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A triple florigen system is essential for flowering and panicle architecture in rice

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1. ABSTRACTS

1.1 ENGLISH ABSTRACT

The transition from a vegetative phase to a reproductive one has always been deeply studied in plants and in particular in the cereals due to the relevance of this trait for seed yield. The transition occurs upon the arrival of a mobile flowering signal, the florigen, at the shoot apical meristem (SAM).
In rice (Oryza sativa), there are two different florigens: *Heading Date 3a* (*Hd3a*), that promotes flowering in short day (SD) conditions and *Rice Flowering Locus T 1* (*RFT1*) that promotes flowering in both short and long-days (LD). They are transcribed and translated in leaves once environmental conditions are optimal, and then transported through the phloem to the SAM. After reaching the shoot they form a heterohexameric complex named Florigen Activation Complex (FAC) binding to two 14-3-3 proteins and to two bZIP transcription factors and activate conversion of the shoot apex into an inflorescence by promoting expression of inflorescence-identity genes.

Here, we show that Hd3a and RFT1 arrival at the SAM activates *FLOWERING LOCUS T-LIKE 1* (*FT-L1*), encoding a third florigen-like protein showing atypical characteristics.

Expression data from qRT-PCR, in situ hybridization and transcriptional marker lines indicated that its mRNA is mainly expressed in the SAM and persists during all developmental stages of inflorescence development. Misexpression of *FT-L1* from meristem- and tissue-specific promoters indicates that only when expressed in the SAM, *FT-L1* has florigenic activity.

Isolation and analysis of EMS and CRISPR mutants showed that FT-L1 strengthens the effects of Hd3a and RFT1 during conversion of the vegetative meristem into inflorescence, in an addictive manner. Furthermore, it controls panicle branching by inhibiting secondary branching and promoting spikelet meristem differentiation.

Thus, we propose a triple florigenic module that promotes a balanced progression of inflorescence identity and determinacy.

1.2 ITALIAN ABSTRACT

Nelle piante ed in particolare nei cereali il passaggio dalla fase vegetativa a quella riproduttiva è sempre stato studiato a fondo per la rilevanza di questo tratto nella resa. La transizione è identificata dall'arrivo di un segnale mobile di fioritura, il florigeno, al meristema apicale del germoglio (SAM).
Nel riso (*Oryza sativa*) sono presenti due diversi florigeni: *Heading Date 3a (Hd3a*), che favorisce la fioritura in condizioni di giorno corto (SD) e *Rice Flowering Locus T 1 (RFT1*) che favorisce la fioritura in giorni sia corti che lunghi (LD). Vengono trascritti e tradotti nella foglia una volta che le condizioni ambientali sono ottimali e vengono trasportati attraverso il floema al SAM. Dopo aver raggiunto il meristema, formano un complesso eteroesamerico chiamato Florigen Activation Complex (FAC) che si lega a due proteine 14-3-3 e a due fattori di trascrizione bZIP e inducono la conversione dell'apice del germoglio in un'infiorescenza con la promozione dell'espressione dei geni correlati.

In questo lavoro mostriamo che l'arrivo di *Hd3a* e *RFT1* al SAM attiva *FLOWERING LOCUS T-LIKE 1 (FT-L1)*, che codifica per una terza proteina florigenica che mostra caratteristiche atipiche.

I dati di espressione da qRT-PCR, ibridazione in situ e marker line per analisi di attività trascrizionale hanno indicato che il suo mRNA è espresso principalmente nel SAM e persiste durante tutte le fasi dello sviluppo dell'infiorescenza. L'espressione di FT-L1 sotto promotori meristema e tessuto-specifici indica che solo quando quest'ultimo è espresso nel SAM ha un ruolo con attività florigenica.

L’identificazione e l'analisi dei mutanti EMS e CRISPR hanno mostrato che FT-L1 rafforza, in maniera additiva, gli effetti di Hd3a e RFT1 durante la conversione vegetativa in infiorescenza. Inoltre, esso controlla la ramificazione delle pannocchie inibendo le ramificazioni secondarie e promuovendo la differenziazione del meristema della spighetta.

Pertanto, proponiamo un triplo modello florigenico che promuove una progressione equilibrata dell'identità e della determinazione dell'infiorescenza stessa.

2. AIM OF THE THESIS

Flowering time in rice (*Oryza sativa*) is determined by the transition from vegetative to reproductive stage. Once the photoperiodic conditions are optimal together with internal signals the florigens are produced and a strong developmental reprogramming occurs.

Florigens *HEADING DATE 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) are produced in the vasculature of the leaf, are able to move through the phloem until reaching the shoot apical meristem (SAM) where they induce the transition to the reproductive phase by forming an heterohexameric complex called Florigen Activation Complex (FAC). At the SAM, *Hd3a* and *RFT1* are mainly responsible for committing the vegetative meristem to an inflorescence meristem fate through FACs that induce conversion of the shoot apex into a panicle by promoting expression of panicle-identity genes.

During my PhD I focused my attention on *Flowering Locus T Like* 1 that encodes a PEBP florigen-like protein and is the closest homolog of the florigen FT of Arabidopsis.
A transcriptional screen at the rice SAM indicated that FT-L1 expression is strongly induced at the SAM by photoperiod and florigens, during floral commitment.

The aim of my thesis is the molecular and phenotypic characterization of *OsFT-L1* during rice reproductive development, to define its role in the floral transition and panicle development.

To study its dependency upon florigens I evaluated its expression in single and double mutants *hd3a* and *rft1*. By mis-expressing it under different tissue-specific promoters I studied where FTL1 carries out its function and where its expression has biological significance. To further study its spatio-temporal expression profile I've used different approaches as *in situ* hybridization assays and transgenic transcriptional marker lines. To determine where the protein accumulates and if it operates in a cell-autonomous manner I have generated translational maker lines.
I have isolated three different mutant alleles in the Italian élite variety Volano, as well as developed CRISPR *ft-l1* single as well as *ft-l1 hd3a* and *ft-l1 rft1* double mutants in the Nipponbare variety. With this material, I assessed FT-L1 function in accelerating the transition from VM-to-IM and from SBM-to-SM.
Through different protein-protein interaction assays I investigated FT-L1 direct interaction with bZIP OsFD7, involved in the fine regulation of panicle development, and demonstrated a FT-L1–OsFD7 module to branching regulation via alternative branch-specific FACs.
Finally, after obtaining the CRISPR *spl14* and *spl17* loss-of-function and gain-of-function mutants, I validated FT-L1 transcriptional regulation by SPLs highlighting its function as an integrator of both the SPLs and photoperiodic pathways.

3. INTRODUCTION

3.1 *Oryza sativa* AS A MODEL SYSTEM

Rice (*Oryza sativa L.*) is an annual herbaceous plant belonging to Poaceae family. It has Asian origin being domesticated 10.000-13.000 years ago near the Pearl River in the south of China[1]. Rice has become staple food, meeting the ever-increasing demand in human food.

There are several species of rice but the most widespread and widely cultivated is *Oryza sativa* which is divided in five groups: *aromatica*, *aus*, *indica*, *temperate japonica* and *tropical japonica*[2]. The two main subspecies used are *indica* and *japonica*, both arising from two separate events of domestication from a common ancestral species, *O.rufipogon*[1].mac

Oryza sativa represents 95% of the rice grown worldwide and it is not surprising that the main rice producers in the world are Asian countries, China in particular. However, it is largely cultivated also in Europe, especially in Spain and Italy. Italy, indeed, is the largest European producer, harvesting rice from Lombardy, Piedmont, Veneto, Romagna and Sardinia[3].

The most common varieties of rice in Europe belong to the group of *temperate japonica*.

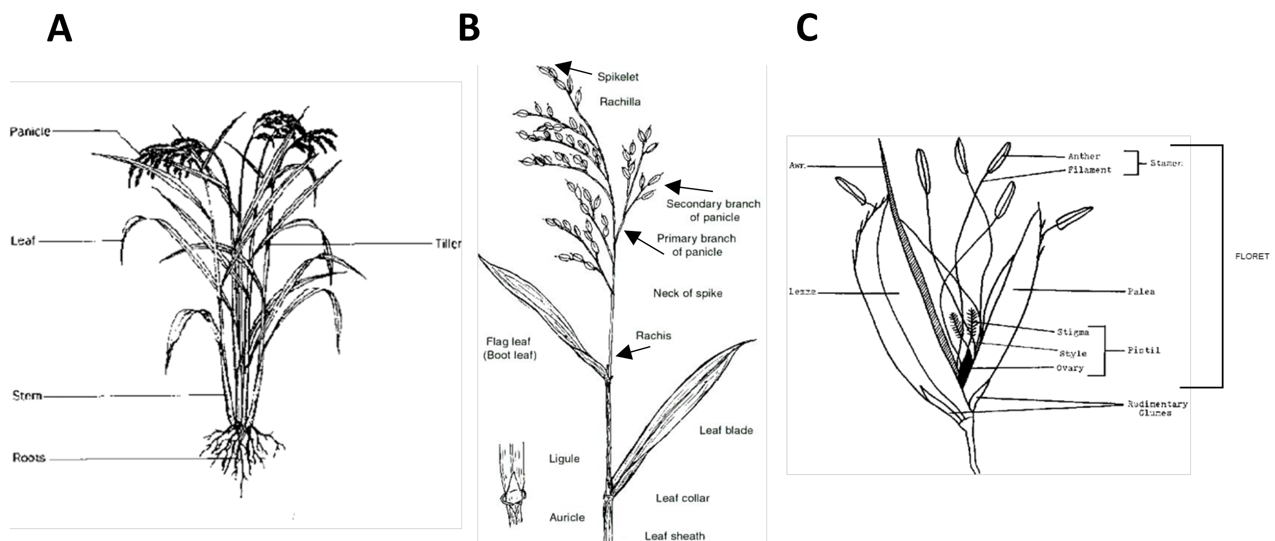

Figure 1 - Rice morphology diagrams.
A. Plant morphology, B. Panicle morphology, C. Spikelet morphology.
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3.1.1 Plant morphology

Rice is an annual monocot plant (fig.1A-C) that develops 60-80 cm height culms, with a remarkable system of gaps (aerenchyma) in the cortical part that also extends to the root system allowing gas exchange.

The leaves, up to 2 cm wide and several decimeters long, are linear and silicate. The inflorescence consists of an apical panicle formed by hermaphrodite flowers wrapped in two glumes. The fruit is a caryopsis.

The architecture of the plant is extremely important from an agronomic point of view, as it determines the adaptability of a plant to cultivation, its harvest-index and its potential yield.

The development of the plant could be divided into embryogenesis, vegetative phase and reproductive phase (fig.2).

The vegetative phase, the longest, is the one in which leaves and tillers are developed. During tillering, at the basal nodes of the main stem, axillary meristems are formed and they grow to form lateral stems resembling the main stem structure. During this phase, the stem and the internodes also grow in number and size by exploiting meristematic cells deriving from the Shoot Apical Meristem (SAM), the vegetative meristem formed during embryogenesis.

When environmental conditions and internal factors become favorable, the reproductive phase can start, normally after 10 or more leaves are produced.

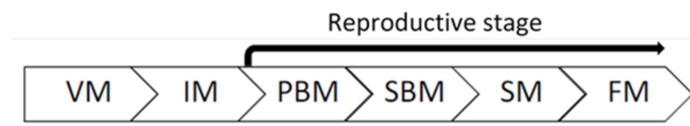


Figure 2 - Meristem developmental stages. *VM: Vegetative Meristem; IM: Inflorescence Meristem; PBM: Primary Branching Meristem; SBM: Secondary Branching Meristem; SM: Spikelet Meristem; FM: Floret Meristem.*

In the shift from vegetative to reproductive stage each lateral culm generates the panicle, which is supported by the highest internode of the culm. In this stage, the vegetative meristem (VM) is converted into an inflorescence meristem (IM) that will generate sequentially new meristems.

These new meristems are: primary and secondary branch meristems (PBM and SBM), then spikelet meristems (SM) with spikelets composed of florets and glumes, and, in the end, floret meristems (FM), with flower organs[4]. Rice flowers are named florets (fig.1C), and they consist of reproductive organs as six stamens and one central pistil (forming the fertile part) and of one lemma, one palea and two lodicules (forming the sterile part). Contrary to most of the Gramineae, rice fertilization occurs with closed flower, particularly in cultivated varieties which are therefore mainly self-pollinating.

When the florets are fully developed, plants can flower with the inflorescence emerging from the last leaf, called flag leaf.

Panicle architecture is mainly regulated by the moment of transition between each meristem type and it must be, therefore, finely regulated since any delay or defects in specification of a meristem identity would cause aberrancy in further meristem determinacy.

During the reproductive phase, a rapid elongation of the internodes occurs to bring the mature panicle out of the culm.

3.1.2 Panicle Morphology

As previously mentioned, panicle's structure is widely responsible for yield. From a morphological point of view (fig.1B), the inflorescence meristem develops from a main axis, called rachis, forming bracts, whose outgrowth is suppressed during development as a marker of reproductive transition[5]. During the

reproductive stage, the rachis aborts and the lateral organs begin to develop directly from it. Branches primordia called primary (PBM) are lengthened, and then the secondary branches (SBM) form on the primary branches in the basal part of the meristem. There could be also higher order branches, as tertiary branches, which start from secondary ones, and so on. Then branch meristems develop spikelet meristems (SM) with the production of glumes to indicate the final transition to a spikelet determination. Finally, SMs are converted to a floret meristem and floret reproductive organs formation begins[6].

A mature panicle is made up of 10 or more primary branches, bearing approximately 150 spikelets in total[7].

3.2 PHOTOPERIOD AND FLOWERING

Flowering induction is one of the major physiological changes that determines the transition from vegetative to reproductive development in most plants.

In cereals, the flowering time is also called heading date referring to the heading moment when the panicle is extruded from the flag leaf to expose the mature flowers[7].

To optimize reproductive success and ensure sufficient seed numbers for species propagation, the flowering period must be tightly regulated through the integration of external inputs such as day length, temperature, water and nutrient availability and internal signals such as developmental stage or the ability to produce certain hormones called florigens[8].

Plants are classified into three categories based on their dependency upon the photoperiod for flowering, which could be more or less stringent. Long-day (LD) plants, flowering when night length falls below a certain threshold, such as *Arabidopsis thaliana;* short-day (SD) plants, which flower when night length exceeds a certain threshold, such as *Oryza sativa* and day-neutral (ND) plants, which flower independently from the photoperiod.

Nowadays, rice grows in a wide range of latitudes from 55°N to 36°S, thanks to a diversification of the flowering time and sensitivity to photoperiod. Rice, indeed, can flower both under SD, or under LD taking more time. This last condition has been promoted also by artificial selection, following the human domestication and expansion of the cultivation area. For its dual capability to flower under both SD and LD, rice is classified as a facultative SD plant[9]. Since fine-tuning photoperiod sensitivity has been a key strategy to allow expansion of rice cultivation to higher latitudes[9], rice must have an optimal response to the photoperiod, managing to measure the hours of light and dark of each season. In fact, it is able to perceive the hours of light and darkness present in the day, thanks to photoreceptor proteins[10], and consequently regulates flowering.

This high sensitivity to light or dark is explained by the External Coincidence Model of Photoperiodism that integrates day/night length measurement and circadian clock activity. According to this model, each species has an innate circadian clock that imposes a 24h rhythm on many biological processes[11].
For SD species such as rice, the External Coincidence Model postulates that when the dark period coincides with a sensitive phase of the circadian cycle, marked by the expression of regulatory proteins, flowering is accelerated. Conversely, if the sensitive phase falls during a period of light, flowering would be inhibited.

For LD species such as *Arabidopsis thaliana*, the External Coincidence Model imposes an opposite sensitive phase of the circadian cycle for flowering. Indeed, flowering of these species is promoted when a sensitive phase of the circadian cycle falls during exposure of the plant to light.

The main flowering activators setting the sensitive phase of the circadian clock in rice are *Heading Date 1 (Hd1*) and *Early Heading Date 1 (Ehd1)* while in Arabidopsis is *Constans (CO).* These master regulators receive the information relating to day-length and translate it into a molecular signal that stimulates inflorescence formation.

Constans (CO)

Constans (CO) is an Arabidopsis zinc finger transcription factor expressed in the leaf vasculature, where it activates the transcription of florigen FLOWERING LOCUS T (FT) in a cell-autonomous manner. CO is regulated by GIGANTEA (Gi), a component of the circadian clock that induces its transcription in the leaf [12,13]. According to the external coincidence model, light has a stabilizing effect on the CO protein, which therefore accumulates only during the day[14].

Therefore, CO activates *FT* transcription to promote flowering only under LD conditions; indeed, *co* mutants flower later than wild type under LD conditions while, under SD conditions, flower as the wild type [12].

Heading Date 1 (Hd1)

Hd1 encodes for a transcription factor, belonging to zinc-finger family as CO. Its protein, indeed, shares a high homology with *A. thaliana* CO and, like CO, is characterized by two B-box domains in N-terminal and by a CCT domain at the C-terminal[15].

Hd1 is expressed through the light-dark cycle controlled by the circadian clock with different trends depending on the different conditions of LD or SD; in LD conditions there is a peak in the messenger accumulation at the beginning of obscurity. In SD conditions, however, the transcript levels accumulate few hours after the night, reaching the maximum expression peak at 24 hours, then it gradually decreases during the rest of the day[16] (fig. 3A[16]).

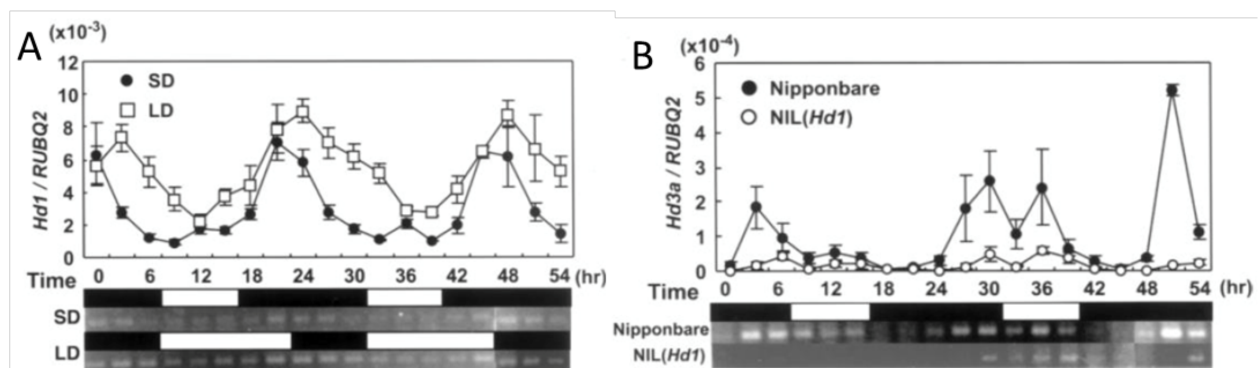


Figure 3 - Relative amounts of Hd1 and Hd3a mRNAs. *A. Relative amounts of Hd1 in Nipponbare in SD and LD conditions. B. Relative amounts of Hd3a mRNA expressed in SD conditions in Nipponbare wild type (black dot) and NIL(Hd1) line, which presents mutant version of the gene hd1 (white dot)* [16].

It is positively regulated by *OsGi*, both under LD and SD conditions. *OsGi* is the rice homolog of *AtGIGANTEA* and, similarly to Arabidopsis, it is also controlled by circadian clock with an oscillatory expression pattern characterized by peaks at the end of the light period. Hd1, like CO, is involved in a GI-CO-FT conserved regulatory module activating, during SD conditions, the transcription of two florigen proteins encoded by *Heading Date 3a* (*Hd3a*) and *Rice Flowering Locus T1* (*RFT1*)[16,8,17].

Furthermore, by the work of Kojima and Hayama, it was demonstrated that in short day conditions *Hd1* works as an activator of its target gene (*Hd3a*) expression (fig. 3B). Indeed, a series of experiments was run on a

nearly isogenic line (NIL) in which a mutated allele for *hd1* was inserted: in these plants, under SD conditions, the expression of *Hd3a* was extremely reduced[16,17].
In the same year, another work by Izawa et al. performed under LD conditions, demonstrated that *Hd3a* mRNA is strongly induced in the *hd1* mutant plants, displaying an early flowering phenotype. Therefore, Hd1 works as a flower inhibitor in LD conditions[18] .

These data suggest a dual functionality of Hd1 in flowering regulation: it induces floral commitment in inductive SD conditions while it represses this transition in non-inductive long-day.

Early Heading Date 1 (Ehd1)

The other floral activator is Ehd1, a transcription factor encoding a B-type response regulator able to activate the expression of *Hd3a* and *RFT1*[19] and to inhibit that of *Hd1* under LD conditions[20] (fig. 4A[18]).

The Ehd1 gene was firstly identified by Izawa as a flowering QTL[18] and then was deeply studied by Doi et al. in 2004[18]. In his work, he demonstrated that this transcription factor is almost exclusively expressed in SD conditions; it also follows an oscillatory trend with two accumulation peaks 2 hours before sunrise and 4 hours after (fig. 4B[18]). The work conducted by Doi et al. studied plants deriving from a cross between the cultivar *Taichung 65* (T65), a double mutant *hd1 ehd1*, and the African variety *Oryza glaberrima* that carries WT Ehd1 alleles.

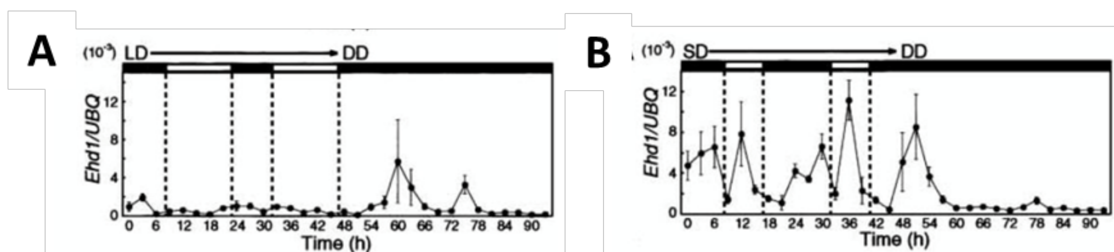


Figure 4 – Expression pattern of Ehd1 in the T65 background under different daylengh conditions.
A. Expression pattern in LD conditions of a diurnal with Ehd1 levels consistently low; two peak were detected in subsequent continuous darkness (DD). B. Expression diurnal pattern in SD conditions with rapid decrease in DD conditions[18]

The research group, observed in *hd1 Ehd1* recombinant plants a strong early flowering phenotype of almost a month compared to the parental T65, grown in LD. Therefore, WT *Ehd1* allele could complement the double mutant phenotype unveiling a different floral activation pathway for *Ehd1* from the evolutionarily conserved one of *Hd1*. In *hd1 Ehd1* T65 plants, moreover, the expression levels of *RFT1* and *Hd3a* increased considerably when compared to parental T65 suggesting *Ehd1* being able to activate florigen expression (fig. 5A,B).

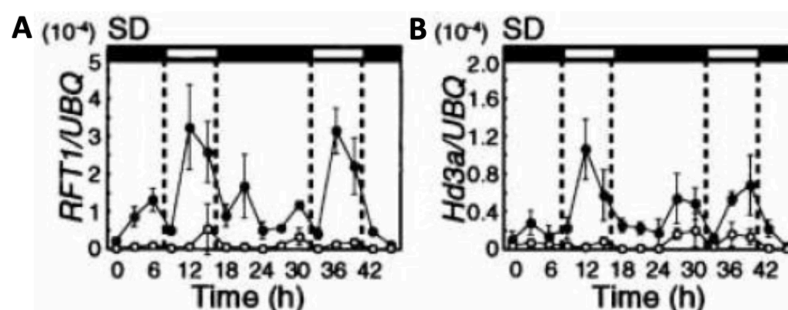


Figure 5 - Expression of RFT1 and Hd3a in NIL(Ehd1) and T65 under SD. *Black dots for NIL(Ehd1) and white dots for T65. After SD treatment, in NIL(Ehd1) expression of RFT1 and Hd3a increased with a diurnal pattern with two peaks. In T65, levels of expression were definitively low*[18]*.*

3.3 FLORIGENS AND ANTI-FLORIGENS

Florigens are small proteins of about 20kDa, produced in response to favorable environmental conditions. They are responsible for flowering and determine plant architecture: these small (about 175 amino acids) and globular molecules are able to move through the phloem and reach the meristematic tissues where they induce the transition of the vegetative meristem into an inflorescence one, promoting the floral commitment[21,22].

They belong to the Phosphatidyl Ethanolamine Binding Protein (PEBP) family. PEBP is a group involved in cell growth and differentiation [23–25] and is divided in three plants cluster (fig.6).

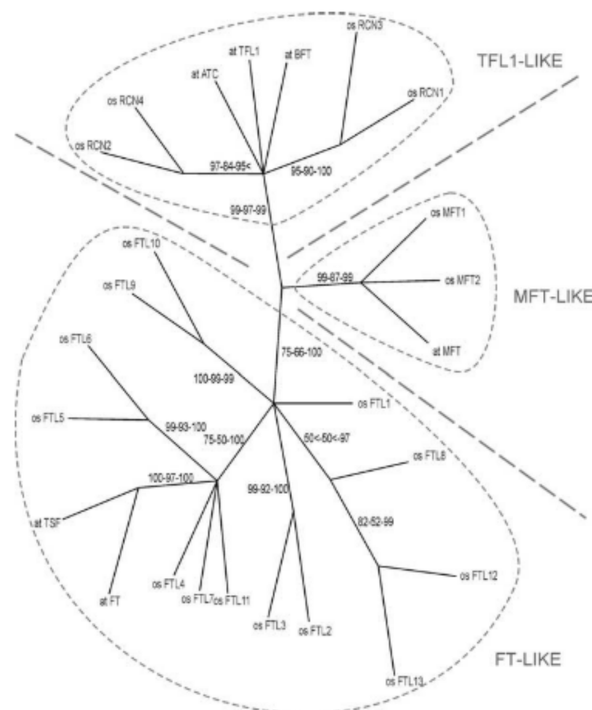


Figure 6 – Phylogenetical representation of PEBP family. *Unrooted Bayesian tree of PEBP genes from rice Oryza sativa (os) and Arabidopisis thaliana (at). Three major classes (TFL1-LIKE, MFT-LIKE, and FT-LIKE) are shown* [33,34].

These three different protein classes include FT-like, comprising those that promote flowering, TFL1-like, the TERMINAL FLOWER1 (TFL1) clade for those that repress flowering [26] and MFT-like, involved in the regulation of ABA and GA signaling pathway[27]. Recent evidence assigns to *OsMFT1* the function of flowering suppressor and spikelet promoter [28].

In rice, there are 13 rice genes in the FT-like family, FT-L1 to FT-L13, 4 TFL1-like and 2 MFT-like genes[23,29,30].

FT- and TFL1-like structures have some specific residues, conserved and necessary for their correct functionality:

- Residue Y85 in FT, Y87 in Hd3a. The mutation in this residue, Y85A substitution in particular, triggers FT conversion to TFL1 and vice versa[26,31];
- Segment B. It confers to FT or TFL1 binding specificity to 14-3-3 determining thus their florigenic or anti-florigenic activity, respectively [29];
- Phosphatidylcholine (PC) binding pocket. Even if FT belongs to PEBP, it doesn't bind PE (phosphatidylethanolamine) but rather the polar-headed PC (phosphatidylcholine) group to allow proper function of FT in promoting flowering - even if it is not clear how this FT-PC interaction triggers it [32].

The rice florigen genes, Hd3a and RFT1, are located on chromosome 6, separated by only 11.5 Kb [8] suggesting a common origin followed by a tandem duplication which occurred after the separation between monocots and dicots [33,34]. This hypothesis that sees Hd3a and RFT1 as two paralogous genes is supported by the presence of an amino acid identity equal to 91% [16].

The action of florigens is defined as *non-cell autonomous* since the proteins, produced in leaf companion cells, act as long-distance systemic signals, in tissues different from those where translation occurs [35–37].

These long-distance proteins are loaded into neighboring sieve element cells to move with the phloem flux, thanks to a conformational change that allows transport through plasmodesmata [38].

Only a couple of genes are known to be involved in florigen transport. To mention one of them, noteworthy is the gene *FTIP*, *AtFTIP1* or *OsFTIP1*, which is required to load FT or RFT1, respectively, in the phloem. In particular, *OsFTIP1* promotes heading under LD conditions trough RFT1 transport [39,40].

Flowering locus T (FT)

FLOWERING LOCUS T is the *Arabidopsis* florigen that encodes a mobile protein that induces plant flowering. It is directly activated by CO at the leaf level and not at the shoot meristem one[41]. Coherently with Arabidopsis flowering time under LD, *FT* transcription increases under these conditions and decreases under SD[35].

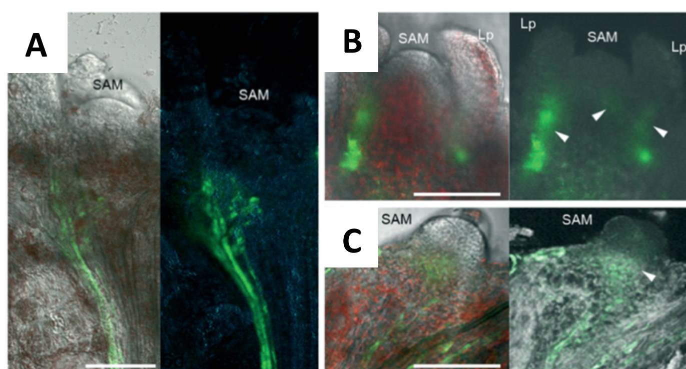


Figure 7 - Confocal analysis of FT:GFP fusion protein in the apical region of SUC2:FT:GFP ft-7 transgenic plants. *Images on the right show GFP signals separated from background emissions. Fluorescence is detected in the provascular tissue and at the base of the SAM.*
A. 6-day-old vegetative plant
B. and C. 10-day-old plant that is induced to flower
Lp, leaf primordium; IM, inflorescence meristem[35].

As a florigen, the FT-GFP fusion protein has been visualized as a long-distance signal. It is a mobile signal that, through symplastic movement, reaches the apical meristem region, where it forms complexes to induce flowering [35,42–44] (fig.7 A-C).

Heading Date 3a (Hd3a)

This florigen is an AtFT rice ortholog [16] and, as previously mentioned, is transcriptional activated by *Hd1* and *Ehd1* in SD conditions with a diurnal peak of expression near dawn and another one 4h from dawn [45].

Tamaki in 2007 conducted a series of experiments aimed at confirming that the Hd3a protein is produced in the leaves and is then able to reach the SAM through the phloem, as for FT. In this work they fused the Hd3a promoter to the GUS reporter gene and confirmed that the GUS transcript is expressed in the leaf vasculature only (fig.8A-C[46]).

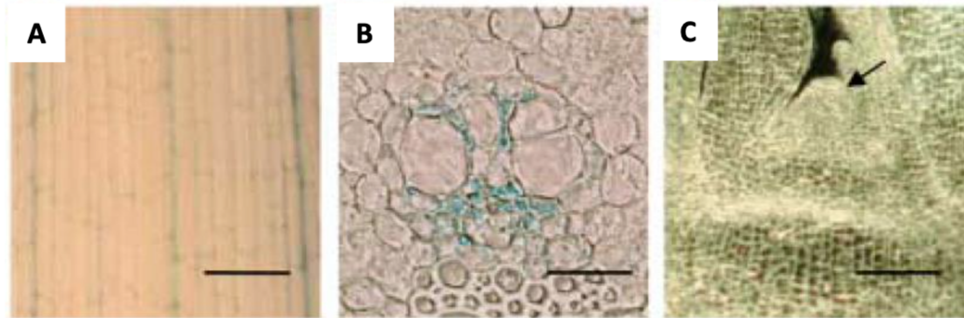


Figure 8 - expression of Hd3a mRNA in rice under SD conditions, visualized through GUS staining of Hd3a:GUS.
A. Leaf blade, expression of Hd3a mRNA in the phloem. B. Transverse section of a leaf blade, expression in xylem parenchyma cells. C. SAM, no expression of Hd3a detected [46].

In particular, GUS reporter activity was localized in the companion cells of the phloem. Furthermore, they expressed Hd3a-GFP fusion protein both under the endogenous Hd3a promoter and under the control of RPP16 promoter (which is a specific promoter of phloem companion cells) obtaining early flowering plants. Thanks to this approach, they pointed out that signal of the fusion protein is detectable both in the vasculature of the leaf and in the meristem, affirming that, differently from *Hd3a* messenger which is expressed specifically in the leaf, the protein is able to travel through the phloem and reach the meristem where it promotes flowering[46] (fig.9[46]).

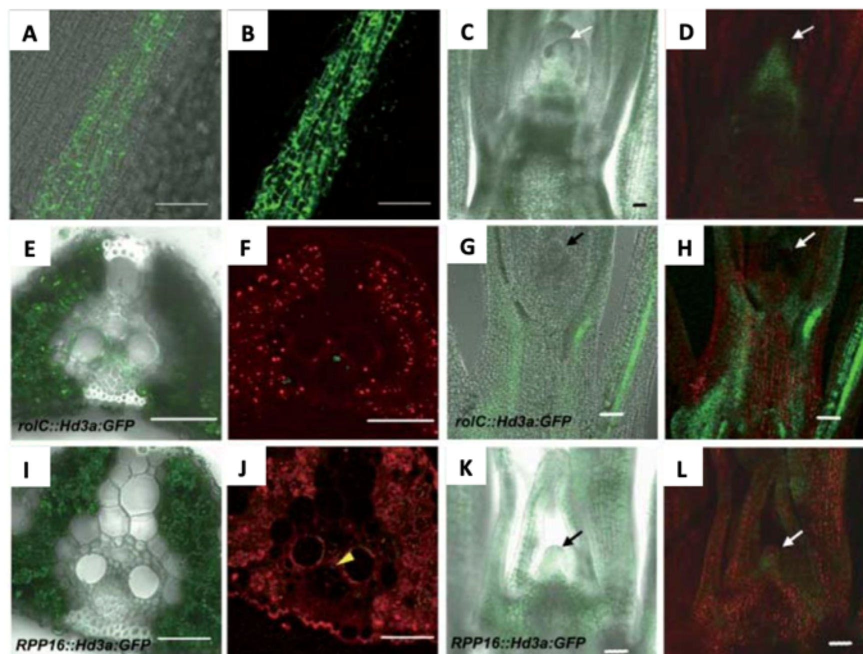


Figure 9 - confocal microscopy of Hd3a:GFP different constructs.
A-D. Confocal images of pHd3a:Hd3a:GFP. E-H. Confocal images of prolC:Hd3a:GFP. I-L. Confocal images of pRPP16:Hd3a:GFP line. A, B. are longitudinal sections of the vascular bundle. C,D,G,H, K, L. are longitudinal sections of the SAM. E, F, I ,J are transverse sections through a leaf [46].

It is only in 2015 that a research group [47] unveiled another function for this florigen, different from flowering promotion. Indeed, in this work, it was investigated the Hd3a protein role in the lateral branches'

development by analyzing *pRPP16:Hd3a* lines that presented an increase in the vegetative branch number. On the contrary when the expression of *Hd3a* is suppressed by a RNAi, a significant reduction in the number of tillers was detected. The authors concluded that Hd3a is a promoter of lateral branching.

Rice Flowering Locus T1 (RFT1)

RFT1 is the closest homologue of *Hd3a* and is regulated by *Hd1* in SD conditions (fig.10A). As with Hd3a, Komiya[34] demonstrated in 2008 that *RFT1* transcript is not present in the meristem, while in plants transformed with the fusion protein RFT1-GFP, under the control of the RFT1 promoter, the fluorescence is detected in leaf vasculature and in the meristem (fig. 10B).

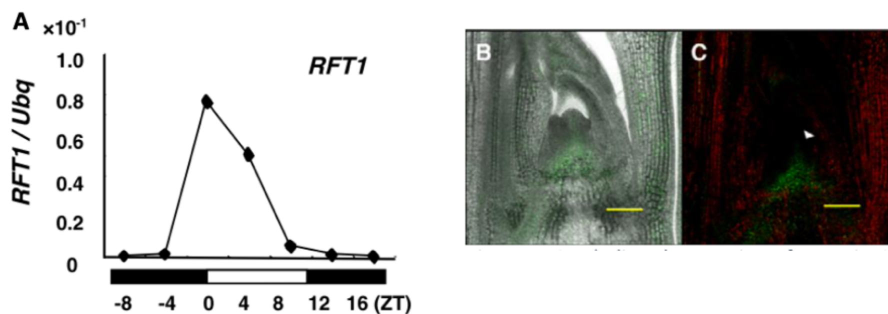


Figure 10 - RFT1 expression and localization pattern. *A. diurnal expression of RFT1 in SD conditions. B-C. Confocal images of the pRFT:RFT1:GFP; longitudinal section of the SAM*[34] *.*

For what concerns *RFT1* expression, evidence unveiled a more complex model for this florigen since it is expressed both under SD and under LD, differentiating it from Hd3a. In the same work the authors investigated whether plants were still able to flower by decreasing the quantity of florigens' transcript, by analyzing in SD the phenotype of two specific RNA interference lines, against Hd3a or RFT1[34]. RFT1 RNAi line flowered comparably to the wild type, while Hd3a RNAi line showed a delay of about one month (fig.11.A)

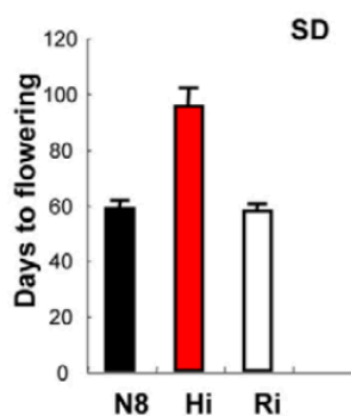


Figure 11 - days to flowering for Hd3a RNAi and RFT1 RNAi lines, compared to wild type N8 (Norin 8) in SD. *Hi stands for Hd3a RNAi, Ri stands for RFT1 RNAi*[34]*.*

These data pointed out that in SD *Hd3a* is promoting flowering, while *RFT1* is not since its absence does not cause any flowering phenotype.

To understand whether there could be an addictive effect with both florigens' transcripts reduced, the same

group constructed and transformed an RNA interference line directed against both *Hd3a* and *RFT1* transcripts, obtaining lines that could not flower even after 5 years[34,48] (fig.12).

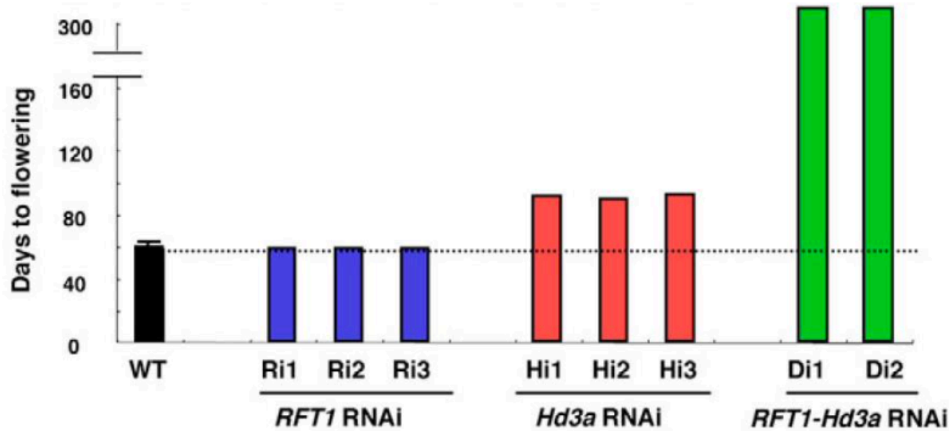


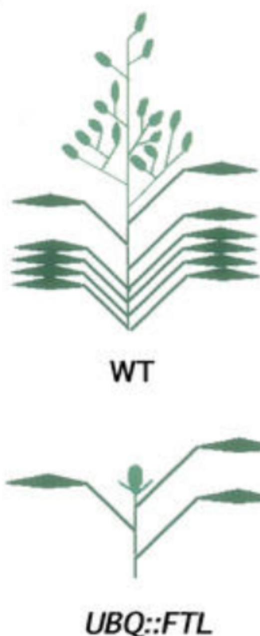
Figure 12 - flowering time of different RNAi lines compared, grown in SD conditions.
Ri: RFT1 RNAi lines. *Hi: Hd3a RNAi lines. Di: RFT1-Hd3a RNAi lines*[37].

These results therefore suggest Hd3a and RFT1 are essential in flowering promotion and underly the incapability for plants to flower in their absence.

3.3.1 Flowering Locus T Like 1 (FT-L1)

During my PhD I focused my attention on *Flowering Locus T Like* 1 that encodes a florigen-like protein and is the closest homolog of the florigen FT. This gene belongs to the family of PEBP and to the same FT-like clade of *Hd3a (OsFT-L2)* and *RFT1 (OsFT-L3*), with which it shares a very similar structure and a high homology in amino acidic sequence[33,37]. This gene, indeed, derives from the same common ancestor of *Hd3a* and *RFT1*[49].

At the beginning of my PhD, except for the phylogenetical evidence, only few data were present in literature about this florigenic molecule and no mutants were available.



To investigate the role of FT-L1 in flower induction, Izawa performed an overexpression of FT-L1 under the control of the maize UBQ promoter [18]. The transformed plants showed internodes' elongation and loss of apical dominance. In addition, strong early flowering was detected during regeneration in tissue culture after the formation of the first 3-5 leaves with terminal floret and reproductive-like organs at the tip (Fig 13).

This evidence led to the hypothesis of a relevant role of FT-L1 in the flowering promotion along with *Hd3a* and *RFT1*.

Figure 13- Schematic representation of pUBQ:FT-L1 phenotypes, compared to the WT*. The wild-type (WT) rice panicle is composed of primary and secondary branches with several florets. pUBQ:FT-L1 plants flower after the formation of the first 3-5 leaves with floret and reproductive-like organs at the tip* [18].

Furthermore, the lab where I performed my PhD, published a data set, obtained through RNA-seq [50] of genes differentially expressed at the SAM by exposure to inductive short-day conditions during floral commitment (fig.14).

Interestingly, among the top ten most transcribed genes at the SAM, there was *OsFT-L1*, together with floral commitment genes such as *OsMADS14*, *OsMADS15* and *OsMADS34* [51,52].

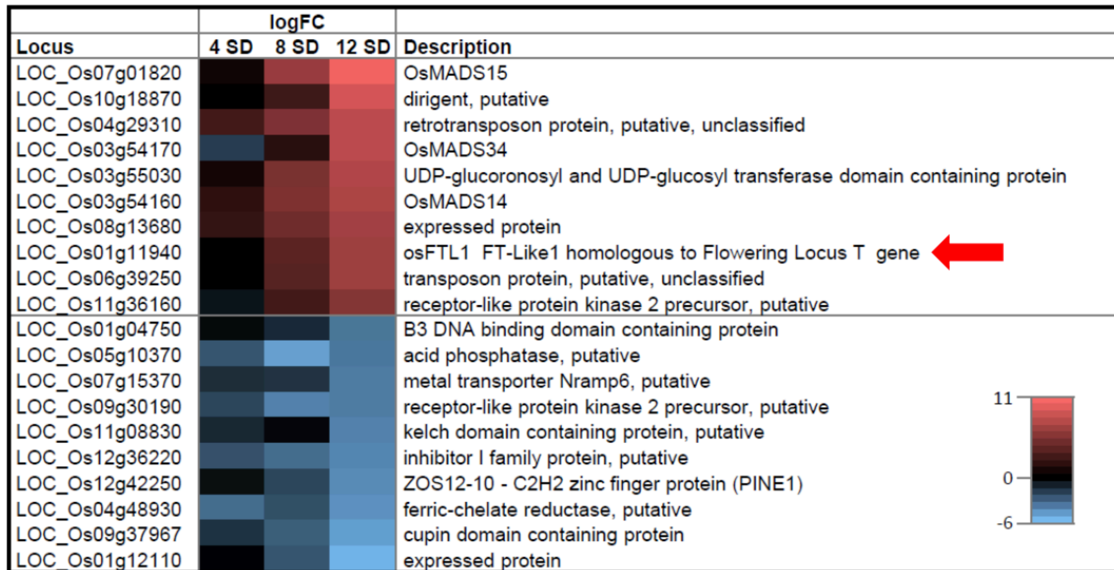


Figure 14 - Heat map showing the most 10 up-regulated and down-regulated genes at 4, 8 and 12 days of photoperiodic induction. *With a red arrow I have highlighted FT-L1 presence in the dataset[50].*

These preliminary data were very interesting and intriguing, since this florigen-like seemed to have atypical features, being directly transcribed at meristematic level, differentiating it from the other leaf florigens.

Furthermore, only in 2021 an article was published calling the attention on its potential role in panicle architecture by interacting with a transcription factor OsFD7 [53] *(see Introduction section 3.5.2 -Alternative FACs)*.

Terminal flower 1 (TFL1)

Within the group of PEBPs there is a cluster composed by TFL1-like proteins detected in the SAM[22,26]. FT and TFL1 are both regulators of floral transition sharing more than 60% homology in the amino acidic sequence: on one hand FT is a florigen, which means that it induces flowering, on the other TFL1 has been identified as a flowering suppressor, as well as inflorescence architecture definer. They therefore have antagonistic roles [1,21,26,54].

When expressed, TFL1 won't let the meristems assume the floral identity and is responsible for indeterminate growth of the inflorescence shoot. Plants with TFL1 overexpressed under a constitutive promoter show a longer vegetative phase and branched inflorescence. *tfl1* loss-of-function produced terminal flowers at the shoot apex (fig. 15A) [39,54,55].

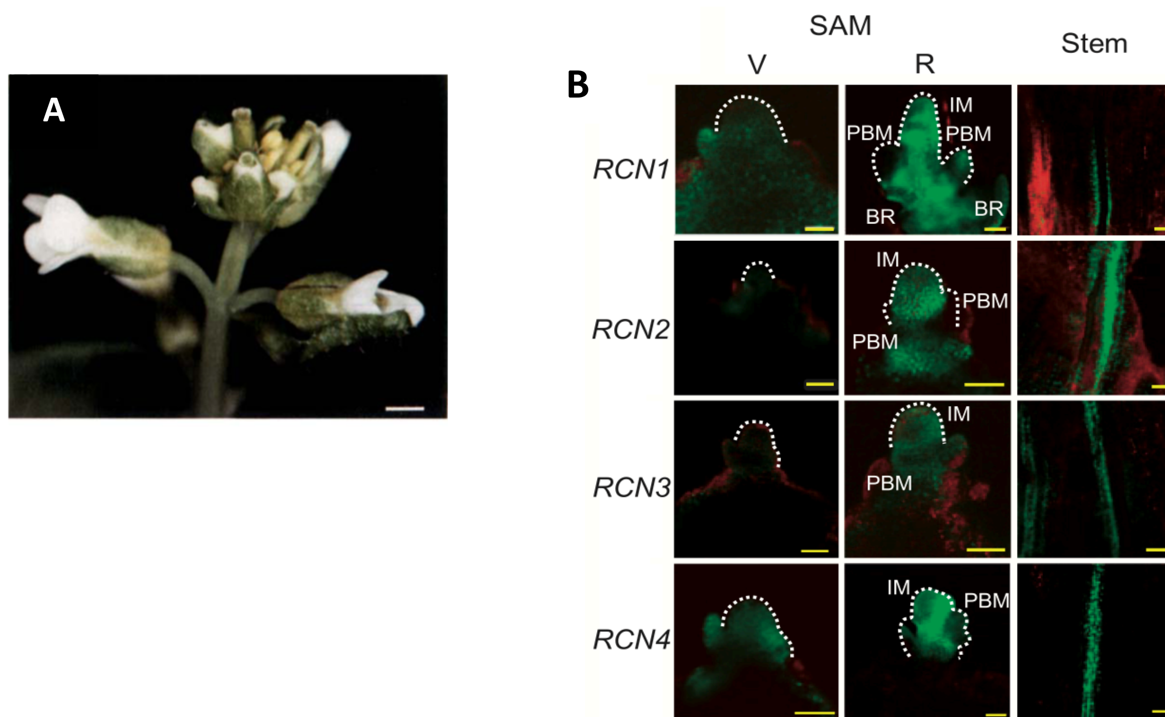


Figure 15 – Arabidopsis and Rice anti-florigens.
A. Lateral views of the main inflorescence apices of terminal flower-2 [54]*. Bars represent 1 mm.*
B. Confocal images of RCN– GFP protein in gRCN-GFP transgenic rice plants in SAM and stem vasculature[56]*.*
V vegetative meristem; R reproductive; IM, inflorescence meristem; PBM, primary branch meristem; BR, bract.
Scale bar = 50 mm.

Rice Centroradialis (RCNs)

In rice there are four anti-florigens, RCN1, RCN2, RCN3 and RCN4 all homologs of TFL1.Their transcript was observed, using GUS reporter plants, in the roots and in the vascular tissues of stem and beneath the SAM[56].

As for the florigens, in RCN–GFP lines, RCN protein localization was detected in the meristem, from the vegetative to the reproductive phase, and in the phloem, in particular at the vasculature (fig.15B)[56].

In a work of Nakagawa, in 2002, the phenotypes of RCN1 and RCN2 overexpressing plants have been analyzed, when expression was driven by the 35S promoter. They described a delay in flowering time and an increase in panicle branch number. In particular, regarding panicle architecture, they pointed out that the number of higher-order branches increased significantly while the number of primary branches was constant[57]. Furthermore, they suggested that the flowering phenotype of delay was caused by delays in phase transition that impose indeterminate growth of the SAM while repressing floral meristem identity genes.

On the contrary, *rcn* knockout plants display a panicle phenotype definitively less dense, with small and less branched inflorescence [39].

In conclusion all these data support the idea that, even if RCNs are not expressed in the SAM, they carry out their function precisely in this tissue by assembling florigen repressor complex that antagonized flowering commitment.

3.4 MOLECULAR CONTROL OF FLOWERING IN RICE

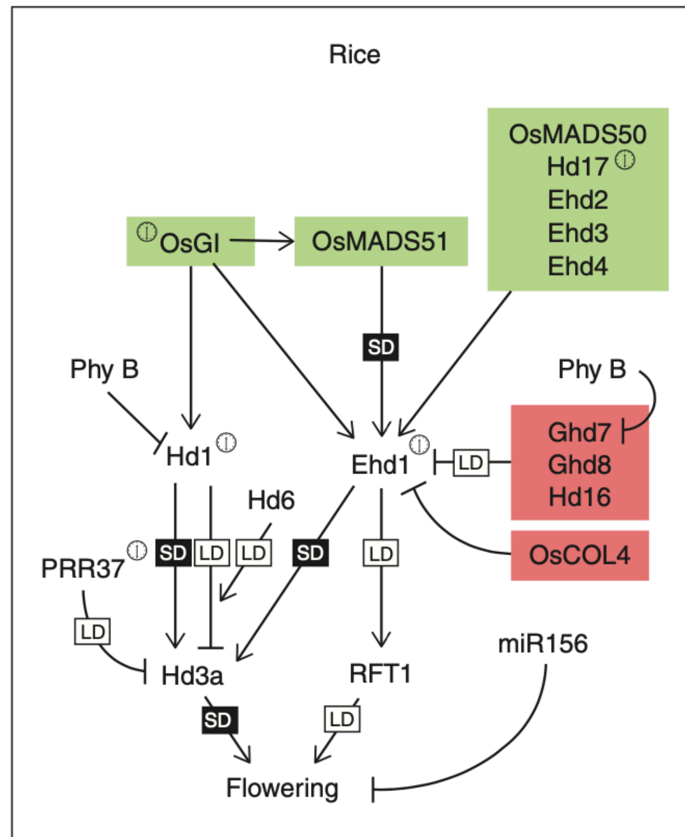


Figure 16 - Brief summary of the genes involved in the flowering regulatory network*. In green the activators of Ehd1, in red its inhibitors. The clock symbol shows a setting by the circadian clock. Arrows indicate transcriptional activation, whereas flat-ended arrows indicate transcriptional repression. SD is for Short Days and LD is for Long Days.* [8].

To briefly schematize what has been described so far about the molecular control depending on the photoperiod during floral commitment, I'm showing here a recap for both day-length conditions (fig.16)

Under inductive SD conditions, Hd1 acts as an activator of the *Hd3a* expression. Due to the similarity with the Arabidopsis CONSTANS-FT system, the regulation module of OsGI-Hd1-Hd3a is believed to be evolutionarily conserved to promote flowering [8,17]. Also, Ehd1 regulates the expression of *Hd3a*, by inducing it.

Indeed, in SD conditions, both *Hd1* and *Ehd1* activate *Hd3a* transcription, promoting flowering. Under non-inductive LD conditions an exclusive rice flowering regulation pathway is activated. *OsGI* induces *OsMADS51* transcription which, in turn, together with *OsMADS50* activates *Ehd1* expression[58]. In these conditions, *Hd1* becomes an *Hd3a* transcriptional repressor while *Ehd1* promotes *RFT1* expression, which, therefore, acts as a major floral activator in LD conditions.
This process is specifically activated during the long day by the coincidence of *Hd1* expression regulated by the circadian clock and the photoperiod mediated by *Phytochrome B*.

Hence, in LD, there are a few flowering suppressors as *Ghd7* and *Ghd8*, regulated by *Phytochrome B*, along with *PRR37*[20]. *Ghd7* particularly, encodes a protein with a CCT domain and is expressed at high levels under long day conditions in which it represses Ehd1 expression[59].

3.5 FLORIGEN ARRIVAL AT THE SAM

Once the florigen is produced in the leaf and transported through the phloem it arrives into the SAM and enters the cytoplasm of the meristematic cells.

The mechanism of florigen unload from the phloem is still not well defined, in particular in rice. However, it is likely that the florigens are unloaded from the end of the vascular tissue and enter into the SAM through the cells just beneath it[46].

At the SAM, florigen promotes the transition of the meristem from vegetative to reproductive. This complex reprogramming of meristem identity, that takes place after floral commitment, is possible upon the formation of the Florigen Activation Complex (FAC).

3.5.1 Florigen Activation Complex (FAC)

This is a heterohexameric complex formed, according to the first model of Taoka, by two molecules of the florigen Hd3a, by two 14-3-3 proteins, GF14c, and by two molecules of OsFD1, a bZIP-type transcription factor (fig.17). Once assembled, this complex is able to bind to the DNA and activate its targets which are thus floral transition markers.

The 2.4A crystallography of the rice complex identified a deep "W"-shaped structure peculiar of the GF14 dimers, forming after the binding to the florigen. At the base of this "W" there are pockets that bind the phosphorylated S192 of OsFD1[60].

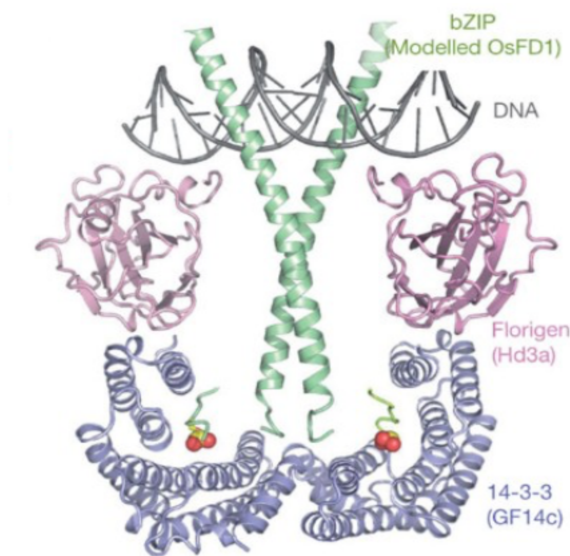


Figure 17 - Modelled structure of the FAC–promoter-DNA complex. *The heteroexameric complex is composed by Hd3a, GF14, OsFD1 and DNA, respectively coloured in magenta, blue, green and grey, respectively* [60].

The molecular mechanism is integrated by the entrance of florigen in the cytoplasm of meristematic cells where it binds to the 14-3-3 that functions as a molecular bridge between the florigen and bZIP transcription factor. The Hd3a-GF14c complex formed in the cytoplasm then enters the nucleus where it binds to OsFD1[31].

This model differs from the one designed for Arabidopsis since, the complex originally described was composed by only FT and FD transcription factor which directly interacted in a complex that allows the activation of downstream genes [42,43].

Taoka et al., were also able to identify all the amino acid residues required for the interaction in the binding interfaces of all components.

Noteworthy for my PhD project, are the residues of the florigen involved in the putative binding with the 14-3-3. Indeed, as shown by green-boxes in the florigens' alignment (fig.18), the amino acids identified and published are all conserved among the florigens as well as FT-L1. Mutations in these residues cause a defect in assembling the complex and result in a delay in flowering commitment leading to a delay in flowering time (the most interesting example for my thesis, is Hd3a P96L or FT P94L for *ft-6*)[32].

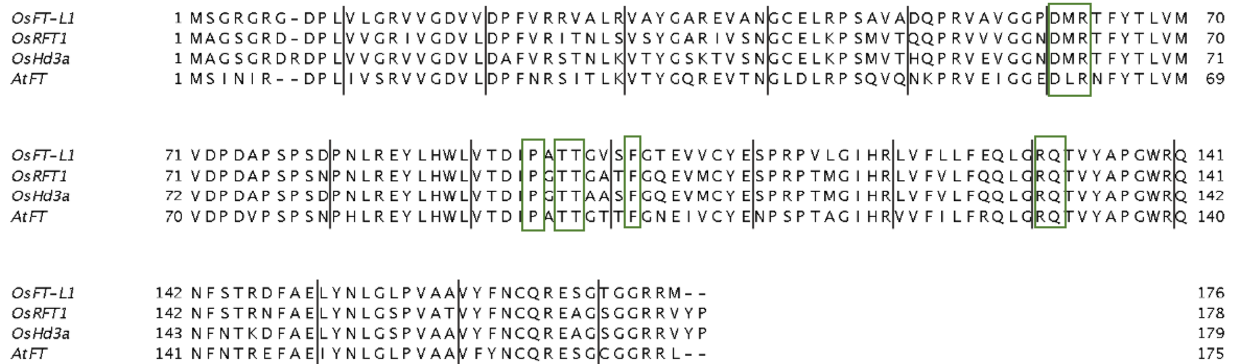


Figure 18: Alignment of FT-L1 with RFT1, Hd3a and Arabidopsis thaliana FT.
Sequences for rice proteins (OsFT-L1, OsRFT1 and OsHd3a) were taken from MSU (rice) and for Arabidopsis from TAIR (AtFT). Green boxes highlight the conserved amino acidic residues involved in binding with Gf14 proteins.

3.5.2 Alternative FACs

At this point, several researchers wondered if it was possible to assemble alternative complexes by varying one of the components at a time since there is a great deal of variability within the various component groups.

A good example of this variability is provided by the 14-3-3 protein family. In 2009, Purwestri 's group [61] identified 8 proteins members of the GF14 family in rice, from GF14a to GF14h, which share 85-95% of amino acid identity. Six of these proteins, from GF14a to GF14f, are expressed in the SAM thus suggesting the possibility for these proteins to form alternative complexes [62–64].

It was possible to corroborate the formation of alternative FACs not only for additional 14-3-3 proteins but also for the two florigens which, carrying out similar functions in different photoperiods, assign great adaptability to the complex.

In 2015, also RFT1 was shown to be able to interact with the 14-3-3, assembling a florigenic complex that promotes the transition under LD conditions, when RFT1 is expressed and induces the flowering[65].

Furthermore, as previously mentioned, it was demonstrated that Hd3a is able to assemble alternative complexes from that of the shoot apex; indeed, Hd3a by activating meristems of the tillers promotes vegetative lateral branch outgrowth[48].

Another work unveiled RCNs capability to assemble alternative and antagonistic complexes by creating interactions with the 14-3-3 proteins, data consistent with high residues' conservation among FT and TFL1[56]. These complex, named Florigen Repressor Complex (FRC), are responsible for inhibiting flowering promotion.

Moreover, in the same work they suggested an existing balance between FAC and FRC, to finely regulate the flowering and inflorescence development in a competitive manner whereby FRC activity is highest in the first vegetative stages, while FAC activity is predominant during and after floral commitment.

Also OsFDs transcription factors plays a non-negligible role in the variability of FAC components and functions.
Indeed, Tsuji et al., in 2013 identified five new *OsFD*, from *OsFD2* to *OsFD6*, all presenting the same conserved SAP (serine-alanine-proline) terminal motif, with serine residues that must be phosphorylated for interaction with Gf14s[66].

The work then focused on *OsFD2* which was shown to be able to interact with Gf14b.To understand the role of this transcription factor a *pUbq:OsFD2* overexpressing line was constructed. Overexpression of *OsFD2* led plants to display alterations in the development of the leaf, with abnormally lengthening culms from different tiller meristems that should be dormant in wt; these culms then never develop panicles reiterating leaves formation with a flag leaf-like morphology. Furthermore, they constructed *pUbq:OsFD2 S164A*, with an alanine residue substitution into the putative phosphorylation site, in order to prevent interaction with the 14-3-3, confirming that serine residue in the SAP motif is necessary for the correct functioning of this bZIP.
In 2017, Brambilla et al. proposed a dual action model of Hd3a and RFT1 proteins that can assemble both florigen activation and repression complexes in leaves, depending on the OsFD involved, to finely regulate flower commitment. In particular, the work was centered on two bZIPs, bZIP42 named Hd3a Binding Factor 1 (HBF1) and bZIP9 (HBF2) that performed as florigens' expression repressors [67].
Indeed, these two transcription factors directly interact, exclusively in the leaf, with Hd3a or RFT1 repressing their expression in order to cause a delay in the flowering time.

Another bZIP recently studied is OsFD4 identified in 2020 as a component of a flowering inducing complex, at the SAM. To corroborate this model, CRISPR *osfd4* mutants were generated; they displayed late flowering by delaying the expression of marker genes involved in floral commitment and inflorescence promotion[68].

The most recent bZIP described is OsFD7 [53].It is expressed in the meristem as well, plays a role in the definition of the panicle through the regulation of the ramifications. By directly interacting with florigens Hd3a, RFT1 and more strongly with OsFT-L1, it carries out its function in the fine regulation of the first stages of development of the panicle and in the floral induction pathway. OsFD7 RNAi lines were characterized by a denser inflorescence architecture, with a later increase in seed sterility maybe due to a floral meristem identity acquired too early (fig.19).

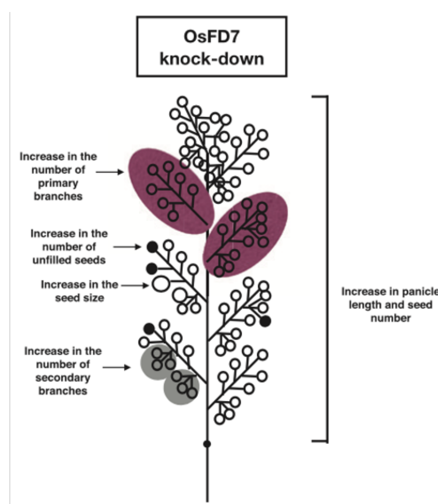


Figure 19 - osfd7 RNAi lines*. These mutants display an altered panicle morphology with an increase in the panicle length, number of primary and secondary branches, and the number and size of seeds*[53].

This last data sustains authors' hypothesis of OsFD7 being a promoter of *SEP* family genes. Moreover, the work reported an interaction OsFD7 -OsRCN1 suggesting a further role for this TF as repressor, together with the anti-florigen, of the flower commitment.

To conclude, the examples provided support the great plasticity and ductility of the FAC complex.

3.6 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes

As previously introduced, rice plays an essential role in the growing food demand, being able to provide 20% of the world's daily nutritive needs [3] and sustaining almost half of the world population[69].

To improve this cereal yield, the agronomic traits that could be exploited have been studied for a long time, in particular the efforts have been directed to obtain low tillers numbers, a greater number of grains per panicle and strong and robust stems and roots[70].

SQUAMOSA PROMOTER BINDING PROTEIN-LIKE proteins play a key function in regulating these characteristics and therefore the architecture of the plant. In particular, these proteins encode for transcription factors of the SPL family[71] which share a strongly conserved DNA binding domain, containing a Squamosa Promoter Binding (SPB)-box, whose core binding motif consists of 4 bases (GTAC) [72].
The stability of SPL proteins is regulated by the E3 ubiquitin ligase IPA1 INTERACTING PROTEIN1 and OTUB1-like deubiquitinase [73], while its transcriptional activity is modulated by *OsSHORT INTERNODES 1* and *DWARF 53* [40,74].

Genes from this family have been reported to be important regulators of several developmental processes. The most relevants include *SPL7*, *SPL17* and *SPL14* - also known as *IDEAL PLANT ARCHITECTURE1 (IPA1)*, one of the major inflorescence development regulators - that best represent this family.

The expression of many *SPL* genes is regulated by *OsmicroRNA*s, as *miR156* and *miR529* [75,76] that bind a specific recognition core causing *SPLs* transcript degradation. It has been demonstrated that *miR156* negatively regulates inflorescences meristem activity in the initiation of reproductive branching, while *miR529* controls panicle development in further stages. Both fulfill their action by regulating SPLs[75].

In particular, *miR156OE* plants revealed that *miR156* regulates both tiller branching and panicle branching, through specific and separate pathways (fig.20A,B)[75].

Figure 20 – Vegetative (tillering, A) and reproductive (panicle, B) branching regulated by miR156.
Plants (A) and panicles (B) at adult stage of WT, MIM156, miR156OE, and the hybrid between miR156OE and MIM156 plants [75].

Overespressors present small panicles characterized by few primary branches and spikelets, independently from the rising tiller numbers[75]. These phenotypes indicate *miR156* as a repressor of inflorescence meristem activity and reproductive branching development.

Point mutations in the *miR156/529* binding site cause *SPL* accumulation leading to the formation of the ideal plant architecture with few tillers and higher number of branches per panicle [72,77,78]. These types of mutations are gain-of-function, dominant (*SPL-D*), while *spl* loss-of-function mutations cause dwarfism and increase tillers' number (fig.21A-C)[79].

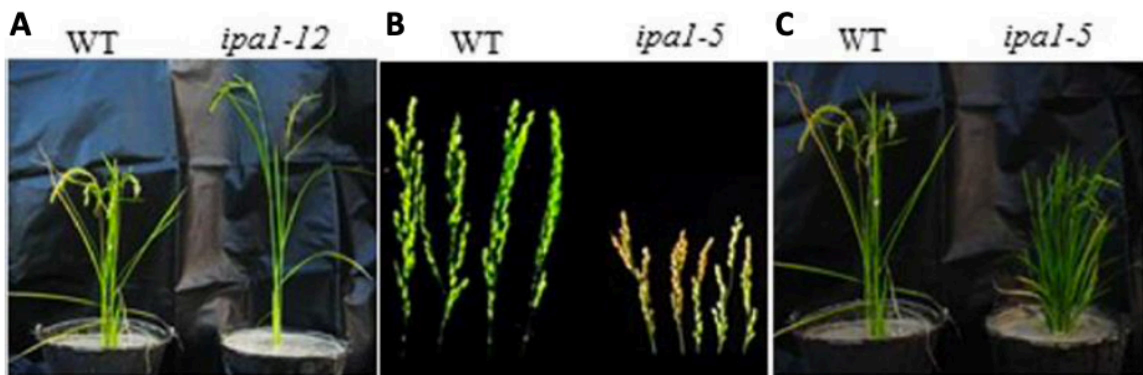


Figure 21- CRISPR ipa1 mutants' phenotypes.
A. Wild type and gain-of-function ipa1-D mutant plants. B. Wild type and loss-of-function ipa1 panicle morphology. C. Wild type and loss-of-function ipa1 mutant plants [79].

In a work by Duan, it has been shown that *SPL14* affects plant architecture by directly binding and activating promoters of genes involved in tillering development suppression and in increasing plant height and panicle branches. Among them are *Teosinte Branched 1 (OsTB1)* and *Dense and Erect Panicle 1 (DEP1)* [74].
Lu's group has demonstrated through a Chromatin immunoprecipitation (ChIP)-seq experiment that, among the genes regulated by *SPL14*, there is also *FT-L1* that is bound 2 kb upstream the FT-L1 transcription start site (fig.22)[72].

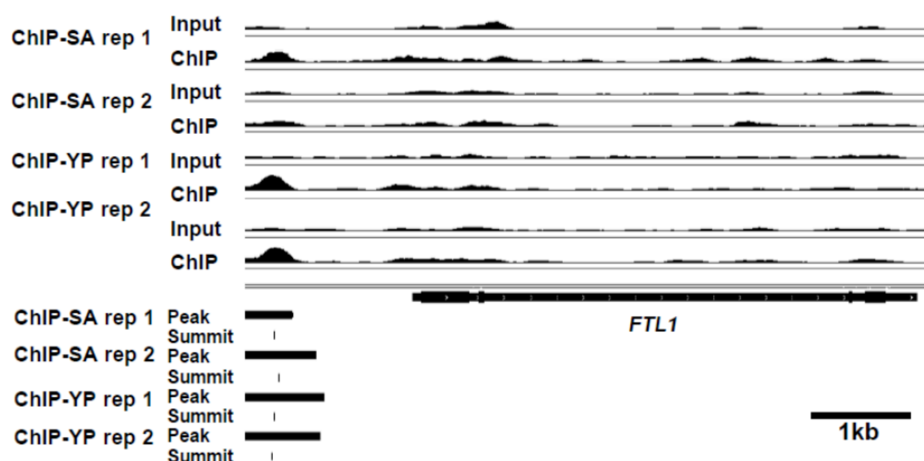


Figure 22 - SPL14 binding profile in the 2 kb upstream of FT-L1 transcription start site, as output of a ChIP-seq experiment [72].

These ChIP-seq experiment and *SPL-D* phenotypes, together with the *ft-l1* mutant phenotypes I obtained, suggested that FT-L1 might function downstream of SPLs, and helped me to design a regulatory model with an epistatic effect between the two genes. In the *Results and Discussion section 4.5*, a further discussion will

follow.
Analysis on IPA1 transcript identified its expression in shoot apex and in young panicle[72].

A more recent work performed by Zhang's group detects *SPL14* and *SPL17* mRNAs specifically in the bracts and not in the inflorescence or branch meristems themselves. The pattern was established in the bracts subtending panicle rachis and both the primary and secondary branches, in later stages[5]. These observations indicated that SPLs might function non-cell autonomously to control branching.

To investigate if there are any redundancy in the SPLs family, Wang in 2015 designed RNAi lines against *SPL14* and *SPL17* (fig. 23A-E).

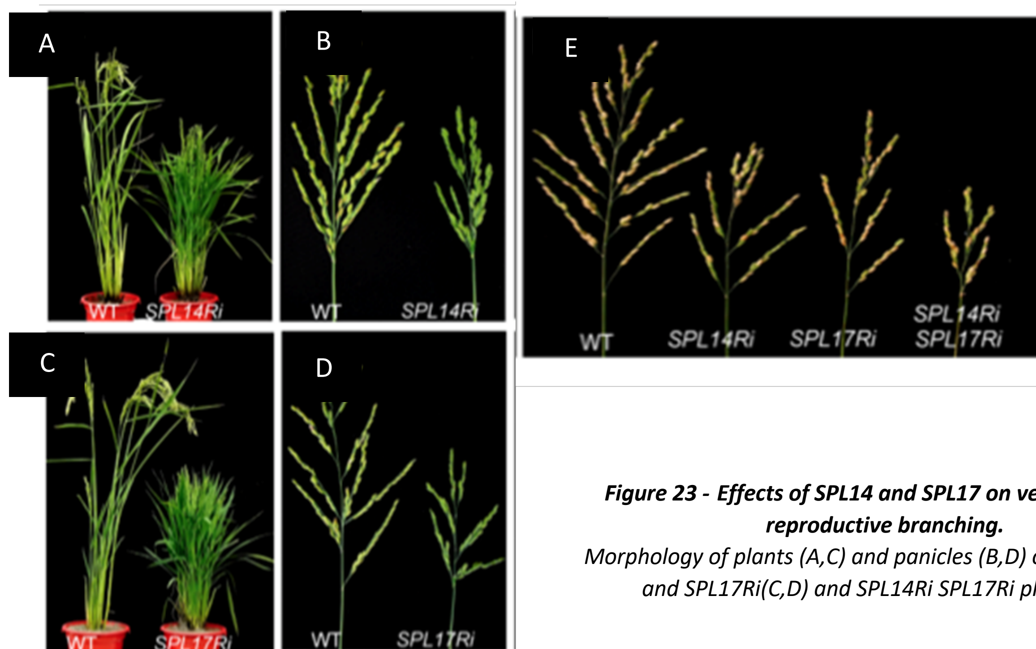


Figure 23 - Effects of SPL14 and SPL17 on vegetative and reproductive branching.
Morphology of plants (A,C) and panicles (B,D) of SPL14R i(A,B) and SPL17Ri(C,D) and SPL14Ri SPL17Ri plants(E)[75].

In both *SPL14RNAi* and *SPL17RNAi* panicles, branches and spikelets diminished, resembling *miR156OE* plants and unveiling an opposite function to that of *OsmiR156* (fig.20). Thus, *SPLs* actively enhance inflorescence activity and promote branch meristems differentiation[75].

Furthermore, RNAi lines shared very conserved phenotypes, implying that *SPL* genes play a redundant function in plant architecture and in reproductive branching.

Double RNAi lines of *SPL14* and *SPL17* enhanced phenotypic effects compared with individual RNAi plants, suggesting that the two genes have an additive effect.

In a more recent work, the same authors confirmed the decrease in branch number also by producing *SPLs* CRISPR lines[5].

To untangle *SPL* genes' function, the group investigated the phenotype in *SPL14OE* and *SPL17OE* lines, observing a decrease in branches and spikelets number per panicle. Interestingly, only the number of secondary branches was reduced suggesting that a precocious determination of secondary branches into spikelet meristems occurs. To corroborate these data, in *SPL14OE*, the expression of *Frizzy Panicle (FZP),* the spikelet meristem marker, increased and, in an *In Situ* Hybridization, it was detected in the branch meristem.

In the overespressors, besides *FZP*, were elevated also the expressions of other spikelet identity markers as *OsmiR172* and *OsMADS34/PAP2*. By ChIP assays the group was able to demonstrate that *SPL14* bound to the promoter of the *miR172* precursors and of *PAP2*.

Moreover, by analyzing the phenotype of plants in which *miR156* were downregulated (*MIM156* lines), they observed panicle branching promotion pointing out that while limited increase in *SPLs* levels promotes panicle branching, very high expression of *SPLs* inhibits it[5].

Taken all this evidence into consideration, the group concluded that development and conversion of BM to SM is attributable to *SPL* genes, which are responsible for branching suppression and spikelet promotion trough *miR172* and *PAP2*[75].

3.7 REPROGRAMMING AFTER FLOWER COMMITMENT

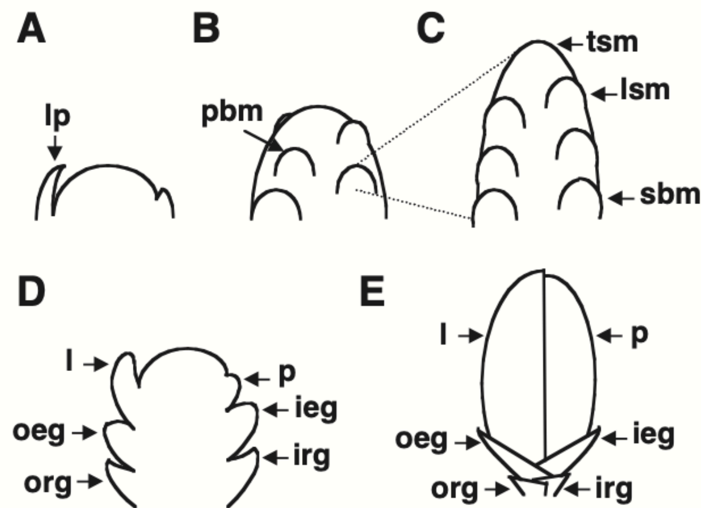


Figure 24 - Rice inflorescence development from vegetative meristem to floret meristem*.*
A. Vegetative meristem (VM) with leaf primordia (lp) emerging from shoot apical meristem. B. Reproductive stage with rachis degeneration and lateral organs development. Primary branch meristems (pbm) emerge from the central rachis C. Branch meristems with both lateral and terminal spikelet meristem (lsm, tsm) at the top and secondary branch meristem (sbm) at the rachis base, following a gradient of determination that decreases from the tip to the base D. Spikelet meristem at floret formation stage. Branch meristem is converted into spikelet meristem with specific identity as terminal spikelet meristem (tsm), outer and inner rudimentary glumes (org, irg), palea (p) and lemma (l), outer and inner empty glume and (oeg,ieg) E. Schematic representation of a mature spikelet. Reproductive organs are surrounded palea (p) and lemma (l) [90].

The transition from the apical meristem of the vegetative shoot to the reproductive ones is precisely controlled both by environmental (short-days, high temperatures) and endogenous signals.
In the conversion to an inflorescence meristem (IM; later becoming rachis meristem, RM), the shoot apical meristem (SAM) undergoes a fully complete reprogramming towards a reproductive growth (fig.24).
After the florigen arrival at the shoot and after the commitment, the meristem suppresses the vegetative program to set the reproductive one. This conversion, which develops along with all meristems' differentiation and determination, leads to a global change in the transcriptome.
The reproductive structure becomes more and more complex.
Due to the dynamic morphological modifications, we can expect substantial changes in a significant number of genes that are involved in the definition of meristem identity[51].

Many research groups have tried to clarify through transcriptomic data analysis and with the use of marker genes, how this reprogramming is organized.

In particular, the greatest efforts to identify pathways and events that characterized each developmental stage have been made by Furutani in 2006, Zhang in 2014, Harrop in 2016 and by a more recent work by Zong et al., in 2022[51,80–82].
Each group clustered the resulting data-sets according to different rationales. Harrop sampled only meristematic regions of young panicles dissected by Laser Micro Dissection (LMD) and then clustered data by gene expressions' changes, dividing them in two different blocks: the first characterized by steady or gradual changing trend, the second showing drastic alterations during the development from one meristem to the following one [81].
Furutani, instead, conducted global transcriptome profiling on developmental stages from phase transition to panicle branch differentiation using a cDNA microarray and forming clusters by inflorescence morphology[51].
In the work by Zhong a single-cell RNA sequencing approach was used to map the identities distribution of cells and global development of the inflorescence sampled at two different stages: the first one with inflorescence < 2 mm and the second with 2–3 mm inflorescence. In this experiment the authors collected branch, spikelet, and floret meristems. After correlating marker genes expression and enriched genes in each cluster, a specific tissue identity was assigned to each cluster that was further divided into inner subclusters[82].

The formation of rice inflorescences is a transitory event producing, in a confined time period, a structure showing a high number of branches[51].
The first morphological sign of inflorescence development is the longitudinal elongation of the shoot apical meristem (SAM) caused by cell division. This stage is followed by the secondary meristems (or axillary, AM) formation.
Not surprisingly, among up-regulated genes in the transition from vegetative meristem (VM) to inflorescence meristem (IM) after the arrival of Hd3a and RFT1 at the shoot apex, several are related to auxin signaling, for instance *OsIAA19* which is involved in cell division[48].
The formation of a new apical meristem identity is accomplished by branches of both primary and higher-order or, alternatively, by determinate meristems, such as the floret meristem, that differentiates floral organs.

As largely describe in the *Introduction 4.5 session*, with the arrival of the florigens at the shoot and the formation of the FAC, target genes of the complex are activated.

Among these early targets, the majority belongs to the MADS-box family, including genes belonging to the *AP1*/*FUL* clade with *OsMADS 14*, *OsMADS 15*, *OsMADS 18,* and to the *SEP* clade with *OsMADS 34/PAP2*[37,48,51,83].
In particular, *OsMADS34*/*PAP2,* regulates the inflorescence architecture by finely controlling the number of primary branches and repressing their early differentiation into secondary branches and spikelets. REF
In an *osmads14 osmads15 osmads18* and *osmads34* quadruple mutant the plants displayed alterations during the reproductive phase with panicle architecture totally reshaped since the VM, instead of becoming an IM, continued to generate leaves[83]. Thus, IM identity is determined by the combined actions of *PAP2* and the three *AP1/FUL-like* MADS-box genes.

Maintenance of the proper size and activity of the meristem is also essential for reproductive organogenesis. Loss-of-function mutants of KNOX genes, such as *OSH1,* have defective inflorescence morphology[80]. Another gene involved in the inflorescence branching maintenance is *TAWAWA1 (TAW1),* that acts preserving IM activity and suppressing the transition to SM identity[84].

Regardless of their future differentiation destiny, branches are all generated as axillary meristems. Their identities are defined in subsequent moments, depending on their position on the inflorescence and initiation timing. Consequently, in order to delineate rice panicle architecture a key feature is the branching pattern[51].

During the early stages of panicle development, apical and axillary meristems' establishment and activity influence branching in two different phases: the number of primary branches is determined by the rachis meristem abortion timing, whereas the branching complexity is specified by indeterminate branch meristems transition[81].

In grass reproductive meristems, auxin regulates axillary meristem initiation and outgrowth by controlling cell polarity establishment and cell elongation[80].
Branches, indeed, are formed in the axils of leaves or bracts, where local auxin maxima lead to lateral organ production. Mutations in auxin transport proteins abolished all inflorescence branching [6].
In rice, auxin accumulates in all branch meristems of the inflorescence, as well as in the primordia of glumes and other floral organs [6].
Auxin flux through the epidermis converges on small regions of the inflorescence meristem, a process that is regulated by the auxin influx carriers and the auxin efflux carriers. For instance, OsPINOID (OsPID)[85,86], an auxin efflux carrier in rice, regulates reproductive and vegetative branch development probably by interacting with LAX PANICLE 1 (LAX1). *LAX1*, indeed, is required for the formation of axillary branches and *lax1* mutants have a dramatically reduced number of inflorescence branches displaying no lateral spikelets[87].

A second regulatory level, during early reprogramming, depends on the *SPLs-miR156/529* module. To obtain the best levels of reproductive branching the expression of *SPLs* and *miRs* genes needs to be carefully regulated as an excessive production of *SPLs,* as well as an underproduction, seriously affects panicle branching. *miR156* and *miR529* binding to the recognition core in *SPLs* mRNAs regulated their expression by degrading their transcript[75].

SPLs, on the other hand, modulate panicle branching by directly regulating the miR172/ AP2 and PANICLE PHYTOMER2 (PAP2)/Rice TFL1/CEN homolog 1 (RCN1) pathways that promote spikelet transition.

Establishment of the IM requires to suppress the vegetative development program. Work from the Zhang's group proposed the integration of MADS-box activation with a new regulatory pathway involving *miR156/529-SPLs-NL1-PLA1* necessary to suppress bract outgrowth in the panicle.
In particular, *SPL14* directly binds to the *NL1* promoter activating its expression. *NL1*, together with *miR156-SPL*, co-regulates *PLA1* expression to suppress bract outgrowth. In particular, *NECK LEAF1 (NL1)* and *PLASTOCHRON1 (PLA)* are both involved in bract outgrowth suppression, in rice. Furthermore, NL1 that encodes a GATA-type zinc finger transcription factor, has a crucial role in maintaining the correct pattern of reproductive branching[88].
This modulated suppression is an essential event required for the shift between vegetative and reproductive branching by a complete transcriptome and chromatin accessibility reprogramming.
Indeed, *spl7 spl14 spl17* and *nl1* mutants exhibit a panicle phenotype with vegetative features to indicate that their transcriptomes are not rightly reprogrammed [5,89].

Between apical and axillary meristems, there is a significant expression switch. A possible explanation for this is based on the difference between the destiny of the RM, aborting after branch meristem (PBM) differentiation, and the axillary meristems, undergoing transition to determinate spikelet meristems (SMs)[81].

Among the genes involved in maintenance of indeterminate branching growth there is *TFL1*, *RCNs* rice homologs. *RCN1* and *RCN2* overexpression delays the conversion from branch to floral meristem and leads to a highly branched inflorescence[57].

SM development.

The transition from inflorescence meristems to spikelet meristems determines inflorescence morphology, and affects the grain yield.

In spikelet meristem determination, two empty glumes are generated after the rudimentary glumes. None of these glumes forms axillary meristems. The spikelet is the basic structure of grass inflorescences, and each spikelet can form one to several small flowers called florets, depending upon the species. In rice, each spikelet gives rise to a floret. This process is controlled by transcription factors, such as MADS-box and AP2 genes.

In this transition from BM to SM, *FRIZZY PANICLE* (*FZP*) plays an essential role, promoting flower formation. In *fzp* mutants, floret formation is replaced by sequential branching reiteration in the axils of rudimentary glumes[90].

In single-cell datasets[82] establishment of flower meristem and floral organ differentiation, were represented by two continuous developmental trajectories. Some floral organs possessed an intermediate identity between non-reproductive leaves and reproductive floral organs. Rudimentary glumes, lemmas and sterile lemmas are likely bract-like organs, with an intermediate organ identity between leaf and flower[82].

At the floret meristem (FM) stage, genes related to epigenetic regulation were detected, including genes leading to DNA methylation and histone modification. Specific enrichment of *DWARF TILLER1* (*DWT1*), encoding the WUSCHEL-related homeobox, and of *E-class genes (OsMADS6* and *OsMADS8)* is related to FM activity maintenance and palea specification. In *dwt1* mutant florets there were organs similar to multiple glumes with fewer stamens and occasionally two pistils[82].

Role of hormones.

In all events of growth, development and differentiation, phytohormones play a key role in the fine regulation of each process.

In particular, the ones that play a major role during the reproductive phase are cytokinin (CK), auxin and gibberellins (GA) by gradients that regulate inflorescence patterning. Indeed, genes related to biosynthesis or hormone signal transduction are showing switch-like behaviors. These lead, respectively, to a gradual transition from IM to BM or SM development, thus enabling pattern shifting during the development of rice inflorescence.

To give some specific examples, *OsAUX1* controls meristem determinacy, during rice inflorescence development, by promoting primary branch elongation and spikelet initiation.

OsMAPK6 modulates CK homeostasis to regulate the number of seeds and their size. Zhong et al built transcriptional marker lines, identifying expression patterns in the IM, BM, and SM[82].

Also involved in CK activation there is, *LONELY GUY LIKE PHOSPHORI- BOHYDROLASE 1* (*LOGL1)*, that 1 catalyzes the final step of cytokinin synthesis within the same cluster with genes increasing in expression between RM and SM[81].

Zhong et al. constructed the OsGASR1pro–eGFP marker line to monitor GA response during rice inflorescence development. Strong signals were observed during early reproductive development, with a decreased expression in ePBM and SBM, and an increased one in SM and FM[82].

In conclusion, *OsMAPK6* and *OsGASR1*, with their dynamic distribution patterns, determine inflorescence architecture.

Role of epigenetics.

In this overview of meristem reprogramming events, epigenetic modifications must be considered as well.

In the work of Wang et al., RNA-seq data of shoot apex and panicle demonstrated that the chromatin status was largely reprogrammed after floral transition. In fact, by comparing differentially accessible chromatin regions (DARs) of vegetative and reproductive meristems, the authors detected 2115 accessible chromatin regions (ACRs) that had increased accessibility in the vegetative shoot apex compared to the panicle, and 2776 ACRs more open in panicle compared to the shoot, assessing massive changes in chromatin accessibility[5].

The same group analyzed chromatin accessibility and transcriptome in the *spl7 spl14 spl17* and *nl1* reproductive meristems. They identified common profiles reminiscent of vegetative development indicating that the reproductive program was not correctly settled. Considered all these data, they affirmed that *SPL*s and *NL1* modulated the chromatin conformation, coordinating the transcriptional reprogramming of SAMs during the reproductive conversion[5,89].

Furthermore, in single-cell RNA sequencing, genes involved in epigenetic modification pathways were specifically activated in the spikelet meristem, suggesting that changes in genomic regulation and protection, accompany meristem transition and may prepare the plant for floret initiation.
In the clusters identified by Zong et al, genes and processes involved in epigenetic regulation, including DNA methylation and histone modification, were enriched in floret cell subclusters. For instance, it was detected *OsNRPE1*, a key regulator of gene silencing, which encodes the largest subunit of RNA polymerase V[91]. These enrichments suggest that changes at the genomic level go along with the cellular transition to reproductive development[82].
Consistently, in 2020, Higo et al. analyzed whole-genome DNA methylation of isolated SAMs in the vegetative and reproductive stages. They concluded that changes in DNA methylation begin at SAM emergency playing a crucial role for rice reproductive development in protecting the genome from harmful Transposable Elements[92].