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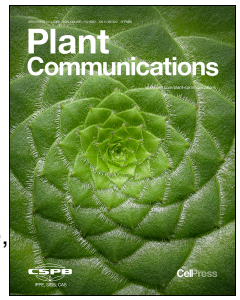

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PII: S2590-3462(23)00121-9

DOI: https://doi.org/10.1016/j.xplc.2023.100610

Reference: XPLC 100610

To appear in: *PLANT COMMUNICATIONS*

Received Date: 2 October 2022

Revised Date: 14 April 2023

Accepted Date: 30 April 2023

Please cite this article as: Vicentini, G., Biancucci, M., Mineri, L., Chirivì, D., Giaume, F., Miao, Y., Kyozuka, J., Brambilla, V., Betti, C., Fornara, F., Environmental Control of Rice Flowering Time, *PLANT COMMUNICATIONS* (2023), doi: https://doi.org/10.1016/j.xplc.2023.100610.

Environmental Control of Rice Flowering Time

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Short summary. Rice flowering time depends on external environmental parameters among which the photoperiod is the most important. Yet, temperature variations, the hormonal balance and occasional stress conditions contribute to modify normal flowering patterns by integrating into the molecular network of regulatory genes.

Abstract

Correct measurement of environmental parameters is fundamental for plant fitness and survival, as well as for timing developmental transitions, including the switch from vegetative to reproductive growth. Important parameters affecting flowering time include day length (photoperiod) and temperature. Their response pathways have been best described in Arabidopsis, that currently offers a detailed conceptual framework and serves as term of comparison also for other species. Rice, the focus of this review, also possesses a photoperiodic flowering pathway, but 150M years of divergent evolution in very different environments have diversified its molecular architecture. The ambient temperature perception pathway is strongly intertwined with the photoperiod pathway and essentially converges on the same genes to modify flowering time. When observing network topologies it is evident that the rice flowering network is centered on *EARLY HEADING DATE 1*, a rice-specific transcriptional regulator. Here, we summarize the most important features of the rice photoperiodic flowering network, with an emphasis on its uniqueness, and discuss its connections with hormonal, temperature perception and stress pathways.

Keywords: rice, photoperiod, temperature, flowering, stress, florigens

Distinctive features of the rice photoperiodic flowering pathway

Flowering time is a key adaptive trait allowing plants to synchronise reproduction with the most favourable environmental conditions. Seasonal changes in day length (photoperiod) follow a sinusoidal curve whose amplitude varies with latitude but, at any given location, is invariant from one year to another. Thus, photoperiod variations offer very stable and measurable parameters to anchor plant reproduction with a specific time of the year, and plant species can be categorized depending upon the photoperiodic regime required to promote flowering. Short day (SD) plants flower when day length falls under a critical threshold, long day (LD) plants flower when day length exceeds a critical threshold, while day-neutral plants do not use photoperiodic cues to time reproduction.

Rice is a facultative SD plant that flowers faster if exposed to day lengths shorter than 13.5h, but can flower also under LD conditions, taking more time (Itoh et al., 2010). Genetic mapping allowed to isolate several flowering time genes, starting with *HEADING DATE 1* (*Hd1*), belonging to the CCT family of transcriptional regulators (Yano et al., 2000). Hd1 shows high sequence similarity to CONSTANS (CO), a flowering promoter central in the photoperiod pathway of Arabidopsis. This feature suggested the existence of an evolutionary shared flowering network, common to monocots and dicots. In Arabidopsis, *CO* transcription is controlled by the circadian clock through *GIGANTEA* (*GI*) and CO is required to activate transcription of *FLOWERING LOCUS T* (*FT*), encoding a mobile florigenic protein (Andrés and Coupland, 2012). A similar network arrangement was demonstrated also in rice, where *OsGI* promotes *Hd1* expression, which in turn promotes transcription of *HEADING DATE 3a* (*Hd3a*), a homolog of *FT*, under inductive photoperiodic conditions (Hayama et al., 2003). The strong homology between genes and their similar arrangement in gene regulatory networks (GRNs), further corroborated the idea of a conserved architecture. However, with more genes being cloned, it became evident that not only rice-specific regulators existed, but also that genes homologous to Arabidopsis flowering regulators, were arranged differently within the flowering network. Therefore, we wish to rediscuss the concept of a shared network and suggest that a strict comparison to Arabidopsis is misleading.

The EARLY HEADING DATE 1 (Ehd1) B-type response regulator was the first rice-specific promoter of flowering to be isolated. Ehd1 induces expression of *Hd3a* and *RICE FLOWERING LOCUS T 1* (*RFT1*) florigens under both LD and SD (Doi et al., 2004; Zhao et al., 2015). This gene occupies a central position in the network, operating as a hub that integrates signals mediated by several genes (Figure 1). All major flowering time regulators cloned after *Hd1*, and including *GRAIN NUMBER, PLANT HEIGHT AND HEADING DATE 7* (*Ghd7*, also known as *Hd4*), *Ghd8* (also known as *Hd5* or *DTH8*), *PSEUDO RESPONSE REGULATOR 37* (*PRR37*, also known as *Hd2*, *DTH7* or *Ghd7.1*) and *RICE INDETERMINATE 1* (*RID1*, also known as *Ehd2* or *OsID1*) encode strong repressors of *Ehd1* that reduce its transcription under LD (Park et al., 2008; Wu et al., 2008; Xue et al., 2008; Matsubara et al., 2008; Wei et al., 2010; Wu et al., 2013; Koo et al., 2013). As a result of this arrangement, and differently from Arabidopsis, LD regulation is characterized by active

repression of florigens expression, with induction of flowering taking place under SD only when transcriptional blocks are released. *Hd1* itself is a LD repressor of *Ehd1*, suggesting that the *OsGI-Hd1* module evolved in connection with, and not in parallel to, *Ehd1*-mediated regulation (Gómez-Ariza et al., 2015; Nemoto et al., 2016). Almost all regulators of flowering cloned to date, either activate or repress *Ehd1* (Figure 1). An additional list of genes not discussed in the main text is provided in Supplementary Table 1.

A second aspect discriminating rice and Arabidopsis, stems from interpretation of the connections between photoperiod measurement and flowering time control. This relationship is summarised by the external coincidence model of photoperiodism, postulating that flowering is induced when a sensitive phase of expression of a circadian-regulated factor coincides with a favourable environmental input (Thomas and Vince-Prue, 1997).

In Arabidopsis, CO is central to this model. Its expression is controlled by the circadian clock that induces a peak of transcription at the end of the light period, only under LD. The presence of light during this phase of the cycle leads to CO protein stabilization and accumulation, *FT* induction and flowering (Valverde et al., 2004; Song et al., 2012). Under SD, peak expression occurs during the night, preventing accumulation of the CO protein. In contrast, Hd1 is not as central to external coincidence, because Hd1 protein abundance follows gene transcription and is not modified by changes in day length or presence of light (Yang et al., 2015). Therefore, Hd1 protein accumulation does not predict LD and SD flowering behaviours, even though it remains possible that post-translational modifications affect protein activity, but not abundance, in a day length dependent manner (Ishikawa et al., 2011).

The accumulation profiles of mRNA and protein of several flowering regulators, depending on the photoperiod, suggest that Ghd7 might be key to interpret external coincidence in rice (Figure 2). Transcription of *Ghd7* is promoted by red light and gated in the morning under LD. Its cognate protein accumulates to reduce *Ehd1* expression and delay flowering (Itoh et al., 2010; Zheng et al., 2019). Under SD, *Ghd7* transcription is reduced and its gate of inducibility shifts towards the night. With the reduction of *Ghd7* expression, *Ehd1* repression is relaxed and a gate for its induction opens during the morning in response to blue light signals mediated by *OsGI* (Itoh et al., 2010). Most importantly, the stability of Ghd7 protein depends on the photoperiod and it does not accumulate under SD, even if overexpressed (Zheng et al., 2019). Ghd7 stability is influenced by direct interaction with OsGI that promotes its degradation in a proteasome-dependent manner. Conversely, phytochromes have a positive effect on Ghd7 stability and mutations in *PHYTOCHROME B* (*PhyB*) or *PHOTOPERIODIC SENSITIVITY 5* (*Se5*), encoding a plastid heme oxygenase essential for biosynthesis of the chromophore of phytochromes, never accumulate Ghd7 (Izawa et al., 2000; Andrés et al., 2009; Osugi et al., 2011; Weng et al., 2014; Zheng et al., 2019). Therefore, the antagonistic activities of OsGI and phytochromes shape the diurnal accumulation pattern of Ghd7, both transcriptionally and post-transcriptionally, and Ghd7 accumulation patterns discriminate between LD and

SD. Thus, a plausible interpretation of external coincidence in rice suggests that it releases LD repression by preventing accumulation of Ghd7. Red and blue light signals have antagonistic effects on the flowering network, both of them converging on *Ehd1* transcription, a behavior substantially different from that of LD species such as Arabidopsis (Figure 2). Post-translational regulation of other important components of the flowering network is still to be evaluated before defining a final model.

A different perspective relates to the evolutionary interpretation of the CO/Hd1 functions, arguing in favor of their different origins (Ballerini and Kramer, 2011). The *CO* gene originated from a tandem duplication of *COL1* and evolved a transcriptional pattern and protein features that made it a key photoperiod sensor. Its appearance can be traced to the common ancestor of the Brassicaceae where it transcriptionally connected to *FT*, and LD flowering induction arose (Simon et al., 2015). Thus, the CO function is a recent acquisition that occurred long after the split between monocots and dicots. Hd1 was likely recruited independently to regulate photoperiodic flowering in rice and the similar network arrangement is most probably the result of convergent evolution. In fact, it is possible that CCT domain proteins are particularly suited to control florigen expression and flowering. Not surprisingly, *Ghd7* and *PRR37* encode CCT domain proteins as central as Hd1 to rice flowering regulation (Xue et al., 2008; Koo et al., 2013; Gao et al., 2014; Nemoto et al., 2016). Finally, a distinctive feature of rice is its evolution of a double florigen system that is essential for flowering under any photoperiod (Komiya et al., 2008; Komiya et al., 2009). Florigens induction is not dependent only upon SD, and RFT1 expression can be promoted also under LD (Komiya et al., 2009). This flexibility in florigens expression allows rice to use both *Hd3a* and *RFT1* in different environments and latitudes (Wang et al., 2021a). A major example of such flexibility is relaxation of day length dependency occurring at higher latitudes and also enhanced by artificial selection, which allowed expansion of the species and of the cultivation area (Takahashi et al., 2009; Goretti et al., 2017). Thus, photoperiodic induction of florigens is fundamental to both SD and LD flowering and, differently from Arabidopsis, no other florigen-independent flowering time pathway has been described to date.

Post-translational aspects of flowering time control

Formation of higher-order complexes

Recent studies are shedding light on higher levels of coordination among components of the photoperiod pathway, dependent upon combinations of protein-protein interactions, higher-order complexes formation and post-translational modifications. This level tunes network outputs by interacting with light quantity and quality signals from the environment.

From this perspective, the Hd1 protein has been the most studied, due to its strong effects on flowering, as well as because of homology to CO which is subject to several levels of post-transcriptional and post-translational regulation (Jang et al., 2008; Song et al., 2012; Song et al., 2014; Sarid-Krebs et al., 2015; Graeff et al., 2016).

The CCT domain of Hd1 is localized at the C-terminus and is necessary for DNA binding and protein-protein interactions, while the N-terminus contains two B-boxes, required for protein-protein interactions and transcriptional regulation (Gangappa and Botto, 2014).

The molecular activity of Hd1 can be explained by its ability to form complexes with other nuclear proteins. Transcriptional repression activity under LD is dependent upon assembly of NUCLEAR TRANSCRIPTION FACTOR Y (NF-Y) heterotrimeric complexes, formed by Hd1, NF-YB and NF-YC. The latter subunits encode histone-like proteins that, upon dimerization, construct a histone-fold domain (HFD) scaffold having affinity for DNA in a non-sequence specific manner. The third element of the trimer confers sequence specificity to DNA binding. The Hd1/NF-Y complex directly binds the *Hd3a* promoter, recognizing *TGTGG* sequences, called CO-Responsive Elements (*CORE*) because identified in Arabidopsis as recognized by CO and present in the *FT* promoter (Adrian et al., 2010; Tiwari et al., 2010; Goretti et al., 2017; Gnesutta et al., 2017; Shen et al., 2020; Lv et al., 2021). Structural studies have then determined the precise conformation of the Hd1/NF-Y and CO/NF-Y heterotrimers bound to DNA, corroborating previous observations (Shen et al., 2020; Lv et al., 2021). The CO/NF-Y structure further suggests a certain degree of flexibility in DNA binding. Specifically, only a *TGTG* sequence is strictly necessary for protein binding in the *TGTGG CORE* of Arabidopsis, whereas the last base does not impact on DNA recognition (Lv et al., 2021). If the same feature were demonstrated for the Hd1/NF-Y heterotrimer, its potential DNA binding sites would expand. However, all DNA interaction studies have been performed at florigen loci. The full repertoire of Hd1 or CO binding sites on a genome-wide scale *in vivo* would help to better define DNA binding properties and possibly identify novel target genes (Figure 3).

The NF-YB/C dimer can also accommodate NF-YA subunits, as well as other CCT domain proteins, including PRR37, PRR73 and Ghd7, expanding DNA accessibility through variation of motifs recognition. While NF-YA/B/C heterotrimers invariably recognize *CCAAT* box elements, how DNA binding specificity would change with incorporation of PRR37, PRR73 and Ghd7 remains to be experimentally assessed (Gnesutta et al., 2018; Shen et al., 2020; Liang et al., 2021).

An additional element of complexity is represented by expansion of gene families. The rice genome encodes for 10 *NF-YA*, 11 *NF-YB* and 7 *NF-YC* genes (Petroni et al., 2012). The combinatorial assembly of their cognate proteins and tissue specificity confer large transcriptional plasticity to the putative complexes, a feature shared with Arabidopsis (Thirumurugan et al., 2008; Kumimoto et al., 2008; Kumimoto et al., 2010).

The Hd1 protein can heterodimerize with Ghd7 to repress *Ehd1* expression, indicating the possibility of interaction also between CCT domain proteins (Nemoto et al., 2016; Zhang et al., 2017). Whether these interactions take place *in vivo* between Hd1/NF-Y and Ghd7/NF-Y complexes, or between individual Hd1 and Ghd7 is unclear. However, biochemical characterization of CO/NF-Y suggests the possibility of multimerization between ternary complexes. Chromatographic studies indicate multiple oligomeric states

for CO *in vitro*, with the most probable being trimeric or tetrameric assemblies (Lv et al., 2021). When the *FT* promoter region containing the *COREs* was incubated with CO/NF-Y in EMSA assays, multivalent binding was observed, and three out of four *COREs* present on the DNA could be simultaneously occupied. These data raise the very interesting possibility that multiple (up to four) heterotrimers assemble on the DNA, recognizing several *COREs* possibly brought in proximity by the multimers. A consequence of this mode of action is that spacing between *COREs* might create a specific syntax read by the multimers, a long-range interaction model that we have already discussed elsewhere (Gnesutta et al., 2018).

Expanding on this concept, it could be speculated that the substitution of Hd1 with PRR37, PRR73 or Ghd7 could lead to a variety of heteromultimers with distinct DNA reading possibilities. Indeed, protein-protein interaction data support the idea that the Hd1-Ghd7 complex contains Ghd8 as well (Cai et al., 2019). Multimerization patterns could soon be demonstrated also in rice. The caveat of this idea is that CO (and possibly Hd1) multimerization takes place via the B-Boxes, which are absent in PRR37, PRR73 and Ghd7. Yet, other regions of the proteins might be able to mediate interactions. For instance, Hd1 and Ghd7 contact each other through the CCT domain of Hd1 and the zinc finger plus central region (but not CCT domain) of Ghd7 (Zhang et al., 2017).

Hd1 promotes flowering and florigens expression under SD, but inhibits them under LD (Zong et al., 2021). When considering protein-protein interactions, this photoperiodic conversion finds a relatively simple explanation, because it clearly depends upon presence of Ghd7 or Ghd8 (Du et al., 2017b; Sun et al., 2022). Under LD, fully assembled complexes repress *Ehd1*, *Hd3a* and *RFT1* transcription. Under SDs, reduced expression of *Ghd8* and instability of the Ghd7 protein deprive the complexes of these components, converting Hd1 into a transcriptional activator. Genetic data support this model because *Hd1 ghd7 ghd8* mutants flower earlier than *hd1 ghd7 ghd8* under any photoperiod (Zong et al., 2021). These data also indicate that Hd1 is intrinsically a constitutive activator of flowering, regardless of day length. Such model might also implicate changes in DNA accessibility (Zheng et al., 2019).

Interestingly, also *CO* has the dual function of LD promoter and SD repressor of flowering (Luccioni et al., 2019). However, differently from rice florigens, expression of *FT* is not increased in *co* mutants under non-inductive conditions. Promotion of flowering by the *co* mutation under SD depends upon reducing expression of *TERMINAL FLOWER 1* (*TFL1*) at the apex, which in turn enhances sensitivity to *FT*. Thus, despite an apparent similarity, the effects of *Hd1* and *CO* under non-inductive photoperiods depend on very different mechanisms (Luccioni et al., 2019).

Protein stability and phosphorylation

Differential protein stability has a central role in the regulation of photoperiodic flowering and the definition of external coincidence. Seasonal and diurnal windows of CO protein accumulation define the timing of *FT* expression. In rice, Hd1 protein abundance cycles with a peak of accumulation occurring

mostly during the day, which is antiphasic compared to mRNA accumulation (Yang et al., 2015). Yet, this pattern is not a consequence of increased stability during the light phase but possibly the result of cycling of *Hd1* mRNA (Figure 2) (Ishikawa et al., 2011). That differential stability is not light-dependent is also corroborated by the similar accumulation patterns observed under SD and LD (Yang et al., 2015; Hu et al., 2022).

Hd1 is targeted for degradation by HEADING DATE ASSOCIATED FACTOR 1 (HAF1), a RING-finger E3 ubiquitin ligase, via the 26S proteasome and by components of the autophagy pathway, including OsATG5, 7 and 8 (Yang et al., 2015; Hu et al., 2022). There is no clear time-of-day effect on Hd1 protein accumulation in *haf1* or *osatg5* mutants as Hd1 levels increase at any time point tested, and in any photoperiod. However, autophagic degradation of Hd1 seems more effective in the dark. These data indicate that diurnal accumulation of Hd1 protein is not shaped by degradation mechanisms or changes in day length.

In addition to protein turnover, phosphorylation is another important step in the post-translational control of Hd1 regulatory activity. HEADING DATE REPRESSOR 1 (HDR1) is a transcription factor that delays flowering by increasing transcription of *Hd1* and reducing that of *Ehd1* and the florigens (Sun et al., 2016). At the post-translational level, HDR1 can bind to the kinase OsK4, which phosphorylates Hd1. These three proteins form a complex in vivo, suggesting that Hd1, possibly in its phosphorylated form, could be involved in a positive loop of self-regulation that involves HDR1 and OsK4 (Figure 3). Additionally, either the phosphorylated or unphosphorylated forms might be preferentially subjected to degradation or incorporation into higher-order complexes.

Florigens as final outputs of leaf regulatory networks

Florigens are small globular proteins belonging to the Phosphatidyl Ethanolamine Binding Protein (PEBP) family, present in all taxa from bacteria to mammals. They are responsible for triggering the flowering process in higher plants, but have also roles in tuberization, nodulation, seed development and as modifiers of plant architecture (Navarro et al., 2011; Chen et al., 2014; Wang et al., 2021b). They are produced in specialized companion cells of the leaves from which they enter sieve elements thought plasmodesmata, and reach distant plant tissues (Chen et al., 2018a).

PEBP having a particularly strong influence on flowering can be divided in two major functional classes, FLOWERING LOCUS T (FT)-LIKE and TERMINAL FLOWER1 (TFL1)-LIKE. In Arabidopsis, FT and TFL1, the founding members of each class, despite sharing an amino acid identity of over 98%, have antagonistic functions. FT promotes flowering by mediating both photoperiod and temperature signals, whereas TFL1 represses it (Wickland and Hanzawa, 2015; Susila et al., 2021).

There are 13 rice genes in the *FT*-like gene family (Chardon and Damerval, 2005). *Hd3a* and *RFT1* are paralogs separated by only 11.5 kb, resulting from a local duplication event that occurred after divergence of monocots from dicots (Komiya et al., 2008). They share a high degree of identity, but their expression

patterns diverged, resulting in partly distinct functions. Both genes are transcribed in response to SD, and their cognate proteins can move to the meristem and trigger flowering (Tamaki et al., 2007; Komiya et al., 2009). Thus, under inductive conditions they are redundant and compensate each other's function. Only *Hd3a* single mutants show a mild delay of flowering. However, the *Hd3a-RFT1* double RNAi never flowers under SD, indicating that, differently from Arabidopsis, the switch to inflorescence development is fully dependent upon florigens (Komiya et al., 2008; Tamaki et al., 2015). Under LD conditions, expression of *RFT1*, but not of *Hd3a*, is induced in leaves. This is sufficient to trigger flowering, albeit later compared to SD and shows how rice facultative photoperiodic behavior is always mediated by florigens. No florigen independent pathway inducing flowering has been described to date.

The closest homolog of *Hd3a* and *RFT1*, *FLOWERING LOCUS T LIKE 1* (*FT-L1*), has florigenic activity, being able to induce flower formation in seedlings grown *in vitro*, when overexpressed (Izawa et al., 2002). *FT-L1* expression is directly induced by Hd3a and RFT1 (Giaume et al., 2023). Its transcripts and protein can be detected at all stages of inflorescence development, in the same tissues, indicating a meristematic cell-autonomous activity (Furutani et al., 2006; Zong et al., 2022; Giaume et al., 2023). Loss-of-function mutants delay flowering and enhance lateness of *hd3a* and *rft1* single mutants. Interestingly, the mutants develop panicles with a higher number of secondary branches, indicating reduced determinacy, and this effect is genetically separable from the control of flowering time. Thus, rice evolved a unique triple florigenic system that times the transition to reproductive growth as well as shaping panicle architecture (Giaume et al., 2023).

Four homologues of *TFL1*, including *RICE CENTRORADIALIS* (*RCN*) *1* to *4*, have been described in rice (Kaneko-Suzuki et al., 2018). Overexpression of *RCN1* and *RCN2* delays flowering and increases the number of panicle branches (Nakagawa et al., 2002), while *rcn* knockout plants possess small panicles with a reduced number of branches (Liu et al., 2013). *RCNs* are transcribed in the vasculature but not in the SAM, differently from *TFL1*. However, the proteins are translocated to the SAM to repress flowering. This mode of action resembles that of the florigens and suggests competition between RCNs and Hd3a and RFT1 proteins at the shoot apical meristem (SAM). It remains unclear how the two opposing activities are balanced when both flowering activating and repressing PEBPs are present at the SAM (Kaneko-Suzuki et al., 2018).

Florigens move through plasmodesmata (PD) to reach distant compartments of the plant. FT is loaded in the phloem by FT INTERACTING PROTEIN 1 (FTIP) (Liu et al., 2012). It has been demonstrated that rice FTIP1 (OsFTIP1), the closest homolog of Arabidopsis FTIP1, is necessary to promote rice flowering under LDs via its specific modulation of RFT1 transport from companion cells to sieve elements. OsFTIP1 interacts with RFT1 and in *osftip1* mutants, RFT1 accumulates to high levels in companion cells, but decreases in sieve elements, suggesting that OsFTIP1 promotes RFT1 export from companion cells to sieve elements in the phloem (Song et al., 2017). While this mechanism is limited to RFT1 transport under LDs, a parallel one

determines Hd3a transport under SDs. *OsFTIP9* encodes a homolog of *OsFTIP1,* and its protein product interacts with Hd3a to mediate its loading into sieve elements (Zhang et al., 2022). Consistent with this function, *osftip9* mutants flower late under SDs but not LDs. Thus, the OsFTIP1-RFT1 and OsFTIP9-Hd3a dimers mirror each other's functions under LDs and SDs, respectively (Figure 3). Whether dimerization could take place also by swapping the interactors between dimers remains undemonstrated. Yet, the interaction of both dimers is strengthened by OsTPR075 a tetratricopeptide repeat (TPR) protein active under both SDs and LDs (Zhang et al., 2022). When mutated, it decreases the amount of Hd3a and RFT1 reaching the apex, leading to late flowering under any photoperiod.

Such mechanisms of transport might require endosomal trafficking mediated by SNARE proteins within intracellular membranes. In Arabidopsis, *SYNTAXIN OF PLANTS121* (*SYP121*) encodes a SNARE protein interacting with QUIRKY (QKY). The SYP121-QKY complex regulates endosomal transport of FT in vesicles directed to the plasma membrane of companion cells. *SYP121* or *QKY* loss of function mutants prevent FT export from companion cells to sieve elements, delaying flowering under LD (Liu et al., 2019a). Endosomal trafficking could be implicated also in florigens transport in rice as OsFTIP1 and OsFTIP9 have been localized in the endoplasmic reticulum (Song et al., 2017; Zhang et al., 2022). However, homologs of SYP121 and QKY in rice have not been studied yet.c

The regulation of florigens loading into the phloematic stream is likely subject to several layers of control. The phosphatidylinositol 3-/4-kinase (PI3/4K) family protein, OsUbDKγ4, reduces OsFTIP1 protein abundance by proteasome-mediated degradation, and accelerates flowering if mutated (Song et al., 2017). How this post-translational mechanism interacts with day length and whether it targets also OsFTIP9 under SDs should be assessed.

Florigens, including FT, Hd3a and RFT1 bind to phosphatidylcholine (PC), a phospholipid more abundant in the outer membrane layer of the SAM, facing the apoplast (Nakamura et al., 2014; Nakamura et al., 2019; Qu et al., 2021). Artificial manipulation of PC levels at the SAM of Arabidopsis modifies flowering, consistent with PC promoting the floral transition in an FT-dependent manner (Nakamura et al., 2014). In rice, a phospholipase D (spPLD) hydrolyses phosphatidylcholine, and the corresponding loss-of-function mutants flower earlier than the wild type, promoting expression of Hd3a and RFT1 targets at the SAM (Qu et al., 2021). Interestingly, the activity of spPLD in delaying flowering depends upon its secretion in the apoplast, suggesting that the PC-florigens interaction takes place out of the cell, mediating aspects of florigens activity that might deal with their transport at the apex. Whatever the mechanism, these evidences indicate that the interaction with PC potentiates the activity of the florigens.

The response of the shoot apical meristem to flowering inductive signals

Variability of florigen complexes

Once translocated to the SAM, the florigens induce its conversion from vegetative to reproductive growth.

The meristem is the ultimate recipient of flowering signals, where integration of several environmental inputs take place. Commitment to a flowering fate is irreversible for most species and must be precisely timed and executed, particularly in annuals whose life cycle ends after a single flowering episode. A proper threshold of inductive signals should be reached before the reproductive switch takes place.

It is still unclear how florigenic proteins move from conductive tissues, mature phloem or protophloem, into meristematic cells at the apex, and how they move within it. However, research in rice, Arabidopsis and several other model and non-model species indicate a common mode of action for florigens. Central to their activity is the Florigen Activation/Repressor Complex (FAC/FRC) (Taoka et al., 2011; Park et al., 2014; Tylewicz et al., 2015; Li et al., 2015; Abe et al., 2019; Collani et al., 2019; Sun et al., 2020; Cerise et al., 2021; Liu et al., 2021). The FAC is an heterohexamer assembled around a dimer of 14-3-3 proteins, forming a W-shaped structure. Upon entering meristematic cells, Hd3a and RFT1 bind to the 14-3-3 dimer in the cytoplasm (Taoka et al., 2011; Zhao et al., 2015). The florigen/14-3-3 complex enters the nucleus where it binds to a transcription factor belonging to the bZIP family, which confers DNA binding properties to the complex. Two florigen molecules rest on the C-terminal regions of each of the 14-3-3 proteins, while the two angles at the base of the W form pockets to which the C-terminal portion of the bZIP binds.

The structure of the FAC has been first shown to contain the OsFD1 transcription factor but it has been later demonstrated that several bZIPs can replace OsFD1 (Tsuji et al., 2013; Jang et al., 2017; Brambilla et al., 2017; Cerise et al., 2021; Kaur et al., 2021). bZIPs act as dimers and the complex orients their DNA binding domain towards the DNA. However, whereas the florigen/14-3-3 dimer was resolved using full length proteins, only nine amino acids of OsFD1 were crystalized, with the structure of the remaining part of the protein being inferred by modelling. Thus, the exact conformation of the bZIP dimer within the FAC still needs to be resolved in more detail.

Similarly to the diversity of bZIPs that take part to formation of FACs/FRCs, a variety of 14-3-3 homo or heterodimers can form the core of these complexes (Cerise et al., 2021). It is still unclear how this plasticity impacts on gene expression. The binding motifs of bZIPs are almost identical both within and between species, as well as for promoters and repressors of flowering (Taoka et al., 2011; Collani et al., 2019; Cerise et al., 2021). Therefore, selectivity of the complexes might depend on additional interacting partners or on the binding syntax (number of and spacing between motives) typical of each promoter (Cerise et al., 2021).

Upon binding to the DNA, FAC targets, promoting inflorescence development, are activated. The most relevant include members of the MADS-box family of transcription factors. In rice, *OsMADS14*, *15*, *18* and *34/PANICLE PHYTOMER 2* (*PAP2*) redundantly control panicle formation (Kobayashi et al., 2012). A quadruple mutant between these genes replaces inflorescence branches with vegetative shoots and no flowers are formed. However, the inflorescence meristem is initiated normally, as indicated by the change from alternate to spiral phyllotaxis which can be observed both in mutant and wild type, as they switch from vegetative to reproductive growth. These observations indicate that *OsMADS14*, *15*, *18*, *34* might not

be the very first factors responsible for conversion of the VM into IM and other targets, activated earlier or in parallel, are likely present.

The FRCs share the same heterotrimeric architecture as the FACs but incorporate elements repressing the floral transition. Most notably, RCNs can replace Hd3a and RFT1, binding to 14-3-3s and delaying transition (Kaneko-Suzuki et al., 2018). Also bZIPs with floral repression function can form FRCs, such as Hd3a BINDING FACTOR 1 (HBF1) and HBF2, even if their activity occurs mostly in leaves and their precise role at the SAM needs to be more thoroughly defined (Brambilla et al., 2017).

Phosphorylation of the C-terminal SAP/TAP motif of bZIPs forming FACs/FRCs is necessary for their interaction with 14-3-3 proteins and mutations in this region reduce the functionality of the complex. Conversely, mutations mimicking constitutive phosphorylation confer stronger flowering promoting activities to FD proteins (Taoka et al., 2011; Collani et al., 2019). In rice, several protein kinases affecting both the functional and interaction properties of OsFDs have been isolated. The calcineurin B-like-interacting protein kinase 3 (OsCIPK3) interacts with, and phosphorylates, OsFD1 (Peng et al., 2021). Interestingly, *oscipk3* mutants show a late flowering phenotype and accumulate less phosphorylated OsFD1 only under LDs, whereas plants grown in SD conditions have a wild type phenotype. Thus, OsCIPK3 specifically affects the assembly of an RFT1/14-3-3/OsFD1 complex under LDs, suggesting that another unknown kinase operates under SDs. The Calcium Dependent Protein Kinases OsCDPK41 and OsCDPK49 interact with, and phosphorylate, OsFD7, which forms FACs with Hd3a, RFT1 as well as with FT-L1 (Kaur et al., 2021). The phenotypic consequences of their mutation are not determined yet, but they could be good candidates contributing to bZIP phosphorylation under SDs. Finally, a high-throughput study interrogating more than 100 interactions between Stress-Activated Protein Kinases (SAPKs) and bZIPs identified SAPK4, 9 and 10 as interactors of OsFD1 (Liu et al., 2019b). Among these, at least SAPK10 can phosphorylate OsFD1, probably targeting the RXXS/T at the SAP domain, even if not directly demonstrated (Liu et al., 2019b). The overexpression of SAPK10 under a constitutive promoter accelerates flowering under both LDs and SDs and elevates transcription levels of *OsFD1* and *OsMADS15*. Collectively, these studies suggest that the kinase-bZIP modules share a common mode of action.

When the SAM is reprogrammed to become a panicle, plant architecture changes to facilitate reproduction. The uppermost internodes, compressed below the SAM during vegetative growth, start to elongate when flowering signals reach the apex. This arrangement ensures coordination between flowering and stem elongation to the extent that, when both are complete, a mature panicle can open its flowers on top of a long stem, above the leaves, releasing pollen to the wind. The Hd3a and RFT1 florigens induce internode elongation by reducing the expression of *PREMATURE INTERNODE ELONGATION 1* (*PINE1*), a C2H2 zinc finger transcription factor that represses growth during the vegetative phase (Gómez-Ariza et al., 2019). *PINE1* is expressed at the SAM and very strongly in basal nodes, where intercalary meristems (IMs) are located. Elevated transcription of PINE1 maintains IMs inactive and their reactivation is thus florigen

dependent. *PINE1* represses growth by reducing stem responsiveness to gibberellins, albeit the exact molecular mechanism involved remains unclear. Equally unclear is how florigenic proteins reaching the SAM create a growth gradient along the stem, whereby the 4th or 5th internode from the apex elongate first, followed sequentially by the uppermost ones (Hoshikawa, 1989). A plausible hypothesis is that the florigens induce secondary signals forming a gradient along the stem. The gradient could depend on auxin which is produced at the shoot tip and transported towards the root (Wolbang et al., 2004). Experiments in which the inflorescence is removed and the decapitated tip is treated with auxin, indicated that this hormone is necessary for stem elongation (Wolbang and Ross, 2001; Wolbang et al., 2004; Yin et al., 2007). Thus, the crosstalk between gibberellins and auxin might be key to interpret PINE1 activity.

Independent work isolated *PINE1* as the gene under a QTL repressing internode elongation in deepwater rice varieties (Nagai et al., 2020). The gene was named *DECELERATOR OF INTERNODE ELONGATION 1* (*DEC1*), and its reduced expression in deepwater rice upon submergence is responsible for rapid internode elongation. This excellent study shows how PINE1/DEC1 activity is central to pathways that lead to internode elongation, independently of the environmental triggers. Also, since variation of expression levels, rather than coding sequence diversity, is responsible for distinct growth behaviours, the regulatory sequences of PINE1/DEC1 could be targeted for breeding efforts aimed at controlling plant growth.

Antagonistic signalling pathways balance the switch to reproductive growth and panicle development

Commitment of the SAM to reproductive growth by FACs is necessary but not sufficient to correctly initiate reproductive growth and complete inflorescence development, and several pathways must contrast their antagonistic forces to reach proper developmental equilibrium.

During or shortly before specification of the inflorescence, the vegetative program must be actively suppressed. Three *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) transcription factors, including *SPL7*, *14* and *17* are necessary for suppressing bract outgrowth and promote inflorescence branching (Wang et al., 2021c). In a triple *spl7 spl14 spl17* mutant, several vegetative shoots develop at positions normally occupied by bracts, replacing the primary branch meristems and indicating that vegetative development extends into the reproductive stage, if not properly blocked. Expression of *SPLs* is regulated by micro RNAs *miR156* and *miR529* at the post-transcriptional level and their ectopic expression mimics the phenotypes of the *spl7 spl14 spl17* mutant (Jiao et al., 2010; Miura et al., 2010; Wang et al., 2021c). Since *miR156/529* act as intrinsic time rulers, creating a spatiotemporal gradient that controls developmental shifts both during vegetative and reproductive growth, the module has a central position in the network controlling transition to inflorescence development. However, how *miRs/SPLs*-mediated suppression of vegetative development interacts with the FAC-dependent promotion of reproductive development is unclear. One possibility is that the two pathways act independently. More likely, florigen signalling might interact with the *miRs/SPLs* module to block vegetative growth, while reproductive meristems are being established (Figure 4). This perspective is supported by the finding that *spl9* mutants have marked

reduction of *RCN1* expression at the apex (Hu et al., 2021). Yet, whether other *SPLs* have a similar effect remains to be tested.

Another point of connection between the SPLs and florigens pathways is at the level of regulation of *OsMADS34*/*PAP2* expression. The florigens and *SPL14* promote its transcription whereas *miR156* reduces it (Kobayashi et al., 2012; Wang et al., 2015). The balance between the two activities certainly impacts on panicle development. In fact, mutations in *OsMADS34* convert panicle branches into shoots (in combination with *osmads14*/*15* and *18* mutants, as described above), increase the number of rachis branches and fails to specify spikelets, which retain vegetative characters (Gao et al., 2010; Kobayashi et al., 2010). Overall, the mutation is unable to establish spikelet meristem identity and prolongs indeterminate growth of the panicle. These phenotypes are partly shared with those of *spl* or florigens mutants and *miR156* overexpressors, suggesting that, despite individual differences, these pathways balance vegetative *vs* reproductive development and determinate *vs* indeterminate growth.

An additional balancing mechanism involves the antagonism between florigens and RCNs. When RCNs reach the SAM, their structural identity with the florigens leads to competition for the formation of FRCs at the expenses of FACs (Kaneko-Suzuki et al., 2018). Mutations in *RCNs* reduce panicle branching and spikelet number, whereas their overexpression causes hyper ramification (Nakagawa et al., 2002; Kaneko-Suzuki et al., 2018). These phenotypes are opposite to those displayed by florigens mutations or overexpression (Tamaki et al., 2015; Giaume et al., 2023). Thus, while florigens control development towards spikelet differentiation, whose direct effect is reduction of branching, RCNs antagonize this trajectory and the resulting equilibrium shapes inflorescence architecture. Artificial modulation of the two opposing forces, by means of genetics, might be of interest for yield increase, if hyper ramification, increase of spikelet number and floret fertility could be associated on the same varieties.

Among *RCNs*, at least *RCN4* is a downstream direct target of OsMADS34/PAP2 and its paralog OsMADS5 (Zhu et al., 2022). Single *rcn4* mutants do not affect branching, likely because of redundancy with *RCN1-3*, but partially rescue the hyper ramification of *osmads34* mutants (Nakagawa et al., 2002; Zhang et al., 2005; Zhu et al., 2022). Thus, RCNs could be placed both at the VM-to-IM transition and at the PBM-to-SM transition as negative regulators, downstream of *OsMADS34*/*PAP2* and *SPLs*.

Finally, one last level of balance is provided by *Delayed Heading Date 4* (*DHD4*) encoding a CONSTANS-like transcription factor (Cai et al., 2021). The DHD4 protein can interact with OsFD1 and competes with 14-3-3s to limit formation of the Hd3a/14-3-3/OsFD1 complex. Mutations in *DHD4* mildly accelerate flowering by inducing the expression of *OsMADS14* and *15* transcription factors. This competition provides a totally new perspective on contrasting forces at the apex because it involves a novel class of proteins not previously implicated as balancing signals (Figure 4).

Selection of flowering time genes during rice domestication and breeding

Flowering time is a trait of major applied interest because it affects two major aspects of rice cultivation: expansion to higher latitudes and adaptation to local environments. The LD regulatory pathway delays flowering (Figure 1), and mutations in major LD repressors accelerate the crop cycle, allowing rice to be cultivated at latitudes with shorter growth seasons (Shrestha et al., 2014). Mutations in *Hd1*, *Ghd7*, *Ghd8* and *PRR37* are widespread in both Asian and European germplasm and have been instrumental to bring rice up to 55°N in China and 45°N in Europe (Gao et al., 2014; Gómez-Ariza et al., 2015; Goretti et al., 2017). Most European varieties share a high degree of genetic similarity with varieties from northern China and mutant alleles of LD repressor genes are largely shared by both germplasms (Cai et al., 2013). Likely, expansion to northern China followed domestication and preceded spread of the crop to Mediterranean Europe. Thus, a common pool of flowering time alleles are under continuous selection by breeders in different areas of the globe (Zhao et al., 2011). Among them, *hd1* mutant alleles are particularly abundant, probably because they confer an adaptive advantage in cultivation also under SD. In tropical regions, functional *Hd1* promotes flowering, shortening the cycle to the extent that varieties would not take advantage of the entire growing season, with severe yield penalties (Kim et al., 2018). An exception to this general rule is represented by varieties harboring functional *Hd1* but non-functional *RFT1*, which are found only in *indica* germplasm cultivated at lower latitudes (Ogiso-Tanaka et al., 2013).

Despite the major effect of single mutations on flowering, loss-of-function alleles of LD repressors are rarely found alone, and, in modern varieties, combinations of multiple mutant alleles are common. This feature could be a consequence of the breeding history of each variety, which is selected to have a flowering time whose cycle length perfectly matches the length of the local cropping season. Additive or epistatic effects depend on the molecular interactions described above, contributing to finely adapt cycle length (Figure 2). E.g., pyramiding of *ghd7* and *prr37* produces the strongest acceleration under LD, because it removes complexes independently repressing florigens expression, allowing access to the highest latitudes.

An additional element of variability which is important for breeding is represented by genes whose mutations have minor-effect on the phenotype. These are instrumental in fine tuning photoperiodic responses and adjusting flowering locally in addition to major-effect ones (Wu et al., 2013; Cai et al., 2021). Sequencing of wild and cultivated accessions belonging to all rice subgroups has uncovered the existence of large natural allelic variation at flowering time loci which can also account for latitudinal expansion (Zhao et al., 2011; Huang et al., 2012). The contribution to phenotypic diversity of several allelic variants has been defined with the use of chromosome segment substitution lines, where the effect of each allele can be unequivocally measured in an almost isogenic background, indicating that alleles don't necessarily fall in the extreme categories of fully functional or loss-of-function (Itoh et al., 2018). Rather, distinct haplotypes can confer varying degrees of photoperiod sensitivity, reflecting adaptation to several geographic areas. These reconstructions of the history of selection give insights about the trajectories of domestication and rice

subgroups differentiation. Several haplotypes are common to all subgroups and represent standing variation. This occurred to the major LD repressors including *Hd1*, *PRR37*, *Ghd7* and *Ghd8*. Other haplotypes arose after subgroup differentiation also taking advantage of introgression events and local genomic rearrangements (Fujino et al., 2010; Itoh et al., 2018). Thus, these studies can also reconstruct gene flow among subgroups and reveal the history of human selection during spread of rice to new environments. Further mining of natural variation will be key in the future to advance flowering time research.

Response of flowering time to variations in ambient temperature

Expansion of cultivation to higher latitudes has exposed rice to lower ambient temperatures during the cropping period. Phenotypic plasticity and artificial selection adjusted the flowering response and adapted rice to the new environments.

Lower ambient temperatures delay flowering under both LD and SD (Luan et al., 2009). In an excellent field study performed across nine LD environments, Guo *et al*. showed that an environmental index derived from temperatures at the early growth stage of rice had a perfect negative correlation with flowering time of a biparental mapping population. Genetic mapping of loci responsible for adaptation of flowering time demonstrated that variation at *Hd1*, *PRR37*, *Ghd8* and *Hd6* accounted for phenotypic variation (Takahashi et al., 2001; Guo et al., 2020). Extending the statistical treatment of environmental data to the 3000 genomes collection allowed to distinguish accessions based on sensitivity of flowering time to temperature change. Accessions with higher sensitivity tended to be distributed to higher, colder latitudes, whereas accessions with lower sensitivity were the majority in equatorial regions. This study showed that temperature can be used as effective predictor of rice flowering time and that genes of the photoperiod pathway mediate between the induction of flowering and ambient temperature perception (Guo et al., 2020). Thus, the LD photoperiod pathway operates also as ambient temperature flowering pathway.

The effect of *Hd1* as LD repressor is enhanced at lower ambient temperature while the *hd1* mutant strongly reduces sensitivity of flowering to changes in temperature (Luan et al., 2009; Nagalla et al., 2021). A similar effect has been observed for *Ghd7* (Nagalla et al., 2021). *PRR37* has opposite effects across a temperature range. When mean ambient temperatures fall below a critical threshold, *PRR37* represses flowering, whereas it reverts to promoter of flowering at higher temperatures (Guo et al., 2020).

Phytochromes act as thermosensors and integrate temperature information into developmental mechanisms (Jung et al., 2016). The reversion of the active Pfr form into its ground Pr state occurs more slowly during the night when temperatures are lower. In rice, *PhyB* enhances the repressor activity of Ghd7 at lower ambient temperatures, consistent with the idea that temperature perception mediated by PhyB is integrated in the flowering network via Ghd7 (Nagalla et al., 2021). Since PhyB interacts with Ghd7 to promote its degradation (Zheng et al., 2019), it could be speculated that at lower temperatures, this mechanism is impaired and that Ghd7 persists in the plant to delay flowering.

Hormonal control of flowering

Of the several pathways that control flowering in plants, hormonal ones are very important only in some species (Blazquez and Weigel, 2000; Trusov and Botella, 2006; Galvão and Schmid, 2014). The role of hormones in rice flowering time has not been extensively studied, and most evidence indicate that the photoperiodic pathway might be the only relevant one. Nonetheless, some hormones can affect flowering, and most importantly shape panicle architecture upon reproductive commitment.

Auxin

The only link between auxin signaling and flowering time is at the level of *OsmiR393*, which targets the auxin receptor homologs OsAFB2 and OsTIR1 (Xia et al., 2012). The overexpression of *OsmiR393* causes early flowering, although it is not clear which genes of the flowering network are responsible for the phenotype and how.

Upon floral commitment, activity of the *DR5:VENUS* auxin reporter has been observed in all panicle meristems and in the developing vasculature of the inflorescence. Moreover, auxin polar transporters colocalize with the reporter during flower formation, and supposedly provide positional information for flower primordia initiation (Yang et al., 2017). Mutants with abnormal auxin content display panicle phenotypes, including anomalous size of the panicle, branching defects and spikelets with altered organ identity (Yoshikawa et al., 2014).

Gibberellins

The role of gibberellins (GAs) as promoters of flowering is well established in Arabidopsis and other species, in which they act at the SAM to induce expression of floral integrator genes (Thomas and Vince-Prue, 1997; Reeves and Coupland, 2001; Eriksson et al., 2006). However, there is poor evidence on the influence of gibberellins on flowering in rice. Treatments with GAs do not modify flowering time, albeit this does not exclude a role in the process. Also, it is unclear whether endogenous (or exogenous) GAs can reach the SAM, since just underneath the apical dome there is a ring-shaped area of expression of *GIBBERELLIN 2-OXIDASE 1* (*GA2OX1*) that is responsible for the inactivation of bioactive gibberellins. The expression of *GA2OX1* decreases drastically upon floral induction, indicating that the SAM could become accessible to GAs during reproductive development (Sakamoto et al., 2001). Overexpression of *GA2OX1* delays flowering in transgenic rice, but this phenotype could be part of a more general and pleiotropic 'GA deficiency syndrome' unrelated to flowering time control (Sakamoto et al., 2003).

Another indirect link between flowering and GA signaling is offered by *Heading date 16*/*Early Flowering 1*/*Casein Kinase I* (*Hd16*/*EF1*/*CKI*, hereafter *CKI*). Allelic variants with reduced activity or knock-down mutants cause early flowering. *CKI* encodes a kinase that phosphorylates the rice DELLA protein SLENDER

RICE 1 (SLR1), thus stabilizing it. Unstable SLR1 could be the cause for the early flowering phenotype (Dai and Xue, 2010). However, CKI phosphorylates also the floral repressors Ghd7 and PRR37 and this modification might be essential for their activity, thus explaining earliness of *ckI* mutants (Figure 3) (Hori et al., 2013; Kwon et al., 2015).

Cytokinins

Cytokinins affect both panicle formation and floral induction. A lack of cytokinin has been associated with a small SAM and abortive inflorescence meristems, leading to smaller panicles with reduced branches (Kurakawa et al., 2007; Ding et al., 2014; Wu et al., 2017; Du et al., 2017a; Song et al., 2018). Conversely, increasing cytokinin content in the inflorescence meristem leads to formation of a highly branched panicle and increases yield (Ashikari et al., 2005).

An elegant model that links cytokinin dynamics to flowering time control has been recently proposed (Cho et al., 2022). Cytokinin signaling is mediated by type-A and -B Response Regulators (RR), and Ehd1 is a type-B RR. Ehd1 works as a homodimer; however, its homodimerization is inhibited by type-A RRs OsRR1 and OsRR2 (Cho et al., 2016). Transcription of *OsRR1* and *OsRR2* increases in response to cytokinin during the vegetative phase. Their cognate proteins can then bind and inactivate Ehd1, reducing transcription of *Hd3a* and *RFT1* and delaying flowering (Cho et al., 2022). During floral commitment, a reduction in cytokinin levels reduces transcription of type-A RRs releasing Ehd1 inhibition and florigens expression.

Abscisic Acid

The effect of abscisic acid (ABA) on flowering mostly relates to its role as environmental stress hormone. The perception of ABA depends upon a group of proteins belonging to the PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-like (PYL)/REGULATORY COMPONENTS OF THE ABA RECEPTOR (RCAR) family (hereafter PYLs), which are essential to transmit the ABA signal (Ma et al., 2009; Park et al., 2009). The rice genome encodes for 13 PYLs belonging to two distinct groups. In a landmark study, Miao et al. showed that different combinations of *pyl1*, *2*, *3*, *4*, *5*, *6* and *12* mutants, belonging to group I, delay flowering to various extent (Miao et al., 2018). Since the same mutations also decrease sensitivity to ABA, a possible interpretation suggests that ABA can promote flowering. This concept is supported by studies with ABA biosynthetic mutants. Disturbing its endogenous levels with both knock-out and overexpressors of the ABA biosynthetic gene, *MAO HUZI 4* (*MHZ4*), causes lateness (Ma et al., 2014). This effect might depend also upon interactions with the ethylene pathway because *mhz4* mutants abolish ABA biosynthesis but enhance ethylene emission. Delayed flowering has been observed also in ABA biosynthetic mutants of Arabidopsis in which the relationship between ABA signalling and flowering regulation has been more thoroughly explored (Martignago et al., 2020).

ABA is antagonistic to GAs in several physiological processes and connections between the two pathways determine the proper hormonal balance. *OsAP2-39* encodes a transcription factor of the APETALA2 family that can directly activate the expression of the 9-cis-epoxycarotenoid dioxygenase *OsNCED-1* (an ABA biosynthetic gene) and increase the level of enzymes responsible for GAs inactivation/degradation. Overexpression of *OsAP2-39* causes a late flowering phenotype that could be recovered by exogenous gibberellins, supporting the idea of GAs as promoters of flowering (Yaish et al., 2010).

Brassinosteroids (BR)

The first evidence of a connection between brassinosteroids (BR) and flowering came with the finding that SDG725, a H3K36 methyltransferase essential for expression of genes involved in BR biosynthesis and signalling, can also affect flowering. In fact, its knockdown leads to a typical BR deficiency phenotype and late flowering. SDG725 promotes flowering by methylating several genes, including *Ehd3*, *Ehd2*, *OsMADS50*, *Hd3a* and *RFT1* (Sui et al., 2013).

More recently, BRASSINAZOLE-RESISTANT 1 (OsBZR1), a positive regulator of BR signalling, has emerged as integrator of flowering time control. OsBZR1 interaction with OsMED25, mediating the recruitment of the RNA polymerase to promote transcription, is essential for OsBZR1 to properly carry out its role in regulating the expression of BR-responsive genes (Ren et al., 2020). Knockdown of *OsMED25* reduces *Ehd1*, *Hd3a* and *RFT1* expression and causes late flowering. Histone deacetylase HDA703 was also identified as interactor of OsBZR1 and promoter of flowering. OsBZR1 binding motifs present in the *Ghd7* promoter recruit the dimer and activity of HDA703 represses its transcription by histone deacetylation, leading to flowering promotion (Wang et al., 2020b).

Ethylene

The *OsETR2* gene encodes for an ethylene receptor expressed in SAM and panicle. When overexpressed, it reduces ethylene sensitivity and causes late flowering, while its knockdown shows the opposite phenotypes. The authors proposed that OsETR2 can delay the floral transition by increasing the transcription of *OsGI* and *RCN1* (Wuriyanghan et al., 2009). Given the interactions of the ethylene pathway with the GA and ABA pathways, the effect on flowering time might be due to more complex interactions between hormones, rather than on single ones (Kuroha et al., 2018).

OsCTR2 is suggested to be a negative regulator of ethylene signaling, but its effects on flowering time are difficult to interpret, since both overexpressor and knockdown lines displayed delayed flowering (Wang et al., 2013). These observations are paradigmatic of the difficulty in studying the dependency of flowering upon hormonal pathways, given their numerous and complex interconnections.

Flowering time under stress conditions

Although transition to flowering is mostly determined by the interaction between the photoperiod and the allelic composition at flowering time loci, other environmental parameters, including abiotic stresses, can modify it. All external stressors eventually converge on transcriptional regulation of *Ehd1*, *Hd3a* and *RFT1*, thus acting as integrators of multiple signals.

Drought stress

A considerable fraction of rice cultivations depends upon rainwater and is therefore subject to fluctuations in water availability. Even when grown in paddy fields, extreme weather events linked to climate change can compromise water supply, imposing drought stress (Figure 5).

Time to flowering can respond in two opposite ways to drought, either decreasing, a response known as drought escape (DE), or increasing. The final effect depends upon the severity of drought. A mild water deficit triggers DE, and earlier flowering is instrumental to complete the life cycle before the stress becomes too severe (Weng et al., 2014; Du et al., 2018; Groen et al., 2020). Conversely, severe drought threatens plant survival, and the flowering delay avoids entering the delicate and energy-consuming reproductive phase (Galbiati et al., 2016; Zhang et al., 2016; Wang et al., 2020a).

In DE, ABA levels increase and induce expression of *bZIP23* which acts as positive regulator of the DE response. *bZIP23* feeds back on the regulation of flowering time genes, inducing transcription of *OsTOC1*, *Ehd1*, *Hd3a* and *RFT1*, while reducing that of *Ghd7* (Du et al., 2018). Genetic analyses indicate that mutations in *PRR37*, *GI* and *EARLY FLOWERING 3* (*ELF3*) delay flowering under mild water deficit compared to wild type controls, showing impaired DE response, and that this happens independently of ABA. The expression of *Ehd1*, *Hd3a* and *RFT1* correlates with flowering time of the mutants. Thus, components of the photoperiod pathway are integrated with DE responses in a complex manner, only partly dependent upon ABA (Weng et al., 2014; Du et al., 2018) (Figure 5). Downstream of florigens, the *OsMADS18* transcription factor has been identified as strongly induced during drought as additional integrator of DE, consistent with the flowering promotive role of MADS box genes at the end of the photoperiodic cascade (Fornara et al., 2004; Kobayashi et al., 2012; Groen et al., 2020).

The flowering delay caused by severe drought is also ABA-dependent but proceeds through a different molecular mechanism. High ABA levels induce expression of the *OsABF1* bZIP transcription factor, a flowering repressor (Zhang et al., 2016). Reducing its expression by RNAi accelerates flowering also under drought stress and induces *Ehd1*. The activity of *OsABF1* depends upon *OsWRKY104* creating an ABA-dependent floral repressive module. Drought stress and ABA also induce expression of *RCN1*, with *rcn1* attenuating the flowering delay caused by stress (Wang et al., 2020a). It remains to be determined if and how the *OsABF1* and *RCN1* dependent mechanisms are integrated and in which tissue. Given that RCNs can form floral repressor complexes with bZIPs, an intriguing possibility is that OsABF1 and RCN1 interact to delay flowering when plants experience severe drought (Figure 5).

Salt stress

Rice cultivation in river deltas is threatened by salinization of soils, occurring when natural events return seawater into the fields. A well described suite of protective mechanisms is activated in response to increasing salinity. Yet, the connections between salt stress and flowering time control are just starting to be explored and indicate that circadian clock components are preferential integrators of these pathways. The Evening Complex (EC) is a central feature of the circadian clock, assembled by LUX ARRHYTHMO, ELF3 and ELF4, and binding to DNA to repress gene expression (Silva et al., 2020). The rice genome encodes for two orthologues of *ELF3* and three of *ELF4*. The *oself4a*, *oself3-1* and *oslux* single mutants are hypersensitive to salt stress, showing reduced survival rates if grown at high concentrations of NaCl (Wang et al., 2021d). Additionally, under SD *oself4a* mutants flower late while single *oslux* and double *oself3-1 oself3-2* never flower (Wang et al., 2021d; Andrade et al., 2022). These observations point to the EC as an integrator of flowering and salt stress signals. Direct targets of the EC include several *PRRs* as well as *OsGI*. OsELF4a, OsLUX and OsELF3-1 can bind the *OsGI* promoter to repress its expression. Mutations in *OsGI* increase rice survival rates upon salt or osmotic stress treatments, increase the concentration of osmoprotectants in leaves, including proline and sucrose, and induce earlier flowering under LD (Li et al., 2016; Wang et al., 2021d). Thus, the EC-OsGI module fine tunes salt tolerance and promotes flowering, representing an interesting target for breeding efforts.

Temperature stress

Rice plants are sensitive to temperature variations, particularly during flowering and grain filling. A 1°C increase in the minimum night temperature is correlated to yield reductions of 10% (Peng et al., 2004). High temperatures can induce early flowering and reduce yield, while low temperatures delay flowering, indicating that temperature and day length measurements coordinately control the reproductive transition. In both cases, temperature perception conveys on transcriptional regulation of *Ehd1* and the florigens (Luan et al., 2009; Chen et al., 2018b). The *qHd1* QTL is a plausible candidate to be part of a, still unexplored, rice thermosensory pathway. Genetic variation at *qHd1* partly explains phenotypic variation of heading dates at high ambient temperatures. The Zhenshan 97 allele of *qHd1* maintains stable heading dates even upon mean temperatures increases. Heading date stabilization is observed when plants are grown at different temperatures but under the same day length, indicating that photoperiod and thermosensory pathways are genetically separable (Chen et al., 2018b). The causal gene underlying *qHd1* has not been precisely mapped yet but the *OsMADS51* transcription factor is a strong candidate. An insertion in the first intron in Zhenshan 97 represents a functional polymorphism, reducing transcription of *OsMADS51*, compared to varieties without insertion. The transcription of *OsMADS51* downstream targets *Ehd1*, *Hd3a* and *RFT1* is also reduced, explaining the flowering delay, particularly at high temperatures.

Functional validation of temperature responses using *osmads51* mutants is still missing. However, syntheny relationships and functional data from temperate grasses suggest that the monocot OsMADS51 clade includes orthologs of FLOWERING LOCUS C (FLC), a major controller of vernalization responses (Ruelens et al., 2013). It is thus tempting to speculate that *OsMADS51*-like genes regulate temperature-dependent flowering, and that in rice, which is missing a vernalization pathway, they have subfunctionalized to control a high ambient temperature flowering pathway.

Nutrients availability

Maximizing yields requires optimal fertilization. Different nutrients have been shown to influence flowering. While supply of K and P accelerates flowering, low or high N fertilization delays it (Ye et al., 2019; Zhang et al., 2021). The *N-mediated heading date 1* (*Nhd1*) gene encodes for a MYB transcription factor whose expression is induced upon N fertilization (Zhang et al., 2021). In the *nhd1* mutant, flowering is delayed under both SD and LD, and transcription of *Hd3a* is reduced. Since NHD1 directly binds to the promoter of *Hd3a*, it lies at the interface between N perception and flowering.

Concluding remarks and future perspectives

In this section we briefly indicate trajectories that we believe should be pursued for advancing flowering time research in its basic and applied facets.

Gene cloning and further refinement of GRNs. More flowering time genes are still to be cloned in the future and placed in GRNs. For these, as well as for many known regulators, precise positioning needs to be thoroughly determined. While expression analyses provide a first mean of placement in the network, more refined genetic analysis can be laborious and time consuming, yet necessary to define complex relationships.

Understanding protein abundance and activity. Transcriptional data are relatively straightforward to produce and sufficient to build GRNs. However, full understanding of network activity will come only after studying regulation at the post-transcriptional level. Protein abundance, modifications, interaction patterns can depend upon day length and be largely independent from transcription. Thus, the study of gene x environment interactions has to determine these features, also for the benefit it can bring to breeding.

Quantitative integration of information. The complex interconnection of genes in GRNs makes it difficult to predict how perturbation of gene activity will impact on the phenotype. This is particularly evident when trying to make quantitative predictions. To this end, in silico models can become a useful tool both for scientists and breeders. Initial models made use of quantitative analyses related to few major regulators to assess latitudinal adaptation, predicting florigens expression and flowering responses (Qiu et al., 2021). The power of these models can increase by integrating more genes, including minor controllers, and by refining algorithms with expression data collected in more environments.

Exploitation of basic understanding for applied purposes. Finally, all the above is useful to guide better breeding, driving selection with molecular rather than phenotypic data, and quickly tailoring new varieties to cultivation environments, possibly also with the use of gene editing technologies. This will be the most daunting task, requiring tight and constructive interactions between scientists and breeders.

Figure legends

Figure 1. Gene regulatory networks controlling rice photoperiodic flowering. The networks represent the transcriptional relationships taking place under LD and SD. Regulatory signals ultimately converge on Ehd1 and florigens transcription. Genes indicated in purple act as flowering inhibitors while green ones act as promoters. Genes indicated in bold have stronger impact on flowering time, as inferred from the effect of the corresponding loss-of-function mutant. Some positive and negative regulators of Ehd1 and Ghd7 have been grouped in boxes, to simplify graphical representation. Arrows and flat-end arrows indicate transcriptional activation and repression, respectively. Light interaction with gene expression is indicated with lightning signs.

Figure 2. Diurnal accumulation patterns of major flowering regulators under LD (boxes on the left) and SD (boxes on the right) show the central position of Ghd7 in the External Coincidence Model for rice flowering. The peak of GI transcription tracks dusk under LD and SD. GI protein interacts with Ghd7 and contributes to its degradation in a 26S proteasome-dependent manner. Transcription of Ghd7 is sensitive to red light with a gate of inducibility (red shading) occurring during the morning under LD. The gate shifts to the night under SD, yet while few publications report reduced transcription under SD, a larger consensus indicates a transcriptional peak in the morning, not different from the one detected under LD. Irrespective of transcription, Ghd7 protein does not accumulate under SD, or in phyB mutants, while shows reduced accumulation in GI overexpressors. Thus, light and photoperiod-dependent regulatory layers determine Ghd7 abundance. Ehd1 expression is gated in the morning by blue light signals (blue shading). OsGI can induce Ehd1 transcription under SD, when not antagonised by Ghd7 protein. The diurnal profile of Ehd1 transcription is also determined by Hd1 and PRR37 that promote its expression under SD and repress it under LD. Finally, Hd3a and RFT1 are transcribed under SD as a combination of Hd1 and Ehd1-mediated induction. Under LD, florigens expression is repressed by Hd1 and induction by Ehd1 is limited. Eventually, RFT1 escapes repression under LD, and is transcribed to promote flowering. Continuous and dashed lines indicate protein and mRNA accumulation patterns, respectively. A clock symbol indicates the gene is under circadian clock control.

Figure 3. Post-transcriptional levels of regulation in the flowering time network. We identified four hubs corresponding to Hd1, PRR37, Ehd1 and the florigens. A, Hd1 hub. Hd1 forms Hd1/NF-Y complexes that

directly repress florigens expression under LD. Repression is released in SD and Hd1 becomes an activator. Hd1 stability depends by HAF1 and by components of the autophagy pathway, including ATG proteins, in the vacuole. Hd1 can be phosphorylated by OsK4 and this modification might impact on Hd1 stability. B, OsPRR37 hub. OsPRR37 can replace Hd1 in a NF-Y complex and repress florigens expression under LD. It can be phosphorylated by CKI and CKIIα. Phosphorylation might affect PRR37 stability or activity. C, Ehd1 hub. Ehd1 is repressed under LD by the Ghd7/Hd1 and OsRE1/OsRIP1 complexes. Phosphorylation is essential for Ehd1 dimerization and activity. OsRR1 interacts with Ehd1 to form an inactive complex and inhibit its capacity to induce expression of the florigens. Phosphorylation of Ghd7 by CKI enhances its repressor activity. D, florigens hub. Activity of the florigens depends on their transport in the phloem which takes place by physical interaction with OsFTIP proteins and OsTPR075. Proteins are indicated by ovals and genes by rectangles. Names of DNA motifs bound by proteins or protein complexes are indicated below the double helix. Red and blue arrows indicate LD and SD regulation, respectively. Dashed arrows/flat-end arrows indicate transcriptional activation/repression. Continuous arrows+P indicate phosphorylation. Continuous flat-end arrows indicate protein degradation.

Figure 4. Balancing signals during the meristematic switch to reproductive growth. Meristems on top represent the approximate stages during which molecular events represented below occur. A, The balance between SPLs and miR156/529 determines the branching pattern and the vegetative features of the inflorescence. B, Florigens transported from the leaves form FACs that induce transcription of MADS box genes and switch the developmental fate of the meristem. DHD4 competes with OsFD1 to bind Gf14 under LD. C, The reproductive switch is antagonized by FRCs, and RCNs transported from the leaves compete with the florigens for binding to Gf14s.

Figure 5. Gene regulatory networks controlling flowering under drought stress. A, a rice paddy field experiencing severe drought during summer 2022 in northern Italy. Drought has been hitting several countries in 2022. B, molecular network controlling *Ehd1* expression in response to mild and severe drought stress. Arrows and flat-end arrows indicate transcriptional activation and repression, respectively. Genes indicated in purple act as flowering inhibitors while green ones act as promoters. Green arrows indicate increased biosynthesis.

Acknowledgments

This work was supported by funding from the Italian Ministry of Foreign Affairs and International Cooperation, Italy-Japan bilateral collaboration on Agrifood #PGR10097.

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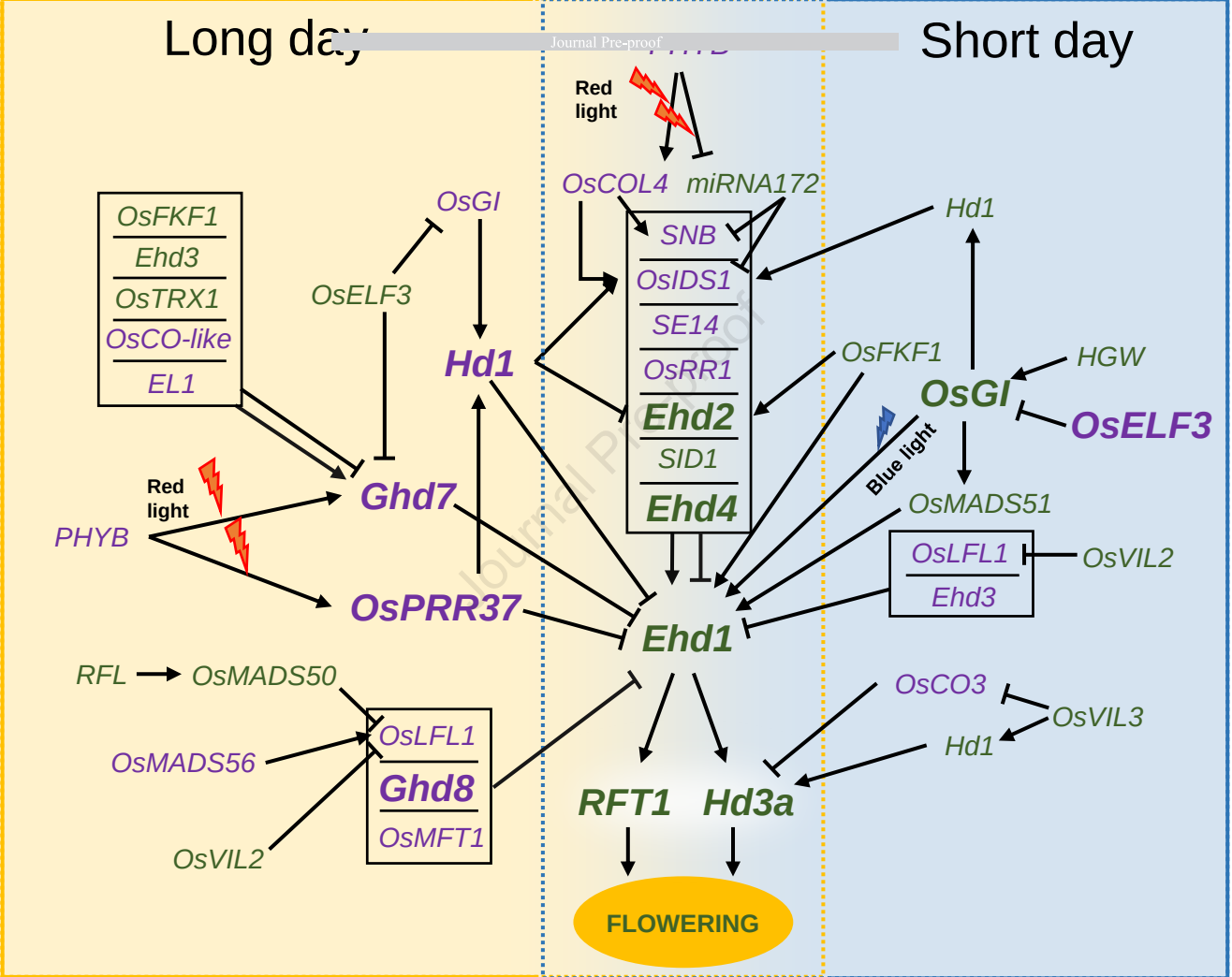
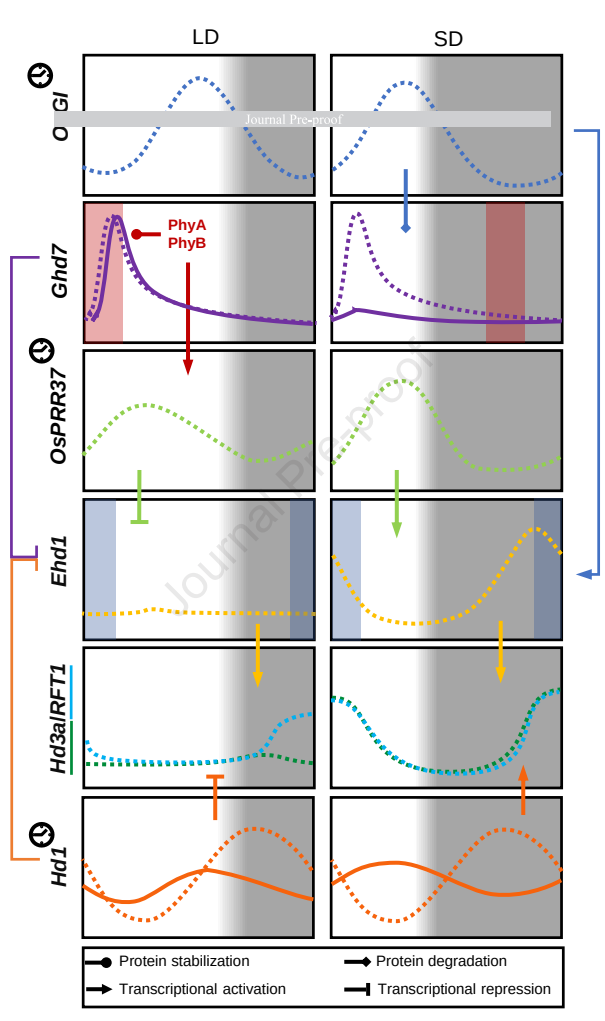
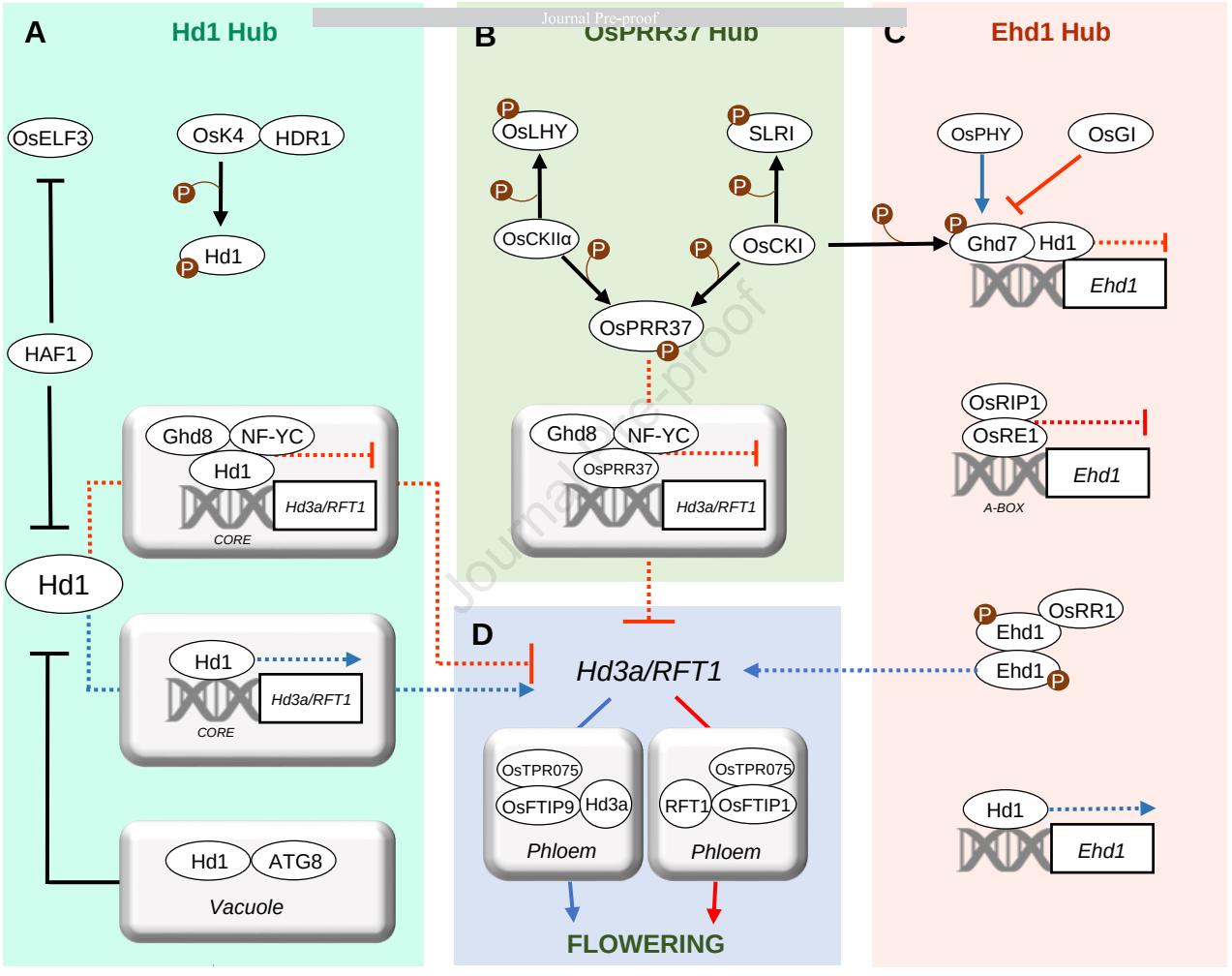
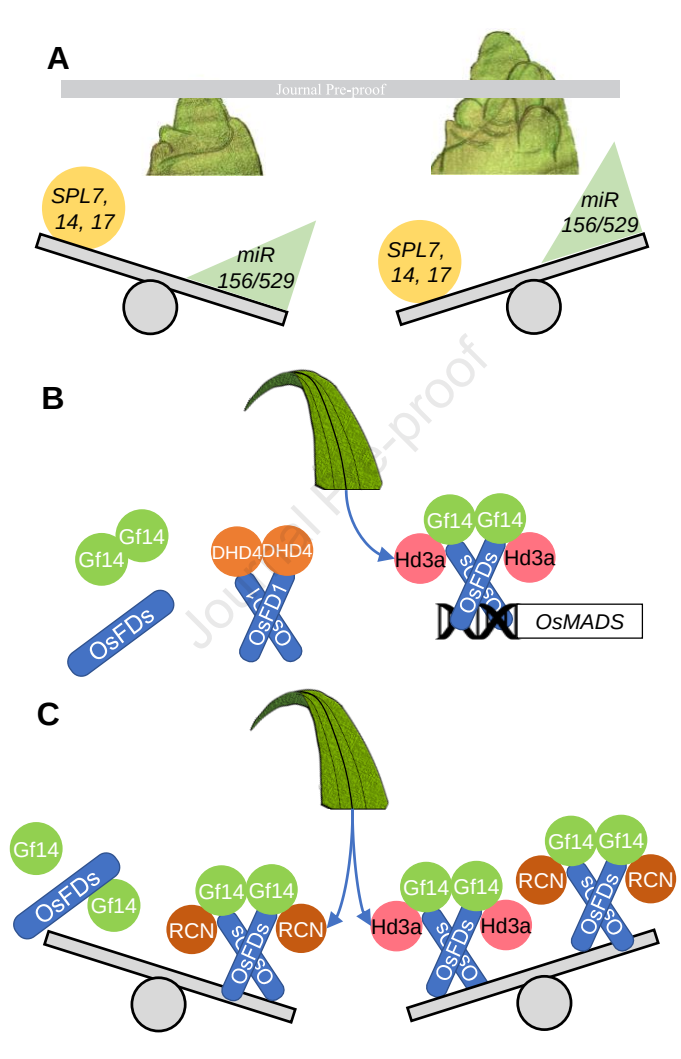



LD
SD
Journal Pre-proof
PhyA
PhyB
Ghd7
OsPRR37
Ehd1
Hd3a/RFT1
Hd1
Protein stabilization
Protein degradation
Transcriptional activation
Transcriptional repression





A

B