *osfd4-1* and *osfd1-1* mutants generating the double *osfd4-1/osfd1-2* mutant. Flowering time experiments under inductive condition demonstrated that no additive effect could detected between *osfd1-2* and the double *osfd4-1/osfd1-2* mutant (Figure 1D), suggesting that *OsFD1* works upstream to *OsFD4*. *[The experiment was conducted with few plants, so we are performing another time the analysis to have a stronger statistic.]*

OsFD4 is expressed exclusively in the SAM – In situ hybridization in progress

Because OsFD1 has a double role in both leaves and SAM during floral transition, we analysed using qRT-PCR the expression of OsFD4 in both tissues. As shown in figure 2A, OsFD4 was not detectable

in leaves whereas a weak expression could be found in the SAM, contrary, as expect, OsFD1 is expressed in both tissues. To better understand the localization of OsFD4 and OsFD1 expression in SAM, we are performing in situ hybridization assay.

OsFD4 interacts directly with RFT1

To verify if OsFD4 could form FACs, we performed protein-protein interaction screening using Yeast Two Hybrid Assay (Y2H). Because the interaction between bZIP transcription factors and florigens could be indirect via 14-3-3 proteins, first we tested the interaction between OsFD4 and the six Gf14s expressed in the SAM, from Gf14a to Gf14f[13]. In yeast all these 14-3-3 proteins interacted with OsFD4, but also with OsFD1 and OsFD3 which was tested as well (Figures 3A and Supplemental Figure 2A). To confirm with another methods this interaction, we used Bimolecular Fluorescence Complementation Assay (BiFC) between OsFD4 and Gf14b which gave the same results of Y2H (Supplemental Figure 2D). Moreover, we verified also if SAM Gf14s can interact also with RFT1 and Hd3a, confirming their role such as molecular bridge between OsbZIPs and florigens[7]. Both florigens interacted with all the Gf14s tested, suggesting that in the SAM OsFD4 can form FACs indirectly via 14-3-3 proteins (Figure 3B).

Because OsHBF1 and OsHBF2 can direct interact with Hd3a without the help of Gf14s[8], we tested direct interactions between OsFD4 and Hd3a or RFT1. Using Y2H, we found that OsFD4 can directly interact with RFT1, but no interaction was detected between OsFD4 and Hd3a, whereas yeast growth was spotted when OsFD3 and both florigens were coexpressed in cells (Supplemental Figures 2E and 2F). These data were recently confirmed independently in another paper[14]. We performed BiFC in a heterologous system to understand localization of OsFD4-RFT1 interaction in the cell and we detected that the fluorescent signal was restored nuclei of *Nicotiana benthamiana* leaves (Figure 3C).

OsFD4 can homodimerize or heterodimerize with OsFD3

Because bZIP transcription factors have to homo- or hetero- dimerize to bind the DNA[11], for this reason we tested the possibility that OsFD4 and OsFD1 could interact each other. Using Y2H, we observed that OsFD4 homodimerized whereas no heterodimerization was found with OsFD1 (Figure 4A). Interestingly OsFD1 did not formed homodimers suggesting that it could act with other bZIPs than itself or OsFD4 (Figure 4A). We chose to verify if OsFD1 could interact with OsFD3 and surprisingly we found that no yeast growth was detectable when we tested OsFD3-OsFD1 interaction

contrary OsFD3-OsFD4 interaction gave a positive result (Figure 4B). To confirm bZIPs interaction and to localize them inside the cell, we performed BiFC experiments. As shown in Figure 4C, OsFD4 and OsFD3 homodimerized and heterodimerized each other, whereas OsFD1 could not interact with itself nor with OsFD4 or OsFD3.

OsFD4 regulates expression of AP-1-like genes

In rice there are four different AP1-like genes, *OsMADS14*, *OsMADS15*, *OsMADS18* and *OsMADS20* which act to promote flowers development[15]. *OsFD1* regulates in the SAM the expression of *OsMADS14* and *OsMADS15*, which are target of the OsFD1-FAC[7]. Using RT-PCR, we decided to analyse the AP1-like genes expression in *osfd4-1* to verify if OsFD4-FAC regulated OsMADS genes during floral transition too. Normally, in the SAM *OsMADS14*, *OsMADS15* and *OsMADS18* increase their level during floral transition, whereas *OsMADS20* is downregulated during this process. For this reason, we performed time course experiments shifting plants from not-inductive long-day conditions to inductive short-day condition and sampling SAMs 0, 6, 12 and 18 Days After Shifting. These time points were chosen based on the progression of floral transition in rice which becomes irreversible after 12 days under inductive conditions which correspond to the maximum expression of florigens in leaves[16]. Comparing *OsMADS* expression of wild type Dongjin and *osfd4-1* plants, we observed that *OsMADS14* and *OsMADS15* level decrease in the mutant, whereas *OsMADS18* and *OsMADS20* were not significantly changed respect to the wild type (Figures 5A, 5B, 5C and 5D). Rice AP1-like genes work together with *OsMADS34*, a SEP-like gene, to promote floral transition[17], for this reason we analysed its expression in *osfd4-1* mutant. Such as expect the level of *OsMADS34* increased in the wild type, whereas a significant downregulation occurred in *osfd4-1* (Figure 5E). To complete our analysis, we checked the expression of *OsMADS14*, *OsMADS15* and *OsMADS34* also in *osfd1-1* mutant and we observed that the expression of these genes was downregulated in the mutant respect to the wild type (Supplemental Figure 3). Taken together these data suggest that OsFD4-FAC, as well as OsFD1-FAC, act upstream *OsMADS* genes to promote rice floral transition.

Use of DAP-sequencing to find new OsFD4, OsFD1 and HBF1 targets

Because only few FAC direct target genes are known and to better understand the role of bZIP transcription factors during floral transition, we decided to perform DNA Affinity Purification sequencing (DAP-seq) on OsFD4, OsFD1 and OsHBF1. DAP-seq is a new method that allows to find in vitro DNA regions bound by transcription factors of interest[18,19]. After the sequencing we found

enrichments in the same consensus motif for OsFD1, OsFD4 and OsHBF1 which shared a core motif of "CACGT" (Figure 6A). At the flank of this motif two bases "GC-" had different probability to be found, also if in OsFD4 the entire motif "GCCACGT" was high conserved (Figure 6A). We used PAVIS to assign a locus to every peak calling genes that contained one or more peaks 5000 bps upstream the ATG or 3000 bps downstream the STOP codon. We observed that OsFD4, OsFD1 and OsHBF1 had an enrichment in 925, 1717 and 15937 genes respectively. Most of the binding sites were in the upstream region of the gene (OsHBF1 35.9%, OsFD1 47.4%, OsFD4 42.8%, Figure 6B), with a major concentration in the 500 bps before the Transcription Start Site (TSS; Figure 6C). To verify the level of redundant targets among OsFD1, OsFD4 and OsHBF1, we crossed target genes using Venn Diagrams. This analysis shown that 698 genes were common regulated by all the tested bZIPs, 220 genes were shared between OsFD4 and OsHBF1, 991 between OsFD1 and OsHBF1, instead no common genes were present in the intersection between OsFD1 and OsFD4 (Figure 6D). Moreover 7, 28 and 14028 genes were singularly targeted by OsFD4, OsFD1 and OsHBF1 respectively (Figure 6D).

DAP-seq method use the entire naked genome to find transcription factor targets which means that no chromatin regulation is added to data collected[18,19]. To be sure that candidate genes were expressed in the tissue of interest, we crossed OsFD1 and OsFD4 datasets with genes expressed in the SAM (data obtained by published RNA-seq[16]) and OsHBF1 and OsFD1 with genes expressed in leaves (data obtained by published RNA-seq[20]) (Figure 6D). Only a minor part of genes found using DAP-seq intersected with RNA-seq data. In SAM OsFD4 and OsFD1 bound 240 common genes, instead unique targets of OsFD4 and OsFD1 were 58 and 320 respectively (Figure 6D). In leaves OsHBF1 and OsFD1 unique targets were 3691 and 7 respectively, instead common targets expressed in this tissue were 566.

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

Thanks to DAP-seq analysis, we found target genes of *OsFD4* and *OsFD1*. We performed RT-PCR between mutants and wild type to verify if *OsFD1* and *OsFD4* targets regulation was influenced. We chose some transcription factors based on the intersection between RNA sequencing data of the SAM and DAP-seq dataset. We analyzed the expression of two genes regulated both by *OsFD1* and *OsFD4*, *LOC_Os08g38590* (*OsMADS62*) and *LOC_Os06g48950* (*OsARF19*), three genes regulated only by *OsFD1*, *LOC_Os01g14440* (*WRKY transcription factor*), *LOC_Os01g64360* (*MYB transcription factor*), and *LOC_Os04g51000* (*RICE FLORICAULA/LEAFY*), and two *OsFD4* target genes, *LOC_Os04g31730* (*B3 transcription factor*) and *LOC_Os07g41580* (*NF-YB transcription factor*)

(Figure 6E, 6F and Supplemental Figure 5). Both *OsMADS62* and *OsARF19* showed a downregulation in *osfd4-1* and *osfd1-1* respect to the wild type, confirming the role of these two bZIPs in their regulation (Figure 6E and 6F). Such as expected, *LOC_Os04g51000* and *LOC_Os07g41580* had a different expression only in *osfd1-1* or *osfd4-1* respectively (Supplemental Figures 5E and 5D), instead the other genes tested were less expressed in both mutants (Supplemental Figures 5A, 5B, 5C). These data suggest that OsFD4 and OsFD1 regulate in a redundant manner some pathways involved in floral transition, whereas their action is not completely overlapped.

OsFD4, OsFD1 and OsHBF1 have different Binding Syntax

OsFD1, OsFD4 and OsHBF1 have different roles in rice floral transition regulation, but despite of that they bind the same consensus motif. To understand if some differences could be found among these bZIPs, we decided to verify the spacing between two consensus motives bound in the DAP-seq based on the transcription factor tested. To perform this analysis we used Position Weight Matrix (PWM) method for DAP-seq data elaboration which allows to find distance between two consensus motives bound by the same transcription factor and to calculate their configuration on the DNA strands (Figure 7A) [21]. We first decided to verify if the method could be applied to rice DAP-seq analysis testing if our data well fitted in the model via ROC curve statistical analysis (Supplemental Figure 4). Our results showed that OsFD4, OsFD1 and OsHBF1 were highly predictable with ROC values of 0,985, 0,971 and 0,9 respectively (Supplemental Figure 4). We performed PWMs for each bZIP and we found that unique profiles were generated. OsFD4 had an enrichment of 4-fold change or more respect to the negative control in the Direct Repeats (DR) 0, Everted Repeats (ER) 1-5, ER18-20, ER44, Inverted Repeats (IR) 34 and IR41 configurations (Figure 7B). OsFD1 showed an enrichment of 3-fold change or more to the negative control in the DR30, ER4, ER16, ER28, ER32-34, ER41-46 and IR38 configurations (Figure 7C). OsHBF1 configuration enrichments were found in DR10, DR18, DR29, DR40, ER3, ER16-18, ER29, ER42 and IR10 with a 2-fold change or more respect to the negative control (Figure 7D). These different enrichments in the spacing between consensus motives could explain the different roles of OsFD4, OsFD1 and OsHBF1 during floral transition.

DISCUSSION

OsFD4 and *OsFD1* collaborate to induce floral transition

FD-like bZIP transcription factors have a crucial role during floral transition because they transduce the florigenic signal in genes expression regulation. In rice leaves, OsHBF1 and OsFD1 are well known to regulate flowering time via Florigen Activation Complexes (FACs) formation. OsFD1-FAC generates a positive feedback loop on the expression of the two rice florigens *Hd3a* and *RFT1* via *Ehd1* regulation, whereas OsHBF1 does the opposite acting in a negative feedback loop manner. Moreover, OsFD1 has a role also in the SAM, where activates the expression of *OsMADS14* and *OsMADS15* promoting floral transition progression. Despite of the central role of OsFD1 in rice floral transition, RNAi plants for this gene have only mild effect on heading date. Here, we present *OsFD4* which is a bZIP transcription factor that controls floral transition in the SAM under inductive and not-inductive conditions (Figure 1A). Contrary to *OsFD1* and *OsHBF1*, the expression of *OsFD4* is detectable only in the SAM suggesting that its function is limited to this tissue. Under short-day conditions *osfd1* mutants showed a stronger delay compared to *osfd4* and no additive effect respect to *osfd1* was detected in the double *osfd1 osfd4* mutant (Figure 1D). These results could be explained thanks to the double role of *OsFD1* in leaves and in SAM and suggest that *OsFD1* acts independently to OsFD4. The expression of *OsMADS14*, *OsMADS15* and *OsMADS34* in SAMs of *osfd4* mutants was significantly reduced to the wild type, suggesting that OsFD4 acts upstream to these genes during floral transition progression.

OsFD4 form FAC with both florigens

The modular structure of Florigen Activation Complexes (FACs)[7] and Florigen Repressive Complexes (FRCs)[7] allows plasticity at these complexes during rice life cycle. PEBPs, bZIP transcription factors and 14-3-3 proteins are the bricks that build these structures. The difference between FACs and FRCs is on the PEBP which forms those complexes, in fact florigens Hd3a and RFT1 are part of FACs, instead RCNs, TFL1-like proteins, assemble FRCs. For now, three different rice bZIPs were published part of FACs, OsFD1, OsHBF1 and OsFD2. The first two have function during floral transition regulation, whereas OsFD2-FAC has role in leaves development. The role of 14-3-3 proteins in FACs building depend of the transcription factor involved, in fact OsHBF1 interact with Hd3a without the help of Gf14c. We tested the possibility that OsFD4 could form FACs with Hd3a and RFT1 and we found that OsFD4 interact directly with RFT1 and via Gf14s with Hd3a. Because RFT1 is the only florigen expressed under not-inductive long day conditions and because *osfd4* showed a stronger flowering delay under this photoperiod conditions respect to the wild type, speculations could be done about a photoperiod-dependent FACs behavior. In any case more evidences must be reported to connect FACs and photoperiodic flowering regulation.

OsFD4, OsFD1 and HBF1 bind the same core motif

We performed DAP-sequencing to find new targets of OsFD4, OsFD1 and HBF1 and to understand the reason why of their different behavior during floral transition. We found that these bZIPs bind the same "CACGT" core motif which differences in the left flank which can add "G-T/C-A" bases. The most conserved motif is the one of OsFD4 which bind the "GCCACGT" motif in a high conserved manner. Recently it was published that in ChIP-sequencing the Arabidopsis thaliana FD can bind "CACGT" core motif too, suggesting that this consensus motif is conserved between rice and Arabidopsis. Interestingly Collani et colleagues performed FD Chip analysis in both leaves and SAM, what they found is that in the SAM the core motif is longer than in leaves ("GACACGT"), this second motif is more like the OsFD4 one. Because OsFD4 is a SAM specific bZIP transcription factor, these data could suggest that there are minor differences in the bind motif of bZIPs in leaves and in SAM.

***OsMADS62* and *OsARF19* are regulated by both OsFD4 and OsFD1 in SAM**

We analyzed the expression of some OsFD4 and OsFD1 targets in wild type and mutants SAM respectively chosen crossing DAP-seq datasets with SAM differential expressed genes during floral transition. Interestingly, in our datasets we cannot find binding of OsFD1 and OsFD4 to *OsMADS14*, *OsMADS15* or *OsMADS34* promoter, otherwise because evidences shown that these genes are direct targets of FAC[7], we can imagine that maybe other proteins stabilize OsFD4 and OsFD1 binding to these region making impossible to find them using DAP-seq assay. For these reasons, we decided to focus our attention on other *OsMADS* genes. We could find that both OsFD4 and OsFD1 can bind *OsMADS30* and *OsMADS62*. We tested the expression change of both MADS-box transcription factors, but only mild changes of *OsMADS30* expression could be found on *osfd4-1* and *osfd1-1* mutants, for this reason we focus our attention on *OsMADS62*, a MADS-box transcription factor of the MIKC is* family[22]. *OsMADS62* expression changes significantly in *osfd4-1* and *osfd1-1* mutants respect to the wild type and phylogenetic analysis find high homology between this gene and MIKC* MADS boxes that regulate pollen maturation in Arabidopsis[23] suggesting that it play a role in floral transition development and maybe in pollen maturation.

Interestingly, we found in our datasets that *OsARF19* is regulated by both OsFD4 and OsFD1. *OsARF19* is an Auxin Response Factor previously involved in leaf angle regulation in rice[24] and highly expressed in floral organs. From RNA-seq data is possible to observed that *OsARF19* is upregulated during floral transition, despite of that in *osfd4-1* and *osfd1-1* mutants *OsARF19*

expression were downregulated respect to the wild type. Auxin is a plant hormone which has a role in cell proliferation and flower development in Arabidopsis thaliana, despite of that its role during floral transition is not completely understood. We focus our attention on *OsARF19* because of its homology with *AtARF7* and *AtARF19*[25], which repress the expression of *GATA, NITRATE - INDUCIBLE, CARBON METABOLISM INVOLVE* (*GNC*) and *GNC-LIKE* (*GNL*)[26]. *GNC* and *GNL* are floral transition repressors and *AtARF7* and *AtARF19* in collaboration with *AtARF2* bind their promoter stopping *GNC* and *GNL* expression in the SAM, for this reason we can hypothesis that also *OsARF19* could have a similar role in rice.

OsFD1, OsFD4 and OsHBF1 Syntax could be the key of their different functions

Recently, DAP-seq data on auxin responsive factors MONOPTEROS (MP) and AtARF2 showed that these transcription factors share the same consensus motif, even if their function is the opposite, in fact, MP activate the transcription of its targets whereas AtARF2 is a repressor. Because ARF usually bind DNA such as dimers, Stigliani et colleagues create an algorithm which analyze the distance between two consensus motives in genes found using DAP. Thanks to this method, they observed that AtARF2 prefers bind Everted Repeats (ER) distant 7/8 bps, whereas MP has a much wider range of preferences[21]. This different behavior could explain the opposite function of AtARF2 and MP and form what they called the "Syntax" of the transcription factor. During the analysis of our DAP-seq datasets we found that despite of their different function OsFD1, OsFD4 and OsHBF1 bind the same consensus motif, meaning that the binding site alone is insufficient to explain their different role. For this reason, we perform the spacing analysis among motives according with the Stigliani et al algorithm. The results show that OsFD1 and OsFD4 which are activators of rice floral transition prefer to bind the DNA in ER conformations, whereas OsHBF1, a repressor, is enriched on ER and Direct Repeats (DR) conformations. These results suggest that in rice bZIPs the space between two motives could determine the binding to the DNA imposing a specific syntax for each transcription factor. Moreover, it is known that bZIP transcription factors must form dimers to bind the same consensus motif meaning that maybe bZIPs could contact the DNA not such as dimers, but maybe such as tetramers, in any case some experiments must be done in order to verify this hypothesis.

MATERIAL AND METHODS

Plant growing conditions, RNA sampling and qRT

Nipponbare and Donjing plants were grown under long-day conditions (14,5 light/9,5 dark) or short-day conditions (10 h light/ 14 h dark). For gene expression experiments, SAM samples were collected

starting at ZT 0 in time courses of several following days. SAMs were manually dissected under a stereomicroscope. RNA was extracted with *TRIzol*® (Termofisher Scientific). c-DNA was transcribed with Im-Prom-II RT (Promega) and qRT was performed with the primers listed in supplementary pimers table. Maxima SYBR qPCR master mix (Termofisher Scientific) was used in qRT PCR experiments.

In situ Hybridization- writing in progress

Protein-protein interaction assays

Yeast two hybrid tests were performed cloning the cds into the vectors pGADT7 and pGBKT7 (Clontech) and transformed into AH109 and Y187 yeast strains respectively. Interactions were tested by mating and growth on selective media -L-W-H -A.

BiFC experiments were performed in tobacco epidermal cells with the vectors pBAT TL-B sYFP-N and pBAT TL-B sYFP-C.

DAP seq method – writing in progress

For DAP-seq library, gDNA was extract from rice leaves, then five micrograms of genomic DNA were diluted in EB (10 mM Tris-HCl, pH 8.5) and sonicated to 200 bp fragments in a covaris S2 sonicator. DNA was purified using AmpureXP beads at a 2:1 bead:DNA ratio. Samples were then end repaired using the End-It kit (Lucigen) and cleaned using Qiaquick PCR purification (Qiagen) according to the manufacturer's recommendations. Purified samples were A-tailed using Klenow 3–5′exo- for 30 min at RT and then purified using Qiaquick PCR purification as described above. Purified samples were then ligated overnight with a truncated Illumina Y-adapter as described in Bartlett et al. Libraries were purified by bead cleaning using a 1:1 bead:DNA ratio, eluted from the beads in 30 µl of EB, and quantified with the Qubit HS fluorometric assay. Separately, HALO-tagged TFs were expressed in an in vitro rabbit reticulocyte TNT expression system and they were immobilized on Magnet HALO-Tag beads, washed, and incubated with the DNA library according to Galli et al 2018. Then read mapping, filtering, and peak calling was done according to Galli et al 2018.

Motif enrichment analysis and spacing/syntax analysis – writing in progess

This analysis was done with François Parcy laboratory and according to Stigliani et al 2019

FIGURES:

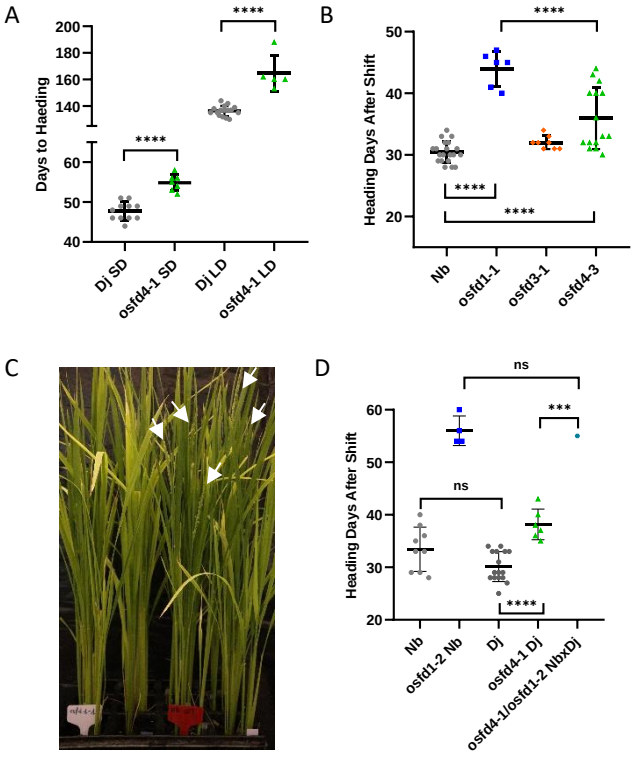


Figure 1:

A) Flowering time of *osfd4-1* mutant under short-day conditions and long day conditions.

B) Flowering time of CRISPR mutant *osfd1-1*, *osfd3-1* and *osfd4-3* under short-day conditions.

C) Picture of osfd1-1 mutants (left) and wild type (right) during flowering experiment under short-day conditions. Arrows indicated panicles.

D) Flowering time of osfd1-2, osfd4-1 and double osfd1-2/osfd4-1 with Nb and Dj wild type.

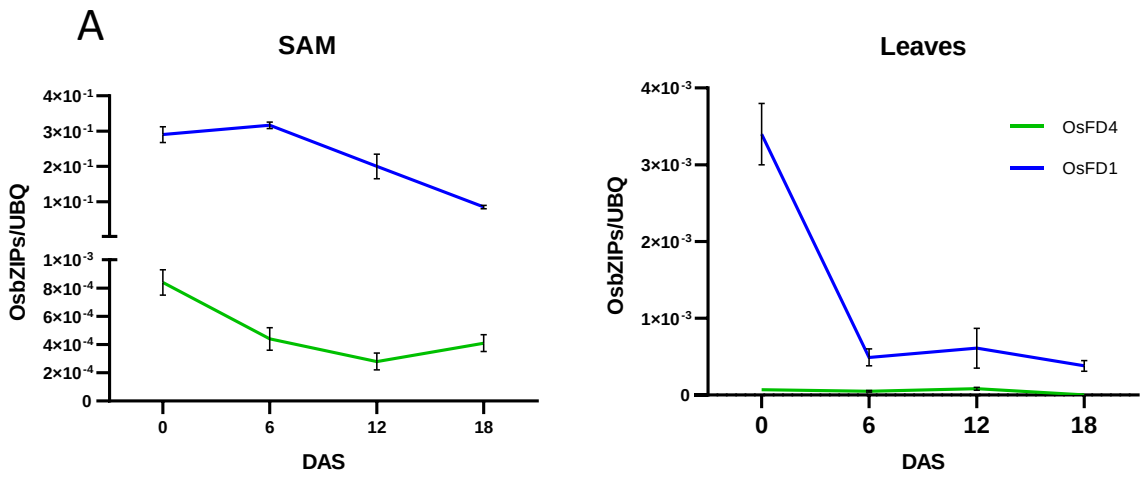


Figura 2 in progress:

A) Expression of OsFD4 (green) and OsFD1 (blue) during floral transition in SAM and leaves tested using qRT-PCR.

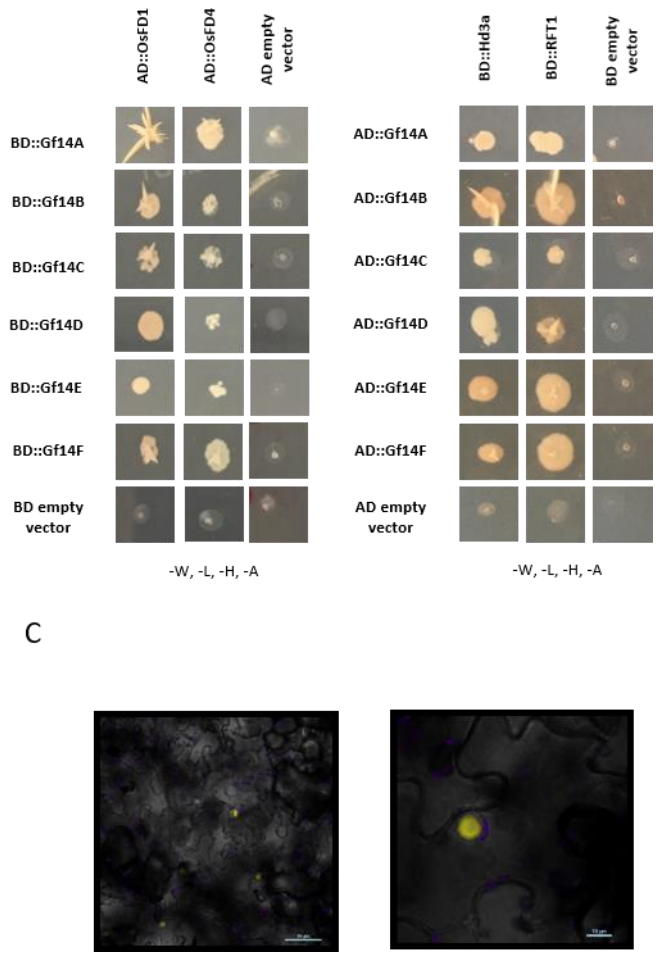


Figura 3:

A) Yeast two Hybrid among Gf14 (from A to F) fused to the Binding Domain (BD) and OsFD1 or OsFD4 fused to the Activation Domain (AD). Yeast were growth on poor medium lacking on W, L, H and A.

B) Yeast two Hybrid among Gf14 (from A to F) fused to the AD and Hd3a or RFT1 fused to the BD. Yeast were growth on poor medium lacking on W, L, H and A.

C) Bimolecular fluorescence complementation between OsFD4 fused with N-terminus of YFP and RFT1 fused with the C-terminus of YFP.

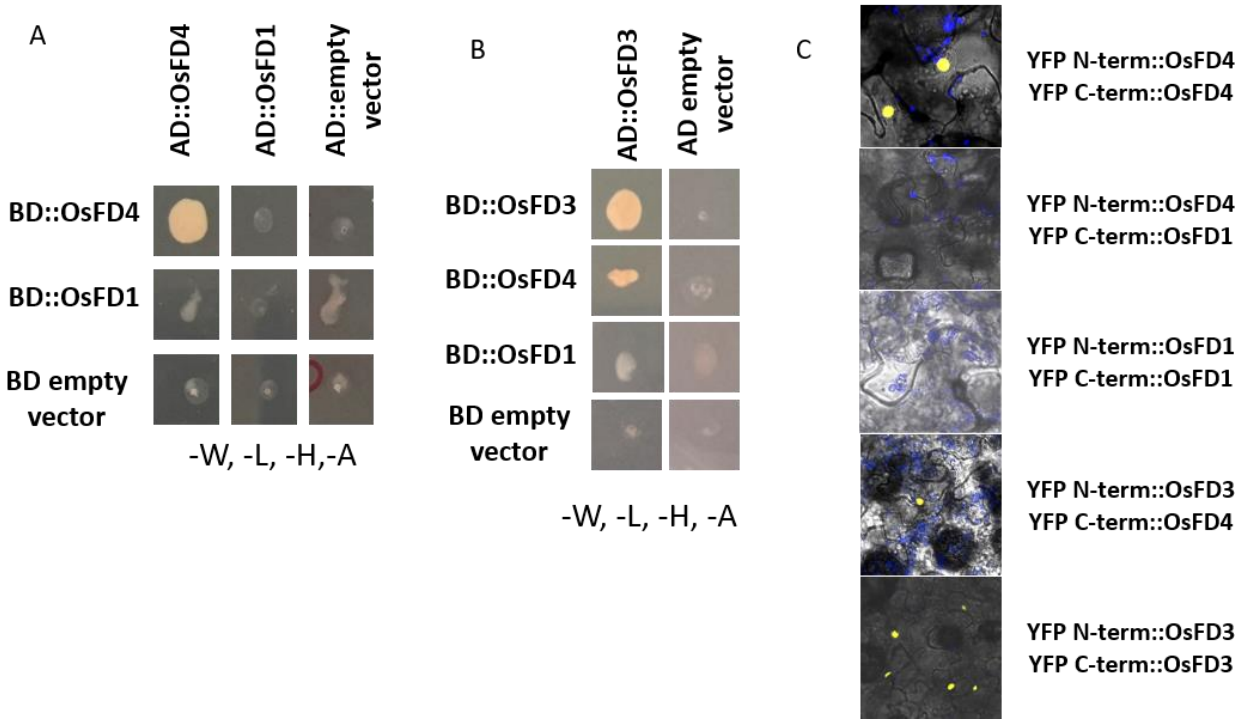


Figure 4:

A) Yeast two Hybrid of OsFD4 and OsFD1 fused with BD or with AD to test homo- or hetero-dimerization. Yeast were growth on poor medium lacking on W, L, H and A.

B) Yeast two Hybrid of OsFD4 and OsFD1 fused with BD or with AD to test homo- or hetero- Yeast two Hybrid among Gf14 (from A to F) fused to the AD and Hd3a or RFT1 fused to the BD. Yeast were growth on poor medium lacking on W, L, H and A.

C) Bimolecular fluorescence complementation between OsFD4 fused with N-terminus of YFP and RFT1 fused with the C-terminus of YFP.

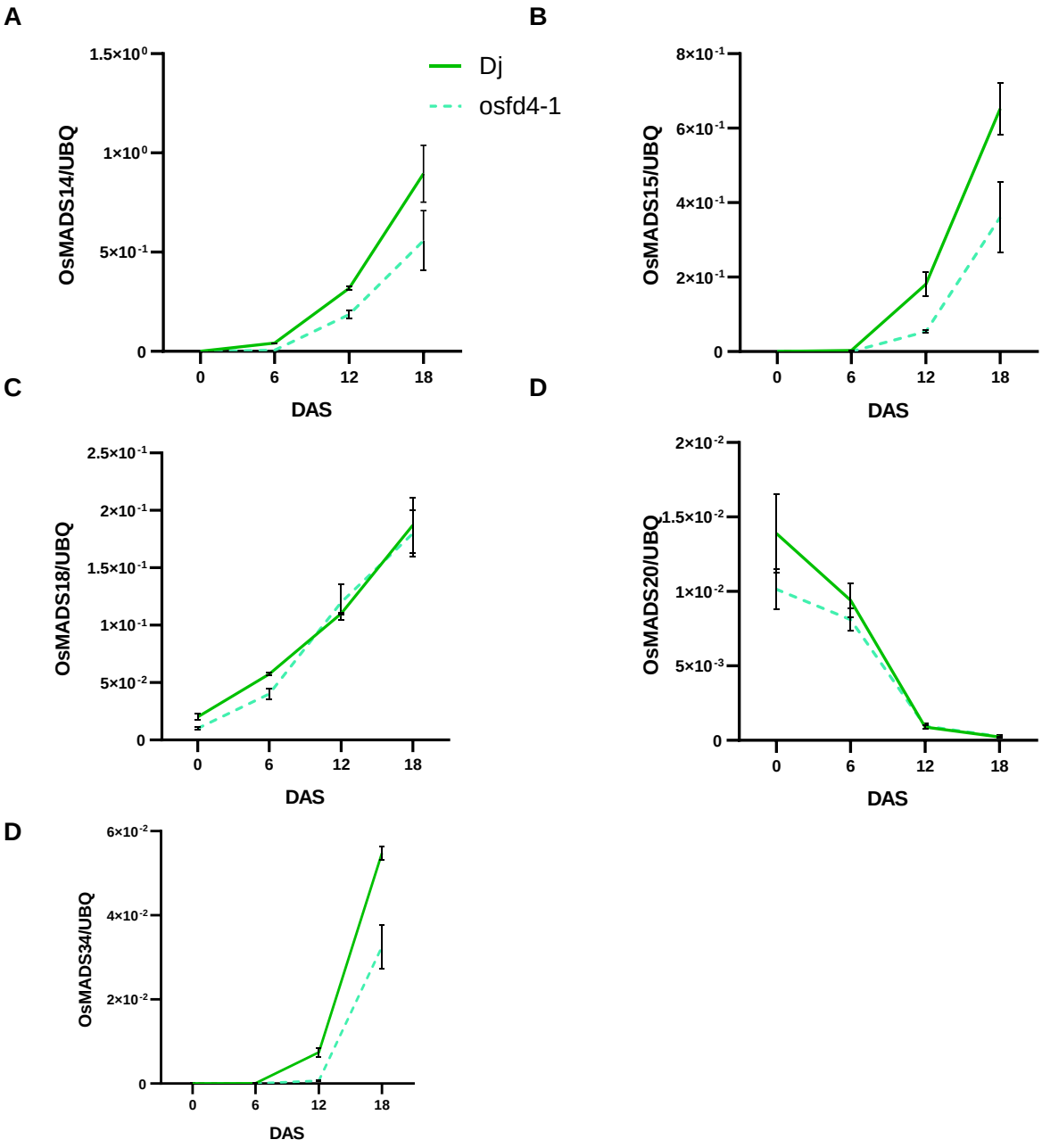


Figure 5:

A) *OsMADS14* expression in wild type and osfd4-1 mutants. B) *OsMADS15* expression in wild type and osfd4-1 mutants. C) *OsMADS18* expression in wild type and osfd4-1 mutants. D) *OsMADS20* expression in wild type and osfd4-1 mutants. E) *OsMADS34* expression in wild type and osfd4-1 mutants. Expressions were tested in plants growth two months under not-inductive conditions and then shifted under inductive conditions. SAMs were sampled at 0, 6, 12 and 18 Days After Shifting (DAS)

A

bZIP	Reads	Peaks	Genes	Motif
HBF1	2.387.145	28.323	15.937	
OsFD1	1.973.636	2.059	1.717	
OsFD4	1.955.263	1.107	925	

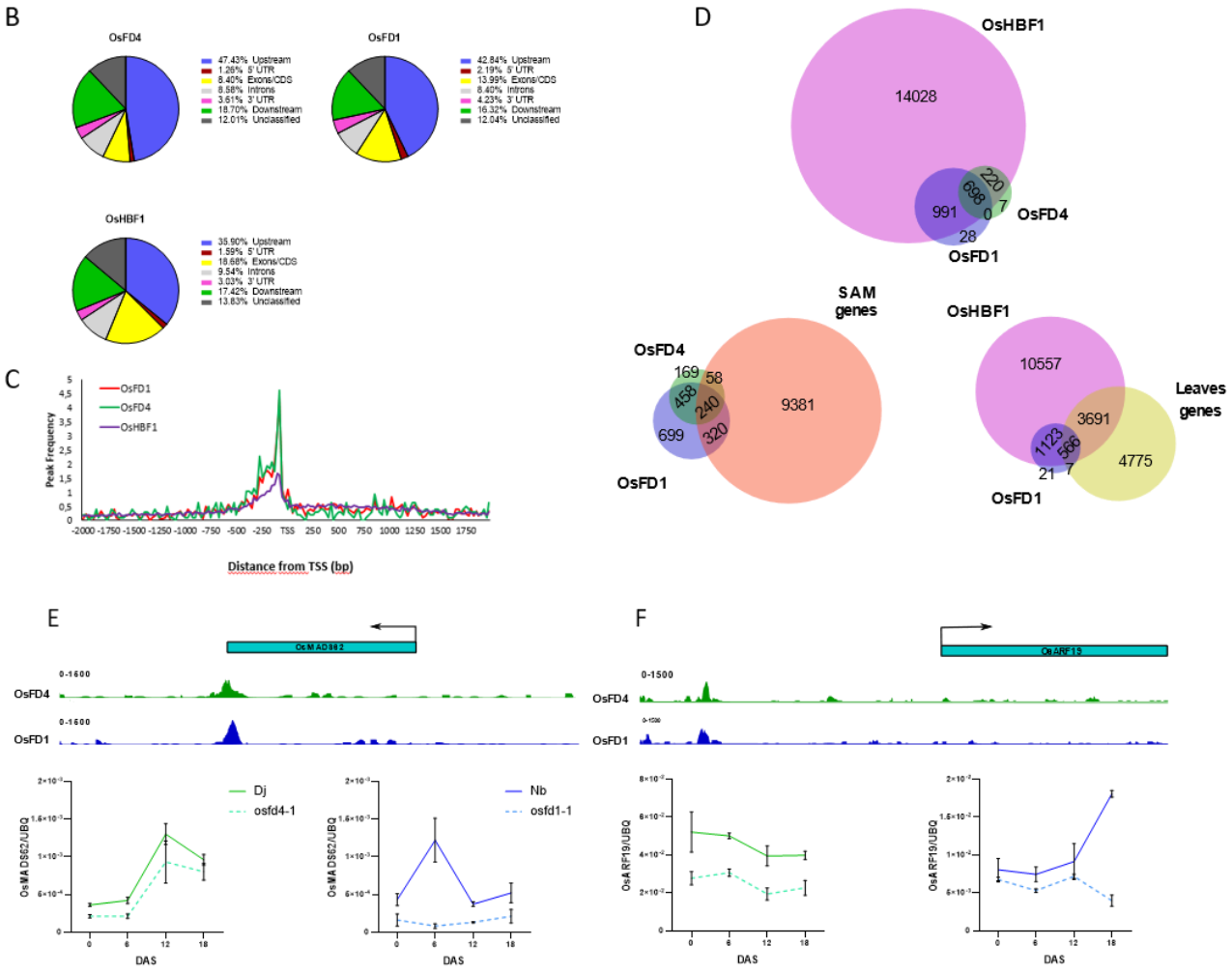


Figure 6:

A) Table with genes found using DAP-seq of OsFD1, OsFD4 and OsHBF1 and their consensus motives

B) Distribution of Peaks in the genes

C) Distribution of Peaks in the Upstream region of genes

D) Venn diagrams among: OsFD1, OsFD4 and OsHBF1 datasets; OsFD1, OsFD4 and genes expressed in the SAM; OsFD1, OsHBF1 and genes expressed in leaves.

E) *OsMADS62* in OsFD1 and OsFD4 datasets and expression of *OsMADS62* in *osfd4-1* and *osfd1-1* mutants

F) *OsARF19* in OsFD1 and OsFD4 datasets and expression of *OsARF19* in *osfd4-1* and *osfd1-1* mutants

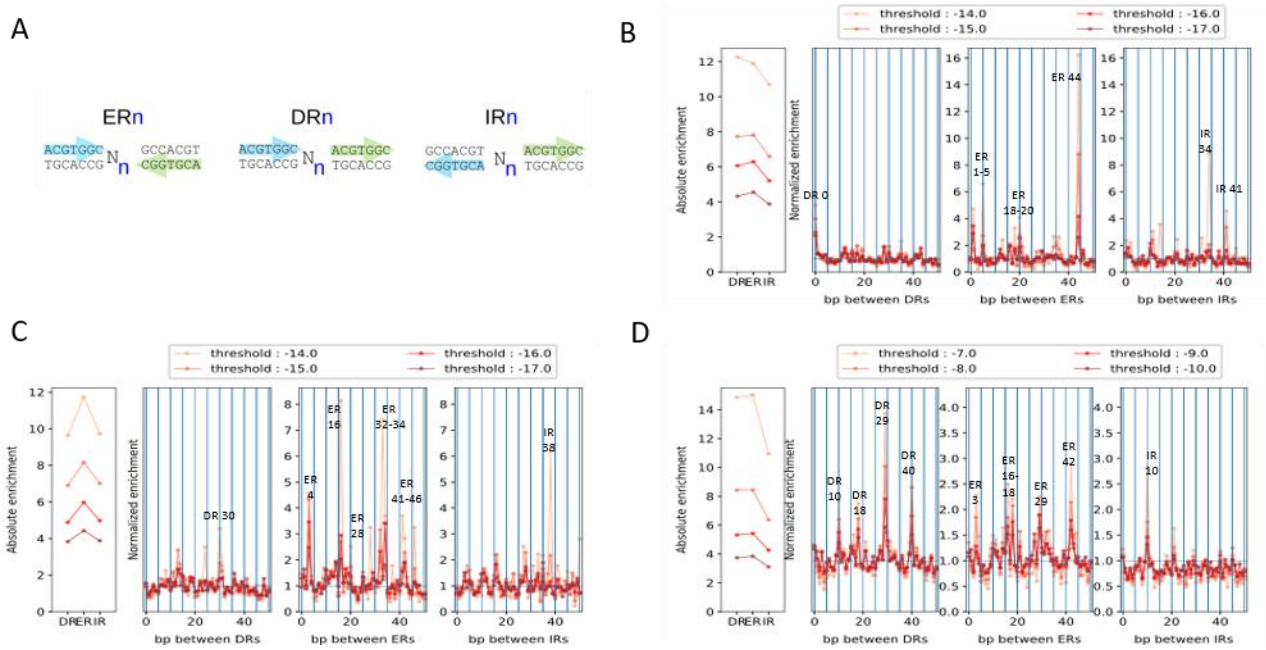


Figure 7

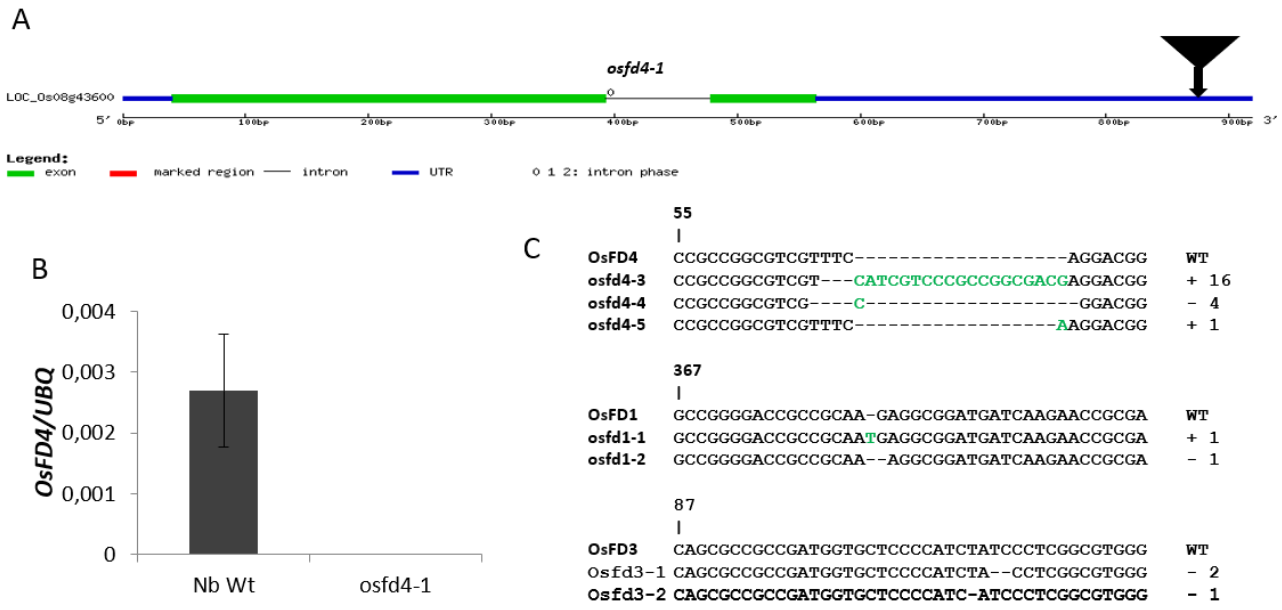
A) Scheme of Everted Repeats (ER), Direct Repeats (DR) and Inverted Repeats (IR) of "GCCACGT" motif

B) Spacing analysis of OsFD4 absolute enrichment and normalized enrichment

C) Spacing analysis of OsFD1 absolute enrichment and normalized enrichment

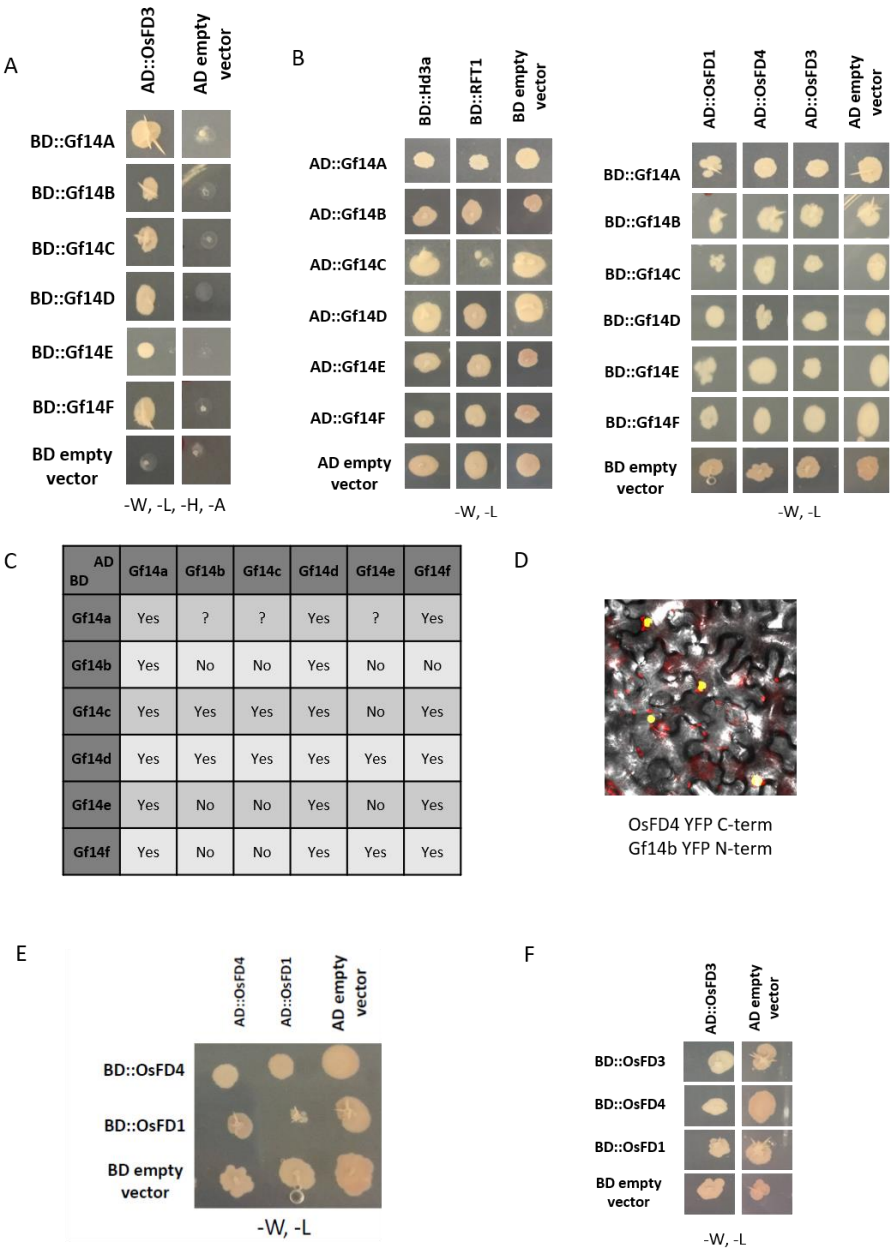
D) Spacing analysis of OsHBF1 absolute enrichment and normalized enrichment

Supplementary Figures



Supplementary Figure 1:

A) T-DNA insertion scheme of *osfd4-1* mutant
B) Expression in the SAM in Dj wild type and *osfd4-1* mutant
C) CRISPR alleles of *osfd4*, *osfd1* and *osfd3*



AD / BD	Gf14a	Gf14b	Gf14c	Gf14d	Gf14e	Gf14f
Gf14a	Yes	?	?	Yes	?	Yes
Gf14b	Yes	No	No	Yes	No	No
Gf14c	Yes	Yes	Yes	Yes	No	Yes
Gf14d	Yes	Yes	Yes	Yes	Yes	Yes
Gf14e	Yes	No	No	Yes	No	Yes
Gf14f	Yes	No	No	Yes	Yes	Yes

Supplementary Figure 2:

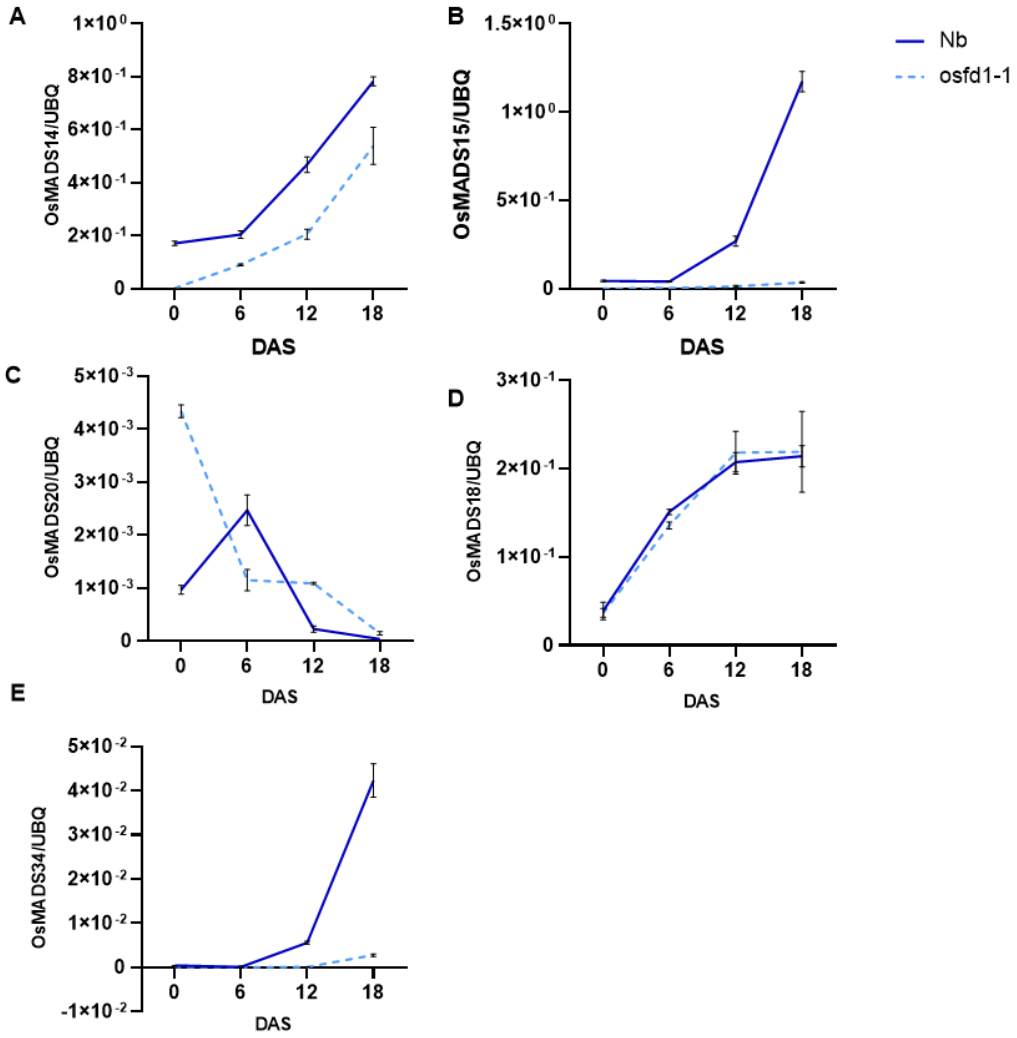
A) Y2H among OsFD3 fused to the AD and Gf14s (from a to f) fused to BD; yeasts were grown on medium lacking W, L, H, A

B) Y2H Mating control of Gf14s and bZIPs interactions and Gf14s and florigens interaction

C) Table of Gf14s which interact each other's in yeast

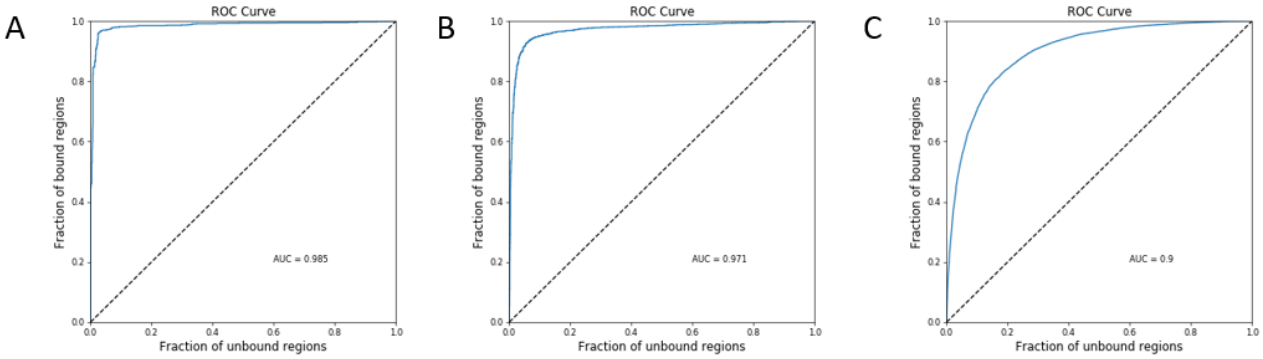
D) BiFC between OsFD4 fused to C-terminus of YFP and Gf14b fused to the N-terminus of YFP

E) And F) Y2H Mating control of bZIPs interactions



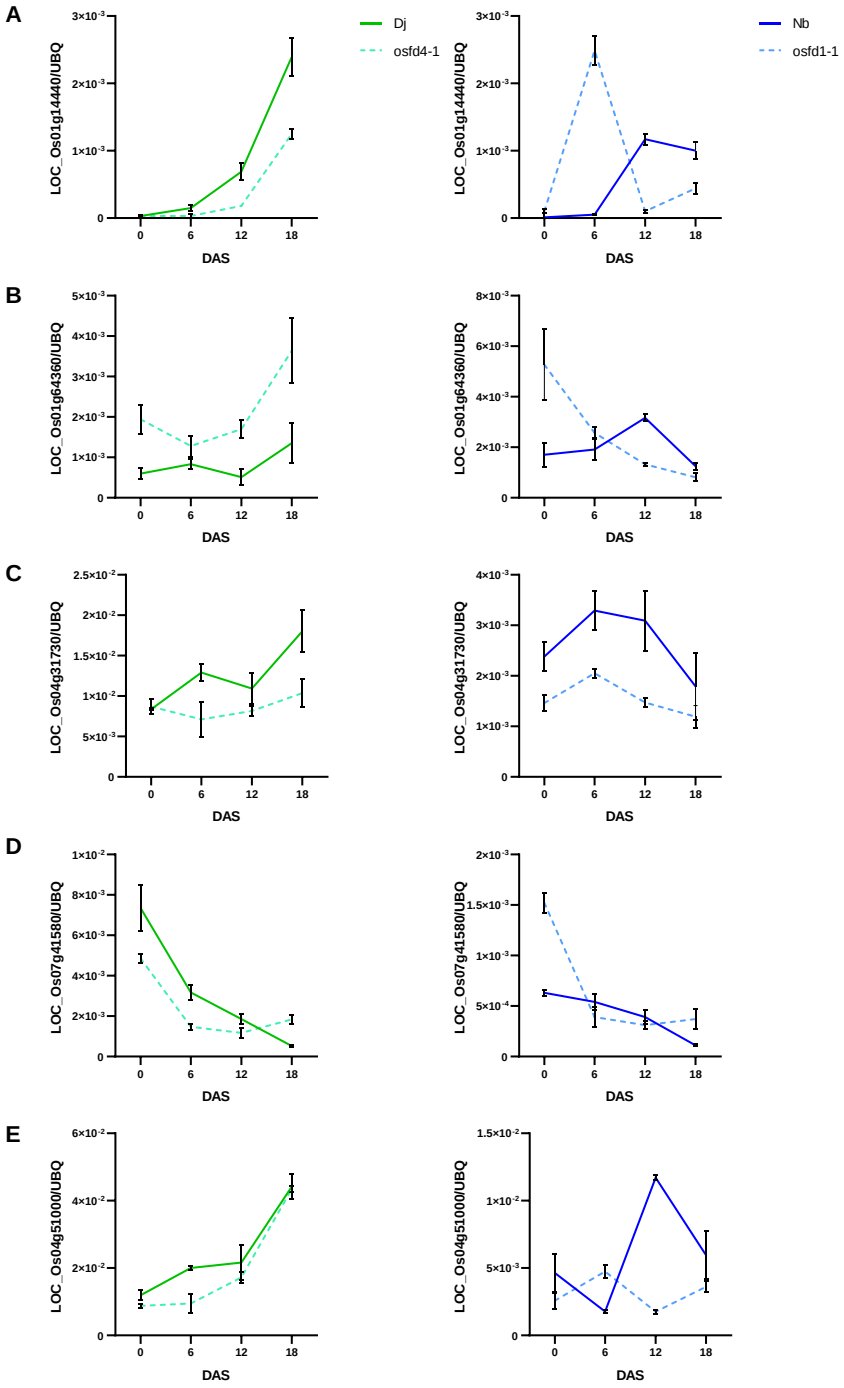
Supplementary Figure 3:

A) *OsMADS14* expression in wild type and *osfd1-1* mutants. B) *OsMADS15* expression in wild type and *osfd1-1* mutants. C) *OsMADS18* expression in wild type and *osfd1-1* mutants. D) *OsMADS20* expression in wild type and *osfd1-1* mutants. E) *OsMADS34* expression in wild type and *osfd1-1* mutants. Expressions were tested in plants growth two months under not-inductive conditions and then shifted under inductive conditions. SAMs were sampled at 0, 6, 12 and 18 Days After Shifting (DAS)



Supplementary Figure 4:

A) ROC curve of OsFD4 PWM analysis
B) ROC curve of OsFD1 PWM analysis
C) ROC curve of OsHBF1 PWM analysis



Supplementary Figure 5:

A) LOC_Os01g14440 expression in osfd4-1 and osfd1-1 respect to their wild type (Dj and Nb, respectively). B) LOC_Os01g64360 expression in osfd4-1 and osfd1-1 respect to their wild type (Dj and Nb, respectively). C) LOC_Os04g31730 expression in osfd4-1 and osfd1-1 respect to their wild type (Dj and Nb, respectively). D) LOC_Os07g41580 expression in osfd4-1 and osfd1-1 respect to their wild type (Dj and Nb, respectively). E) LOC_Os04g51000 expression in osfd4-1 and osfd1-1 respect to their wild type (Dj and Nb, respectively). Expressions were tested in plants growth two months under not-inductive conditions and then shifted under inductive conditions. SAMs were sampled at 0, 6, 12 and 18 Days After Shifting (DAS)

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