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"Interactomics for rice flowering: a Proximity Labelling approach"

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ENGLISH ASBTRACT

Flowering regulation in rice (*Oryza sativa*) involves a complex induction pathway that senses environmental inputs in the leaves and transmits signals to the shoot apical meristem (SAM) for reproductive differentiation. The transcription factor Hd1, homologous to Arabidopsis CONSTANS, is pivotal photoperiodic sensor, promoting flowering under short-day (SD) and repressing it under long-day (LD) conditions. Hd1 function is modulated through the interaction with other flowering regulators, such as Ghd7 and NF-Y proteins, to activate or repress the transcription of the florigen genes *Hd3a* and *RFT1*. A recently characterized florigen-like protein, called FTL1, contributes to meristem differentiation during inflorescence and panicle development, relying on interactions with OsFD transcription factors. During my PhD, I studied Hd1 and FTL1 function with traditional transcriptional and biochemical techniques, as well as through innovative proteomic approaches. Proximity Labelling (PL) was employed for the first time in our laboratory to explore novel protein-protein interaction of Hd1 and FTL1. PL relies on fusing TurboID (TbID), an optimized biotin ligase, to the protein of interest to obtain specific tagging of its interactors *in vivo*. Identification of the proximal proteome is enabled by selective precipitation of biotinylated proteins and mass spectrometry analysis. Our research has established an efficient PL protocol in agroinfiltrated *N. benthamiana* leaves and transgenic rice plants expressing TbID-fused proteins. TbID-fused FTL1 and Hd1 were successfully created, and protein analyses confirmed their stability and enzymatic efficiency, helping refining the optimal PL pipeline. A preliminary PL analysis was carried out on Hd1-TbID using agroinfiltrated *N. benthamiana* leaves. Furthermore, application of PL on rice FTL1-TbID floral meristems yielded >3000 proteins, with 102 identified as potential FTL1 interactors, revealing associations with chromatin, RNA metabolism, and cell structural elements (cell wall, plasmodesmata and microtubules). Ongoing research promises additional proteomic information, while validation experiments are underway to verify the novel information retrieved on FTL1 localization and function. With these experiments, we demonstrate that PL can expand our understanding of rice flowering regulating network, and that the obtained data can be a valuable resource to maximize the breeding of cultivated rice varieties.

ITALIAN ABSTRACT

Il controllo della fioritura nel riso (*Oryza sativa*) si snoda su un complesso meccanismo di induzione che integra gli stimoli ambientali nelle foglie e trasmette un segnale al meristema apicale della pianta per indurne la transizione riproduttiva. Il fattore di trascrizione Hd1, omologo di CONSTANS in Arabidopsis, è un sensore fotoperiodico che promuove la fioritura in condizioni di giorno corto (SD) e la reprime in condizioni di giorno lungo (LD). Hd1 interagisce con altri regolatori della fioritura, come Ghd7 e le proteine di tipo NF-Y, per attivare o reprimere la trascrizione dei geni florigenici *Hd3a* e *RFT1*. Una proteina florigenica di recente caratterizzazione, chiamata FTL1, contribuisce alla differenziazione del meristema durante lo sviluppo dell'infiorescenza e della pannocchia, interagendo con i fattori di trascrizione di tipo OsFD. Durante il mio dottorato ho studiato Hd1 e FTL1 con tecniche di analisi proteica e trascrizionale, oltre che con approcci proteomici innovativi. Per la prima volta nel nostro laboratorio, abbiamo utilizzato il proximity labelling (PL) per esplorare le interazioni di Hd1 e FTL1 con altre proteine. Il PL si basa sulla fusione della TurboID (TbID), una biotin-ligasi ottimizzata, alla proteina di interesse con lo scopo di marcarne gli interattori *in vivo*. L'identificazione del cosiddetto proteoma prossimale è resa possibile dalla precipitazione selettiva delle proteine biotinilate e dalla successiva analisi di spettrometria di massa. Con il nostro lavoro abbiamo sviluppato un efficiente protocollo di PL su foglie di *N. benthamiana* agroinfiltrate e in piante di riso transgeniche che esprimono le suddette proteine di fusione. Le analisi preliminari su Hd1-TbID e FTL1-TbID hanno confermato la loro stabilità ed efficienza enzimatica. Successivamente, l'intero protocollo di PL è stato applicato su Hd1-TbID utilizzando foglie di *N. benthamiana* agroinfiltrate. Inoltre, l'impiego del PL su meristemi florali di riso esprimenti FTL1-TbID ha identificato più di 3000 proteine, di cui 102 sono candidate come interattori di FTL1 e lo associano alla cromatina, al metabolismo dell'RNA ed agli elementi strutturali della cellula (parete cellulare, plasmodesmi e microtubuli). Attualmente sono in corso degli esperimenti di convalida di queste potenziali interazioni di FTL1, finora sconosciute. Con questi esperimenti dimostriamo l'ampio raggio dei dati ottenuti mediante il PL, e che le nuove e dettagliate informazioni sullo sviluppo fiorale possono essere una risorsa preziosa per il miglioramento varietale del riso.

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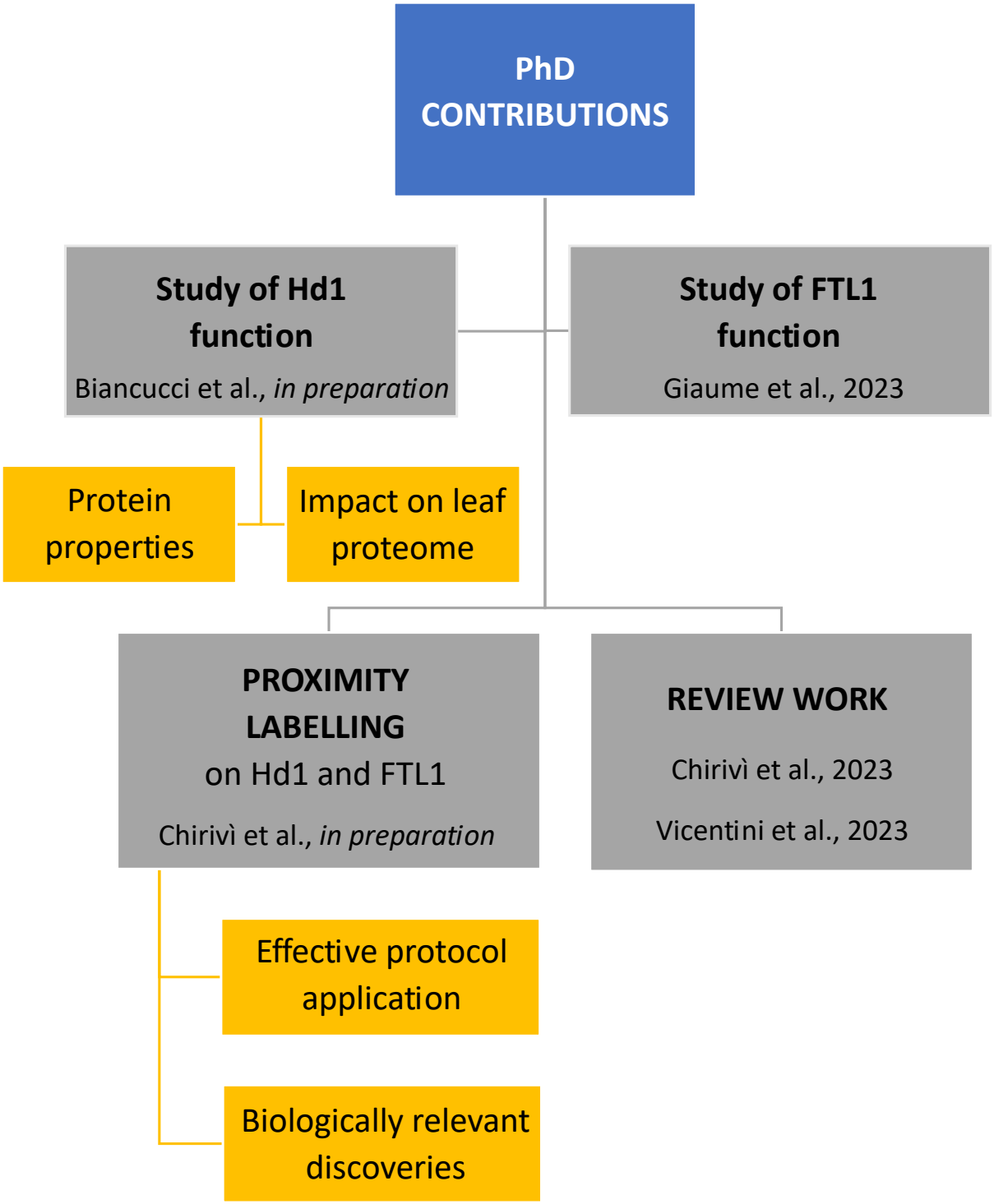

INTRODUCTION

1. Global importance of rice

Rice (*Oryza sativa*) cultivation holds paramount economic and social importance globally, serving as a cornerstone of sustenance and livelihood for billions of people. Having a vast economic weight, rice cultivation has shaped the lifestyle of entire civilizations, since the early moment of its domestication in China and Southeastern Asia, from its wild ancestor *O. rufipogon* 10,000 years ago. Following population growth and subsequent increase of productivity demands, rice cultivation has fostered technological innovation and the breeding of new varieties over time [1,2].

Nowadays, it contributes to global food security and represents a major income source for millions of farmers. The rice industry forms the backbone of rural economies in numerous developing countries, supporting an entire value chain that goes from farming to distribution, contributing to poverty alleviation and overall economic development [3–5].

Europe's rice history is relatively recent compared to other world regions, given that its agricultural practice was introduced during the Middle Ages. Even so, in countries such as Italy, Spain, and France, rice production significantly contributes to regional economies and fulfilment of dietary demands [6]. Around 75% of rice cultivation surface in the European Union is localized in Italy and Spain, with a production of milled rice that is equivalent to 0,4% of the global one. In Italy, annual rice production is of approximately 1,4 million tonnes [5–7] (Fig. 1 and 2).

Figure 1. **Rice-growing areas in the EU.** Since being introduced by the Moors during the Middle Age, rice cultivation is spread over a few countries in Southern Europe. Main producers are Italy and Spain, hosting 75% of the total cultivation surface. Adapted from Kraehmer et al., 2017 [6].

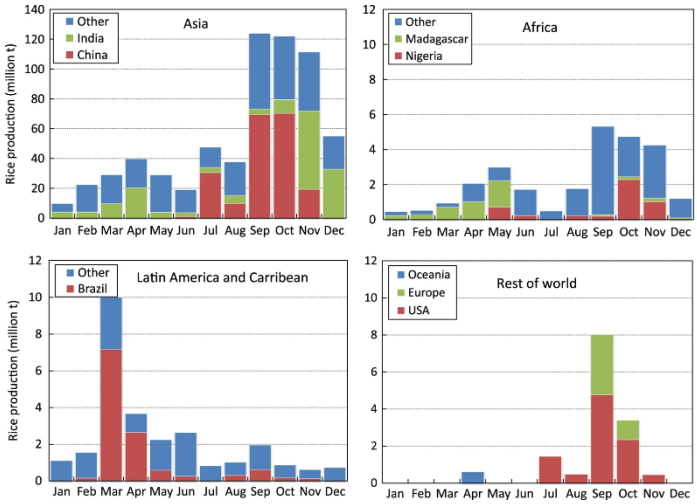

Figure 2. **Rice production by geographic region and months of harvest**. Major rice-producing countries are in Asia, where in many cases this cereal can be harvested twice a year. In comparison, Europe is a small rice producer, but its harvest amounts are similar to those of biggest countries, such as USA. Adapted from Laborte et al., 2017 [5].

All rice in Europe is irrigated, and water provision is a relevant issue for the agricultural and economical sustainability of the productive system. Main worries are also represented by pathogen proliferation, amongst them the infection by hemibiotrophic fungus *Magnaporthe oryzae* [6,8,9]. Against tangible climatic challenges, breeding and technological efforts are being made to enhance yields and sustainability [6,10,11].

From a research perspective, the small size of the diploid rice genome has made it a focal point for the study of biological processes in cultivated monocotyledons. Rice is indeed phylogenetically close to other widespread cereal species, such as wheat, maize and sorghum [12].

Long selective breeding has crucially shaped rice genome over history. The *O. sativa* species has been traditionally subdivided into two main groups subspecies, *japonica* and *indica* differing in growth features, grain size and cultural needs. However, recent genomic analyses have led to reconsider this classification, identifying five distinct groups: *indica*, *tropical japonica*, *temperate japonica*, *basmati* and *aus* [13]. The varieties cultivated in Europe are classified as *temperate japonica* [8]. From early domestication events, rice have spread to distinct climatic areas of the globe. Photoperiod sensitiveness is a major selection trait for rice adaptability to northern latitudes and temperate climates [14]. Photoperiod-responsive genes play a crucial role in regulating the plant's reproductive processes to ensure proper flower development and seed production based on the duration of daylight [15]. By default, rice is a facultative short-day (SD) flowering plant: it flowers faster under short-day conditions, that is, when daylength decreases below 13,5 hours [16]. Due to its tropical origin, rice cultivation at

temperate climates is only possible during the one warm season, making synchronization of flowering time an essential requirement for its affordability [5,8].

2. Photoperiodic-responsive genes: a focus on Hd1

European and North-Asian rice varieties present different combinations of non-functional alleles for one or more flowering repressor genes that allow them to flower under long-day (LD) conditions. Some of these genes are *Heading date 1* (*Hd1*), *Grain number, plant height, and heading date 7* (*Ghd7*) and rice *Pseudo-Response Regulator 37* (*OsPRR37*) [17,18].

In *Arabidopsis thaliana* (from here on, just Arabidopsis), a LD-flowering plant, photoperiodic-dependent flowering is controlled by the regulatory axis made up by the circadian clock gene GIGANTEA (GI), the zinc-finger transcription factor CONSTANS (CO) and the florigenic protein FLOWERING LOCUS T (FT). [19,20]

In rice, OsGI activates the transcription of CONTANS homolog Hd1 to further induce the transcription of FT homologue *Heading date 3a* (*Hd3a*)[21,22]. However, this conserved regulatory axis is not central to the rice gene network, which is more complex and presents distinctive traits that are briefly listed below (Fig. 3).

- **Rice contains unique and monocot-specific regulatory elements that distinguish its flowering induction pathway**.

Besides Hd1, flowering initiation is controlled by the B-type response regulator *Early heading date 1* (*Ehd1*), which integrates different kind of stimuli into the floral induction pathway. Under LD, *Ehd1* expression is inactivated by rice major flowering repressor Ghd7, as well as by Ghd8, OsPRR37 and Hd1 [18,23–25]. The role of *Hd1* in this transcriptional pathway is unique to rice, whereas homologues of Ghd7, Ghd8 and OsPRR37 have similar roles in other monocotyledons [26–30].

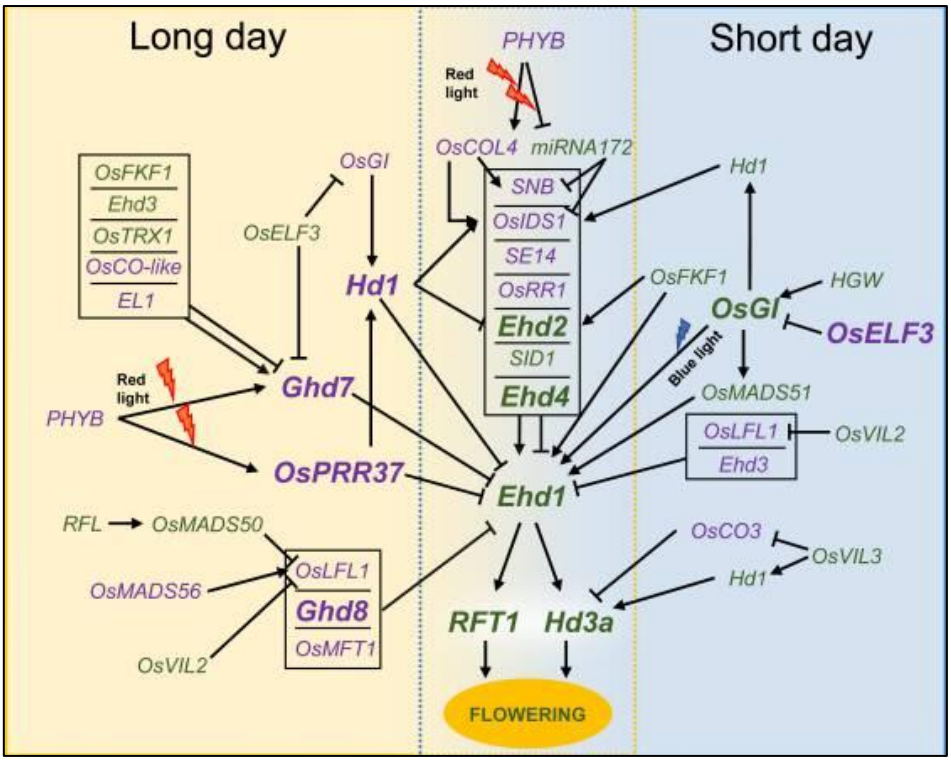

Figure 3. **Rice flowering induction pathway is intricate and finely interconnected.** Light stimuli are integrated into a multi-component gene network that allows flowering under SD conditions and inhibits it under LD conditions. Transcriptional interactions are represented by straight (activation) and flat (repression) arrows. Adapted from Vicentini et al., 2023 [31].

- **Rice and Arabidopsis homologous genes have non-specular functions.**

Rice has two main FT-like genes, *Hd3a/FTL2* and *RICE FLOWERING LOCUS T* (*RFT1/FTL3*), encoding mobile proteins that are transported from the leaves to the shoot apical meristem (SAM)to start the reproductive differentiation. The two genes are paralogues, and their transcription is induced by Ehd1 and Hd1 under photoperiodic inductive conditions [22,23,32]. *Ehd1* itself is directly activated by Hd1 under SD conditions and repressed under LD conditions [33,34]. OsPRR37, which represses Ehd1 transcription, is also able to form repressive complexes at the florigen promoter regions to inactivate their expression under LD conditions. In Arabidopsis, the homologous genes *PRR3* and *PRR7* are circadian clock regulators and relate differently to flowering time. Indeed, they have been proposed to induce flowering indirectly, by binding and stabilizing CO under photoperiodic inductive conditions [22,35]. Finally, the circadian clock protein OsGI, whose Arabidopsis homologue is upstream of CO, is also able to bind *Ehd1* promoter during

daytime, therefore inducing its expression in a manner that is independent to Hd1 [16].

- **In rice, Hd1 has assumed a unique role, acting both as flowering promoter and flowering repressor.**

In rice, the photoperiodic-responsive network is set to prompt the reproductive transition under SD conditions, and a robust control of flowering time is provided by the antagonist roles of Ghd7 and OsGI (Fig. 4). Under LD, Ghd7 protein expression is higher and gated at the morning, and *Ehd1* is repressed. Under SD, the gate of *Ghd7* inducibility falls during night time, relaxing *Ehd1* transcription, which is promoted by OsGI [16,36]. In this multi-factorial scenario, Hd1 has a coordinative, highly diversified role (Fig. 4). Hd1 is equally transcribed under both photoperiod conditions, according to a diurnal rhythmical pattern. Little information is available about the protein accumulation profiles. However,, there seems to be nolight- or daylength-dependent degradation mechanism, differently from Arabidopsis CO [37]. Therefore, the functional reversion of Hd1 activity does not seem to rely on post-translational (de)stabilization. Under SD, Hd1 binds to Hd3a promoter to induce flowering [22]. On the other hand, it has a major role under LD conditions, when it can form different kind of complexes with other transcription factors to repress transcription of *Hd3a*. One of these complexes is the NUCLEAR FACTOR Y (NF-Y) -type (Fig. 5). Usually consisting of three subunits (NF-YA, NF-YB, and NF-YC, with the B and C subunits forming histone-like heterodimers), these complexes play a crucial role in regulating gene expression in eukaryotic cells by recognizing *CCAAT* boxes contained in several gene promoters [38,39]. Interaction of Hd1 with NF-YB and NF-YC subunits confers specificity for *TGTGG* sequences, known as CO-responsive elements (COREs) [40,41]. These sequences are found in the florigens promoter regions and can be alternatively bound also by OsPRR37, which is also able to form complexes with NF-YB and NF-YC dimers [42]. Above-cited Ghd8 gene encodes for an NF-YB protein, which is a confirmed Hd1 interactor [43]. Under LD, Hd1 also interacts with Ghd7, and the two are able to bind *Ehd1* promoter to repress its transcription [34].

Prof. Fabio Fornara's laboratory at the University of Milan has been studying rice genetics for more than 10 years, focusing on genes controlling flowering time and panicle development.

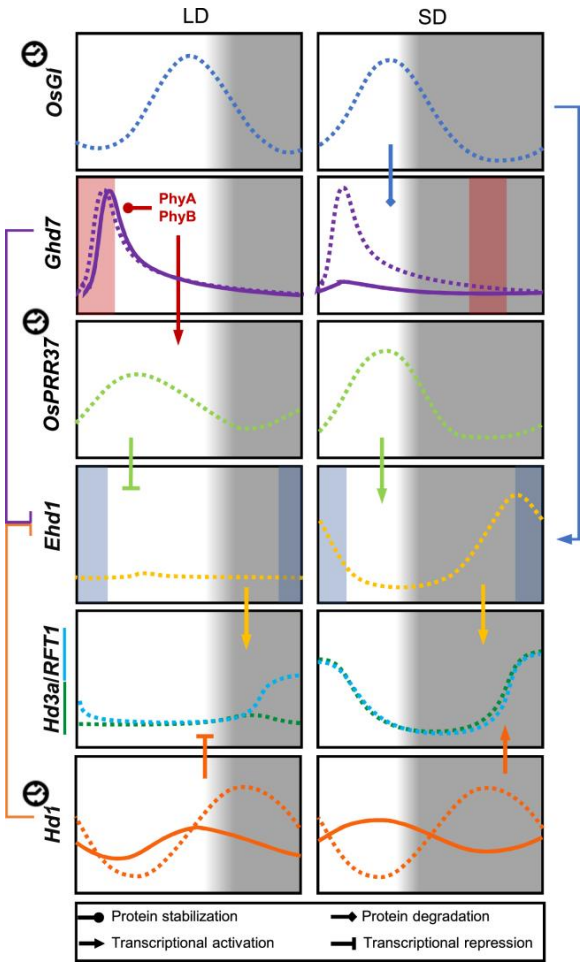

Figure 4. **Diurnal accumulation patterns of major flowering regulators under LD and SD conditions show the central position of Ghd7 in the external coincidence model of rice flowering**. The transcription of GI peaks at dusk in both long-day (LD) and short-day (SD) conditions, while its protein forms a complex with Ghd7, leading to 26S proteasome-dependent degradation. Ghd7 transcription responds to red light, exhibiting an inducible morning gate under LDs; this gate shifts to the night under SDs, with a consensus indicating a morning transcriptional peak.Ehd1 expression is morning-gated by blue light, and OsGI can induce Ehd1 transcription under SDs when unopposed by Ghd7. The diurnal profile of Ehd1 transcription is modulated by Hd1 and PRR37 under SDs, with Hd3a and RFT1 being transcribed in SDs through Hd1- and Ehd1-mediated induction, while under LDs, florigen expression is repressed by Hd1, allowing eventual RFT1 transcription to promote flowering. Adapted from Vicentini et al., 2023 [31].

Since then, high-quality information has been delivered on photoperiodic regulation, definition of floral meristem identity and other processes conferring traits of agronomic interest in rice.

Before my arrival to the laboratory in November 2020, transcriptomic analyses carried out on wild type rice plants and Hd1 knockout mutants *hd1-1* had suggested that Hd1 was able to control the transcription of genes that are not related to flowering induction. Among these, several genes belonging to the phenylpropanoid biosynthetic pathway resulted to be differentially expressed in the mutant compared to the wild type. At the same time, metabolic analyses on leaf cell walls and salt stress experiments had pointed out that cell wall composition and tolerance to osmotic stress are affected in the *hd1-1* mutant.

These findings were the starting point of a new research line, aimed at proving that the cooperative role of Hd1 expands to other unreported pathways.

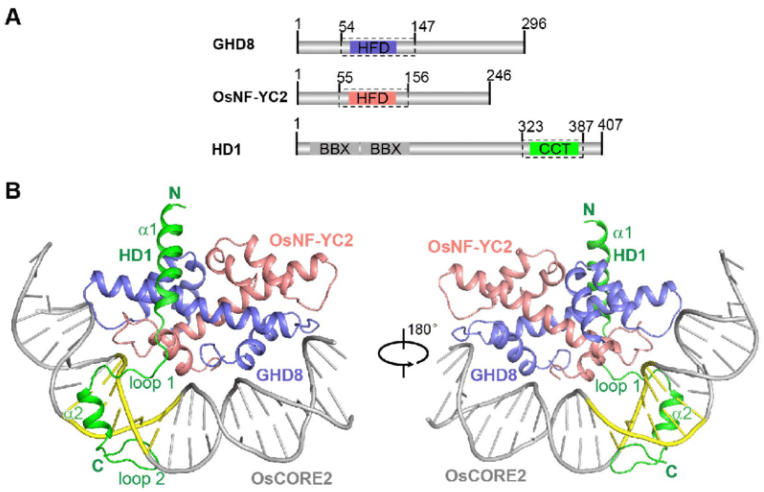

Figure 5. **Structure of DNA-bound HD1 CCT domain /GHD8/OsNF-YC2 complex.** Hd1, GHD8, and OsNF-YC2 functional domains are highlighted in dotted boxes (A). The crystal structure of the complex reveals a heterotrimer formed by HD1 CCT domain GHD8, and OsNF-YC2, depicted in green, violet, and salmon colours, respectively. DNA OsCORE2 is presented in gray with 'CCACA' nucleotides highlighted in yellow (B). Modified and adapted from Shen et al., 2020 [41].

The results of this research have not been published yet and are included in Biancucci et al. (*in preparation*). My personal interventions to this work are exposed in the first part of this thesis. Firstly, I took part in studying Hd1 protein stability and new DNA-binding properties. I then incorporated a total leaf proteomic approach to the research.

3. Flower meristem differentiation and the role of FTL1

The ultimate consequence of photoperiod-induced floral transition is the development of inflorescences, called panicles, where sexual reproduction takes place and seeds are produced. Before this, rice plants proliferate in a vegetative phase, producing leaves and several culms, called tillers [44]. The SAM, an undifferentiated pool of cells which forms during embryogenesis, leads the main-stem growth, while lateral tillers develop from the remaining axillary meristems at its basal nodes. Transitioning from the vegetative to reproductive stage involves each of the culms generating a panicle in their highest internode region. This happens through SAM transformation into an inflorescence meristem (IM), sequentially generating new kind of meristems until floral organs development [15,44]. These new meristems encompass primary and secondary branch meristems (PBM and SBM),

spikelet meristems (SM) comprising florets and glumes, and floret meristems (FM) housing flower organs. The IM arises from a main axis known as rachis, which aborts and allows the development of lateral organs structured hierarchically (primary, secondary, and even tertiary branches). SMs emerge on branch meristems, eventually giving rise to reproductive organs. The timing of transitions between meristem types crucially determines the architecture of the mature panicle (e.g. size, number of branches, number of seeds) [45,46] .

At my entrance in prof. Fabio Fornara's laboratory, I helped in the study of a recently characterized homologue of Hd3a and RFT1, called FTL1. The results of this research have been published in Giaume et al, 2023 [47].

Like Arabidopsis FT and rice florigens, FTL1 is a member of the phosphatidylethanolamine-binding protein (PEBP) family, which groups proteins present in all living kingdoms. Despite their common ability to bind hydrophobic ligands, the members of the PEBP family have numerous distinct functions and are involved in varied biological processes [48–50].

In angiosperms, PEBPs play a crucial role in regulating flowering time and are phylogenetically divided into three clades: FT- , MOTHER OF FT AND TFL1 (MFT)-, and TERMINAL FLOWER1 (TFL1)- like proteins [48]. Rice genome contains 13 FT-like genes, and among them are Hd3a and RFT1 [51]. Their function is partially redundant, but presence of the two proteins is required for flowering. Moreover, RFT1 expression can be induced under LD conditions, even if later than under SD conditions, making rice a facultative, rather than a strict, SD-flowering plant [52,53].

After migration to the SAM and entrance into the cell, rice florigens are known to intercept 14-3-3 proteins in the cytosol to later relocate into the nucleus to form florigen activation complexes (FACs) [54]. These heterohexameric complexes contain two florigens, two OsFD transcription factors and two 14-3-3 proteins working as a molecular bridge between the others (Fig. 6). OsFDs belong to the bZIP family and give specific DNA recognition properties to the complex [55–58]. The FAC is therefore a means through which Hd3a and RFT1 give rise to a transcriptional reprogramming of the SAM to become an IM. As a proof of it, the florigens are necessary to induce a set of well-studied MADS-box genes activated at the start of the transition, allowing the acquisition of reproductive identity [57,59].

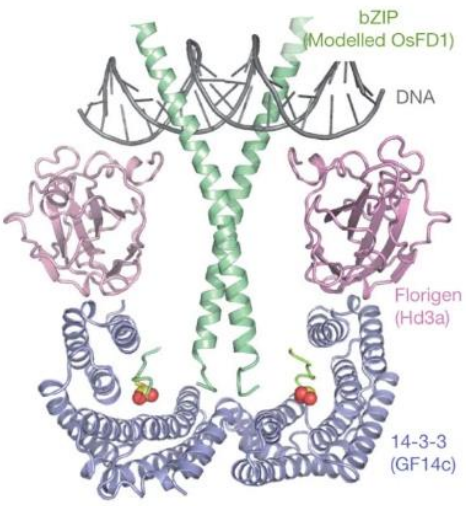

Figure 6. ***Proposed structural model of rice FAC****.: Modelled structure of the FAC–promoter-DNA complex. Hd3a, GF14, OsFD1 and DNA are coloured in magenta, blue, green and grey, respectively. Modified and adapted from Taoka et al., 2011* [54]*.*

Research in our laboratory has shown that mutations of *FTL1*, the closest homologue of *Hd3a* and *RFT1* among all rice *FT-like* genes, had effects on flowering time and on panicle architecture traits [47]. FTL1 mutant lines are late flowering and develop panicles with a higher number of branches and spikelets. As a consequence, they produce more seeds, even if the ratio fertile:sterile seeds is lower than in the wild type. It was demonstrated that FTL1 protein is synthesized directly in the SAM upon floral induction, where it promotes initiation of the IM, forming transcriptionally active FACs. Interaction with the 14-3-3 proteins, as well as with OsFD1, 4 and 7 was tested and confirmed through BiFC and FRET-FLIM assays. Analysis of FTL1 non- functional mutants shows that the expression of flower-identity genes OsMADS1, 5 and 15 is reduced [47].

Finally, confocal microscopy on meristems from FTL1-YFP reporter lines and *in situ* hybridization shed light on the specific localization and persistence of FTL1 transcript and protein in the developing meristem (Fig. 7) [47]. Standing on these experiments, FTL1 is transcribed and translated in meristem tips of IM to FM stages. The coincidence of RNA and protein localization lead to thinking that FTL1 has cell-autonomous activity.

According to this research, flower commitment would be primarily induced at the SAM by FACs containing Hd3a and RFT1. The localized activation of FTL1 expression in reproductive meristems would trigger the formation of FACs containing FTL1, leading to subsequent meristematic transitions and ultimately contributing to panicle determinacy.

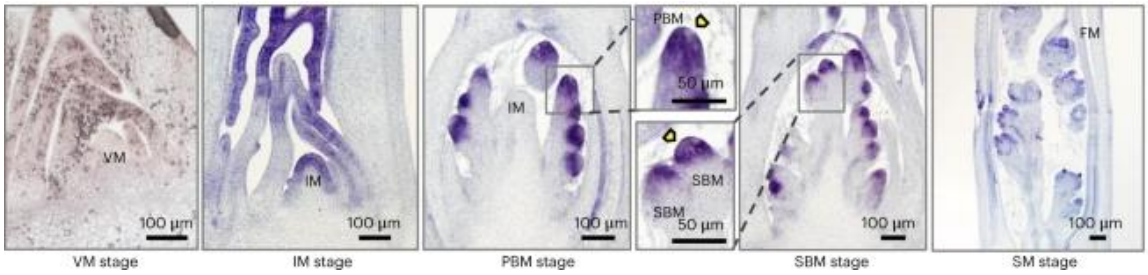

Figure 7. **In situ hybridizations of FT-L1 on wild-type meristems at the vegetative meristem (VM), IM, PBM and SBM stages.** FTL1 starts to be expressed at IM stafe. A tip-localized expression, indicated by yellow arrowheads, is evident in the examination of PBMs and SBMs. FTL1 transcription is finally visualizable at FM stage, on well-developed floral organs. Modified and adapted from Giaume et al., 2023 [47].

I contributed to this research by generating FTL1-YFP reporter lines, to be used for confocal microscopy experiments, through rice calli transformation. I also carried out transgenic plants genotyping and *in silico* phenotyping of panicle architecture in *ftl1* mutant lines.

4. Interactomic approaches to rice flowering networks: Proximity Labelling

The principal scope of my PhD project was the implementation, for the first time in rice, of a cutting-edge interactomic approach, called proximity labelling (PL), applying it to the study of Hd1 and FTL1 flowering regulators.

PL is a powerful, recently developed technique in molecular biology, enabling the identification of protein-protein interactions (PPIs) of a protein of interest, also at the subcellular level within living cells. The protocol requires the expression of a biotin ligase fused to the target protein of interest. Biotin ligase is an enzyme that catalyzes the covalent attachment of a small labeling molecule - biotin - to nearby proteins *in vivo* [60–62] . PL offers several advantages over traditional proteomics, such as the ability to study PPIs in their native cellular environment, as they are before cell lysis and sample processing. This method provides temporal and spatial information about protein associations, independently of their stability or duration, aiding in the understanding of dynamic cellular processes, like those often occurring during plant development (Fig. 8).

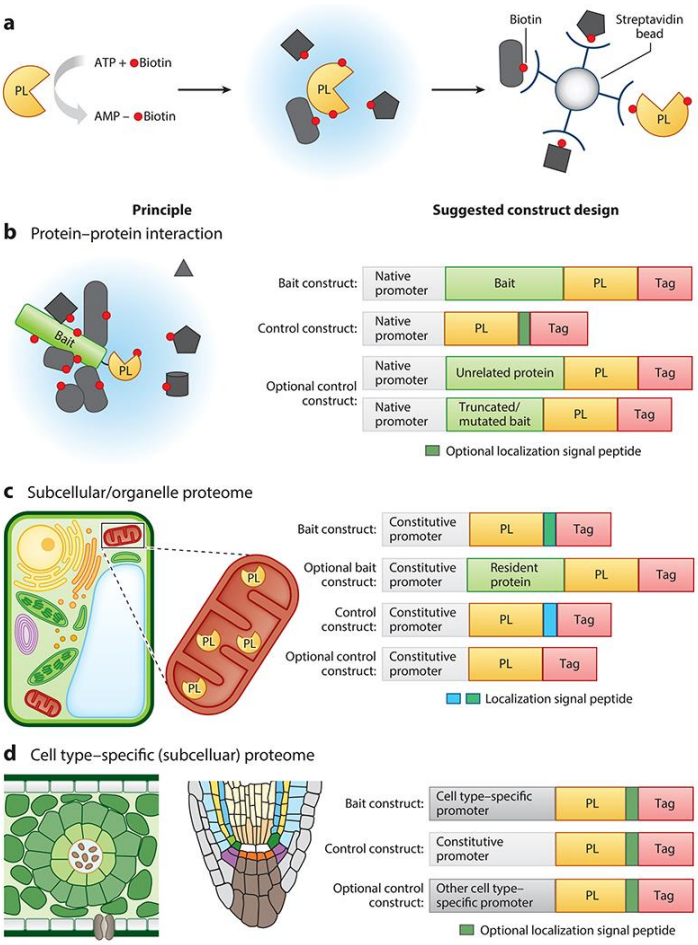

Figure 8. **Proximity labelling (PL) serves as a versatile tool for investigating PPIs, subcellular and organelle proteomes, and cell type–specific proteomes.** (a) In PL, biotin ligase reacts with biotin and ATP, forming a reactive intermediate that biotinylates nearby proteins, facilitating their subsequent enrichment and mass spectrometric analysis using streptavidin beads. For protein–protein interaction studies (b), the biotin ligase is fused to an endogenously expressed bait protein, to study its interactors. (c) In subcellular contexts , PL can be applied to specific cellular compartments, revealing insights into protein landscape. (d) Cell type–specific proteomes are explored using PL with strategically designed constructs, employing tags that allow visualization of distinct cell types in plants. Adapted from Xu et al., 2023 [61].

Moreover, the technology allows the investigation of interactions that might be disrupted during traditional cell fractionation procedures [60–62].

In my PhD research, we employed an ameliorated biotin ligase, called TurboID (TbID) obtained by directed evolution of BioID, a previous engineered version of the original *E. coli* enzyme. This mutated version results in a considerably more robust and faster labelling process [63,64]. Indeed, one distinctive feature of TbID is its accelerated kinetics, enabling researchers to capture protein-protein interactions over shorter labelling times, compared to previous versions, and at room temperature [63,65].

PL methodology involves the following steps: i) expressing the TbID tag fused to a protein of interest; ii) activating the enzyme with exogenous biotin supplementation; iii) isolating biotinylated proteins through selective immunoprecipitation with Streptavidin-coated beads, for subsequent mass spectrometry (MS) identification. This streamlined workflow facilitates efficient and accurate profiling of PPIs for a protein of interest, providing a

picture of the nearby proteome (TbID labelling radius 10nm) in a defined moment of plant growth and development.

During my PhD, TbID-fused FTL1 and Hd1 have been cloned and tested in *N. benthamiana*, rice protoplasts and rice transgenic plants. Here, I present detailed data on fusion protein stability, both in transient over expression systems and in native rice tissues. Also, I present data on protein biotinylation efficiency and on its dependence on a series of internal and external parameters.

Effective protocol application delivered two independent PL proteomic datasets, and more will be produced in the next months. A first, less specific, PL dataset on Hd1-TbID in *N. benthamiana* has served to validate the reliability of the technical pipeline and provide hints on new possible candidate genes controlling flowering time in rice.

The second dataset, containing thousands of proteins, has been obtained from flowering meristems of rice FTL1-TbID transgenic plants, through a similar protocol not envisaging biotin supplementation. The analysis of the candidate interactors has largely opened our view on FTL1 properties, in terms of both cell localization and protein function. A part of the data is coherent with previous literature on FT-like proteins. However, a new series of experiments will be set up to validate interactors with the highest priority.

The information derived from PL implementation for the study of rice flowering genes is considerable and will possibly stimulate future research during next years.

Part of the here-presented experiments have been carried out in the laboratory of Prof. Cristina Ferrándiz, at IBMCP-UPV (Valencia, Spain), where I stayed during 6 months thanks to an EMBO short-term fellowship award.

RESULTS

1. New perspectives on Hd1 function

At the beginning of my PhD, most of the work on Hd1 was focused on the study of protein stability and on its ability to bind DNA, other than already reported florigen promoter regions.

The pACT:FLAG-Hd1 transgenic line of the *O. sativa sub. japonica* Nipponbare (NB) variety was used to evaluate protein stability and eventual variations in its abundance at different day times, in a 24h time frame and after growth under long days (LD) conditions. As shown in Fig. 1b, overexpressed FLAG-Hd1 protein is constantly present and equally abundant at all considered timepoints, suggesting that it is not subject to time- or light-dependent protein degradation.

This is in contrast with wild type Hd1 protein levels, whose accumulation follows specific fluctuation in a daily rhythm, as shown by Yang *et al.* (2015). Quantification of *Hd1* transcription in the same plant material used for protein preparations, indicated that protein abundance follows gene transcription (Fig. 1a). This suggests that gene expression, rather than protein specific degradation, is the mechanism through which Hd1 abundance over a daytime is controlled. This aspect differentiates Hd1 from CO.

Sun et al., 2016 reported that Hd1 is phosphorylated by the HDR1-OsK4 complex. In our experiments, a finest western blot visualization of FLAG-Hd1 under LD and short day (SD) conditions indicates that the FLAG-Hd1 protein is present in two forms of slightly different mass, which is suggesting the existence of a post-translationally modified version of Hd1, compatible with phosphorylation. Also, a high band, consisting with a large mass agglomerate (around 250 kDa), in both photoperiodic conditions, is visible and may indicate a homopolymerization event which was not reported by other researchers before (Fig. 1). The fact that this alleged complex is visible on a denaturing polyacrylamide gel leads to thinking that the interaction is strong and stable in time.

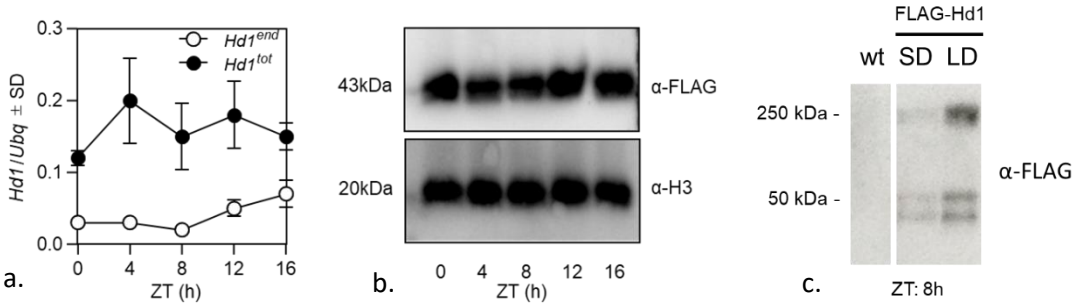

Figure 9. **Expression pattern of Hd1 and FLAG-Hd1 stability during the day.** Wild-type *Hd1* transcription follows daily rhythmic fluctuations in the wild type (a). Under long day conditions, FLAG-Hd1 protein expressed under the control of the actin promoter is equally detectable at all the considered timepoints (daylight at ZT 0, 4, 8, 12 and nighttime at ZT16) and is correlated to transcription (b). At reduced band intensities, a double band is visible, compatibly with FLAG-Hd1 phosphorylation, in both SD and LD conditions.

Leaf RNA-sequencing, carried out on *hd1-1* and wild type rice plants, has demonstrated that Hd1 controls the expression of several genes depending on photoperiodic conditions (Fig. 10). A number of genes belonging to the phenylpropanoid pathway was particularly enriched in the transcriptomic dataset, so that we decided to investigate whether Hd1 could directly control the transcription of non-florigenic genes under the two photoperiodic conditions. Realtime PCR and ChIP-PCR were carried out on two selected genes encoding two enzymes involved in the phenylpropanoid biosynthesis: *PHENYLALANINE AMMONIA LYASE 4* (*PAL4*) and *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*) to validate and extend RNA-Seq results. RR-PCRs performed during a time course under both LD and SD, showed that, in the *hd1-1* mutant, PAL4 is upregulated under LD and downregulated under SD conditions, when compared to the wild type. On the other hand, CAD is only slightly downregulated in *hd1-1* under

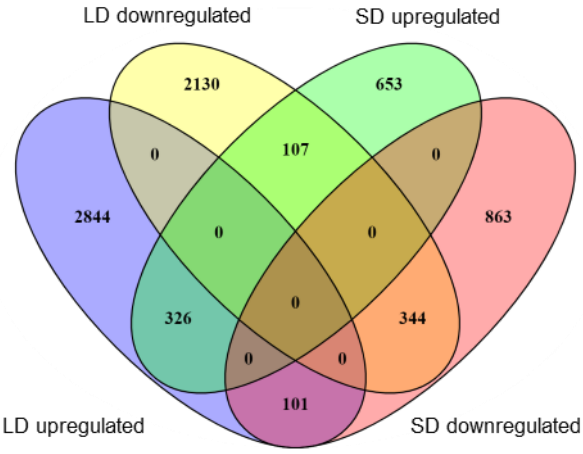

Figure 10. **Differentially expressed genes in *hd1-1* plants under SD and LD conditions.** The Venn diagram shows the proportion of genes that are either up or downregulated in the *hd1-1* mutant under photoperiodic inductive and non-inductive conditions, compared to wild type rice. From Biancucci et. al, *in preparation.*

LD conditions. In both cases, transcription has rhythmic fluctuations during the day that are compatible with circadian regulation (Fig. 11a). These data indicate that the transcription of the two genes is diurnally controlled.

We took advantage of the line expressing FLAG-HD1 to conduct ChIP-PCR and verify whether Hd1 was able to bind the promoter regions of PAL4 and CAD (Biancucci et al. *in preparation*).

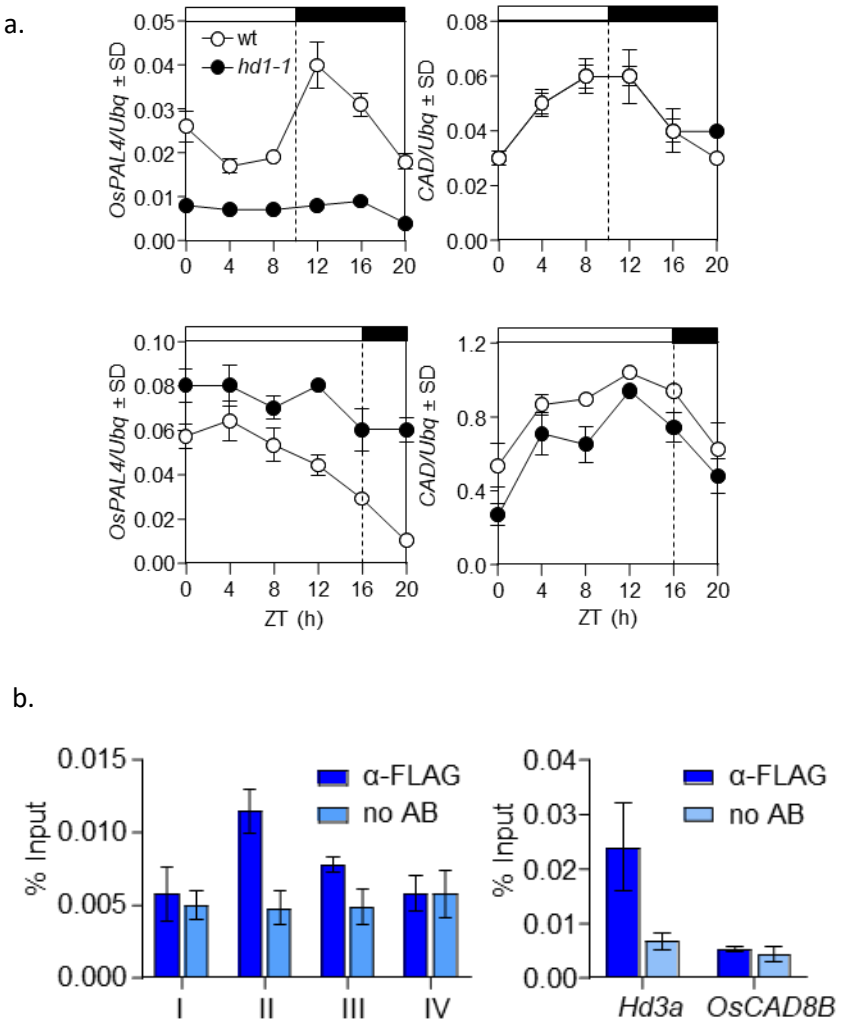

Figure 11. **OsPAL4 and CAD expression patterns and ChIP-PCR.** PAL4 and CAD have a transcription pattern partially dependent on the presence of Hd1 and on the photoperiod. A dramatic effect of Hd1 absence on PAL4 expression is particular evident in *hd1-1* mutant (a). ChIP-PCR performed on pACT:FLAG-Hd1 lines shows that FLAG-Hd1 is able to bind PAL4 promoter in the region covered by primer pair II (ca. 2000 bp upstream of the coding sequence). By contrast, CAD8 promoter is not its direct target (b). The *OsCORE2* region in the *Hd3a* promoter was used as positive control.

Since the FLAG-HD1 protein is abundant and stable across a LD, we decided to sample leaves at ZT1, using the *Hd3a* promoter region around *OsCORE2* as positive control [17]. Only *PAL4* resulted to be a direct target of Hd1, in a region ca. 2000 basepairs from the gene ATG sequence, which was identified by using specifically designed primer sets (Fig. 11b). Therefore, it might be possible that transcriptional regulation of PAL4 and CAD genes is directly regulated by Hd1 binding on promoter regions in the former, and by indirect mechanisms in the latter.

Transcriptomic analyses had shown that >6000 genes are differentially expressed (either up- and down-regulated) during the photoperiodic shift in the *hd1-1* mutant when compared to the wild type. These genes constitute around 30% of the total RNA-seq dataset, and their biological role could be reconducted to several cell processes (Biancucci et al. *in preparation*).

The hypothesis that Hd1 could influence leaf metabolic pathways, emerging from these experiments, lead us to consider total leaf proteome analysis as complementary to transcriptional studies. This approach was employed to unveil possible effects of Hd1 which were not reflected into transcriptomic data. For this purpose, wild type, pACT:FLAG-Hd1, *hd1-1* NB rice lines were grown under SD and LD conditions. For all the rice lines, leaf material was collected from 1-month-old, LD-grown plants, as well as 15 days after shift to SD conditions. Total leaf proteins were extracted and subjected to mass spectrometry (MS) whole proteome analysis (Fig 12).

More than 6000 proteins were identified in our leaf samples, and differential abundance was calculated in the different lines and photoperiodic conditions (Supplementary table 1). The dataset was then analyzed through distinct bioinformatic approaches. The volcano plots represented in Fig. 3 are a good way to visualize the enrichment profiles when different lines and conditions are compared, since they consent to quickly notice differences in the distribution of large protein datasets. In the scattered plots represented here, identified proteins are subdivided in “hit” and “candidate” for each comparison, in a decreasing order of enrichment (see Methods), or non-differentially abundant (“no hit”). The number of differentially abundant proteins is visibly higher when two photoperiodic conditions are compared for the same line, indicating that day length is the first factor promoting leaf proteomic changes in our experiments.

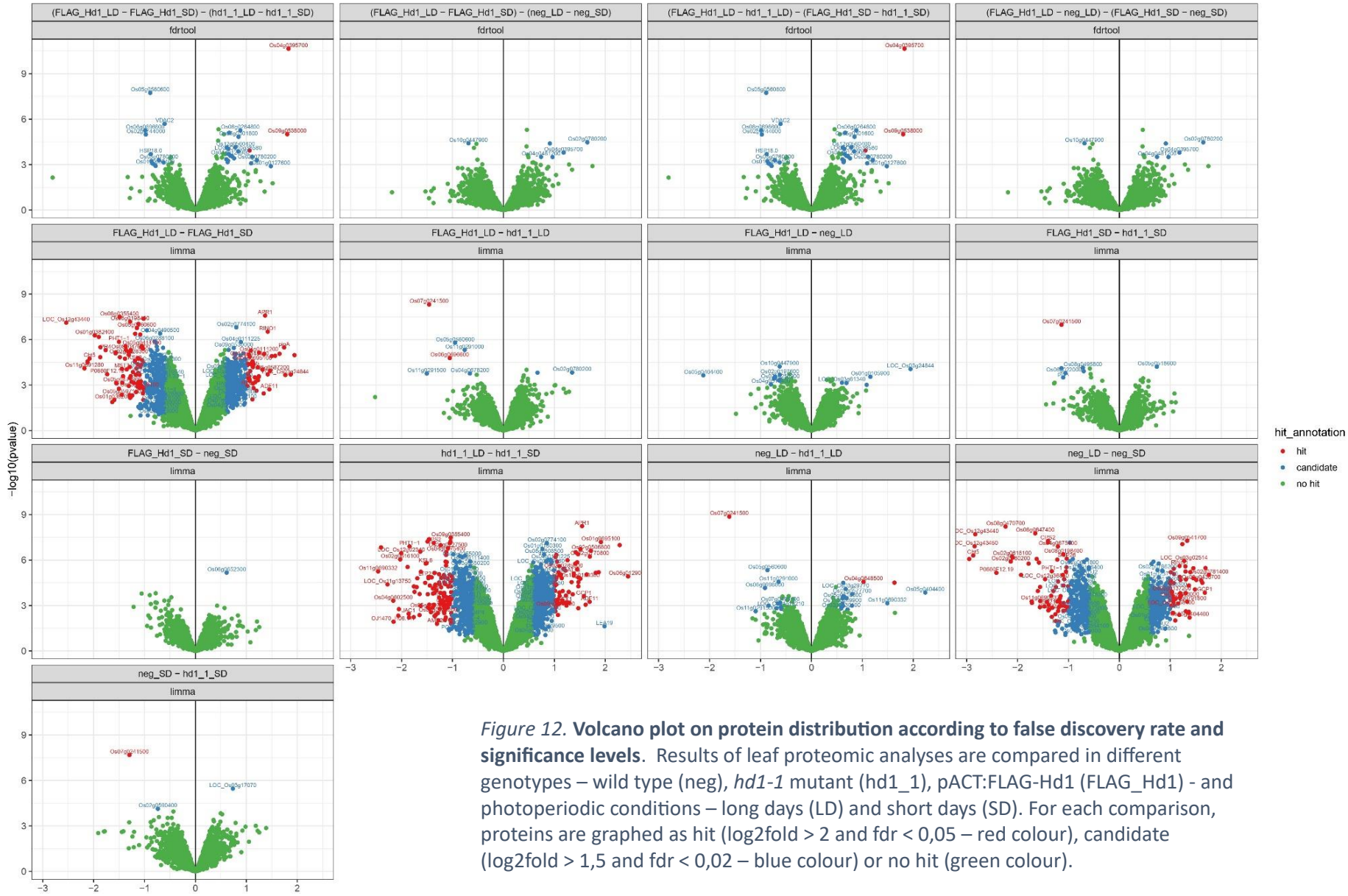

Figure 12. **Volcano plot on protein distribution according to false discovery rate and significance levels**. Results of leaf proteomic analyses are compared in different genotypes – wild type (neg), *hd1-1* mutant (hd1_1), pACT:FLAG-Hd1 (FLAG_Hd1) - and photoperiodic conditions – long days (LD) and short days (SD). For each comparison, proteins are graphed as hit (log2fold > 2 and fdr < 0,05 – red colour), candidate (log2fold > 1,5 and fdr < 0,02 – blue colour) or no hit (green colour).

Secondly, the absence of Hd1, followed by its overexpression, caused proteomic changes when shifting the plants from LD to SD conditions. Percentages of differentially abundant proteins between the two photoperiodic conditions over the total detected proteome are the following:

- 10% in the wild type
- 15% in *hd1-1* mutant
- 11 % in pACT:FLAG-Hd1

Considering that the protein coverage of the dataset is less than 1/3 of the transcriptomic one, it could be inferred that many proteins encoded by differentially expressed genes in the hd1-1 mutant during the photoperiodic shift cannot be visualized due to their lower abundance or to the low range of the MS analysis. It cannot be excluded either that proteomic changes do not finely mirror transcriptomic ones, as the latter could be post-translationally rebalanced.

KEGG and GO analyses were performed on hit and candidate proteins to observe if the quantitative changes observed also reflected in qualitative alterations of specific biosynthetic pathways (Fig. 13 & 14).

The rectangle chart in Fig. 13 clearly shows how specific categories are differentially represented in the *hd1-1* mutant, such as biosynthesis of aminoacids, biosynthesis of cofactors and glyoxylate and dicarboxylate metabolism, whereas others, such as primary and secondary metabolism, or nitrogen metabolism show little variation between the different genotypes.

GO analysis, graphed in the Dotplot of Fig. 14, partially overlaps with these findings, being the synthesis of organonitrogen compounds more represented in the *hd1-1* mutant. Notably, the category hemicellulose synthesis is overrepresented in pACT:FLAG-Hd1 when compared to *hd1-1*.

When referring to GO functional categories, GDP-L fucose synthase is largely represented in pACT:FLAG-Hd1 when compared to the negative control. This enzyme belongs to the oxidoreductases family and is important to guarantee the availability of activated sugars for the synthesis of cell wall glycans [66].

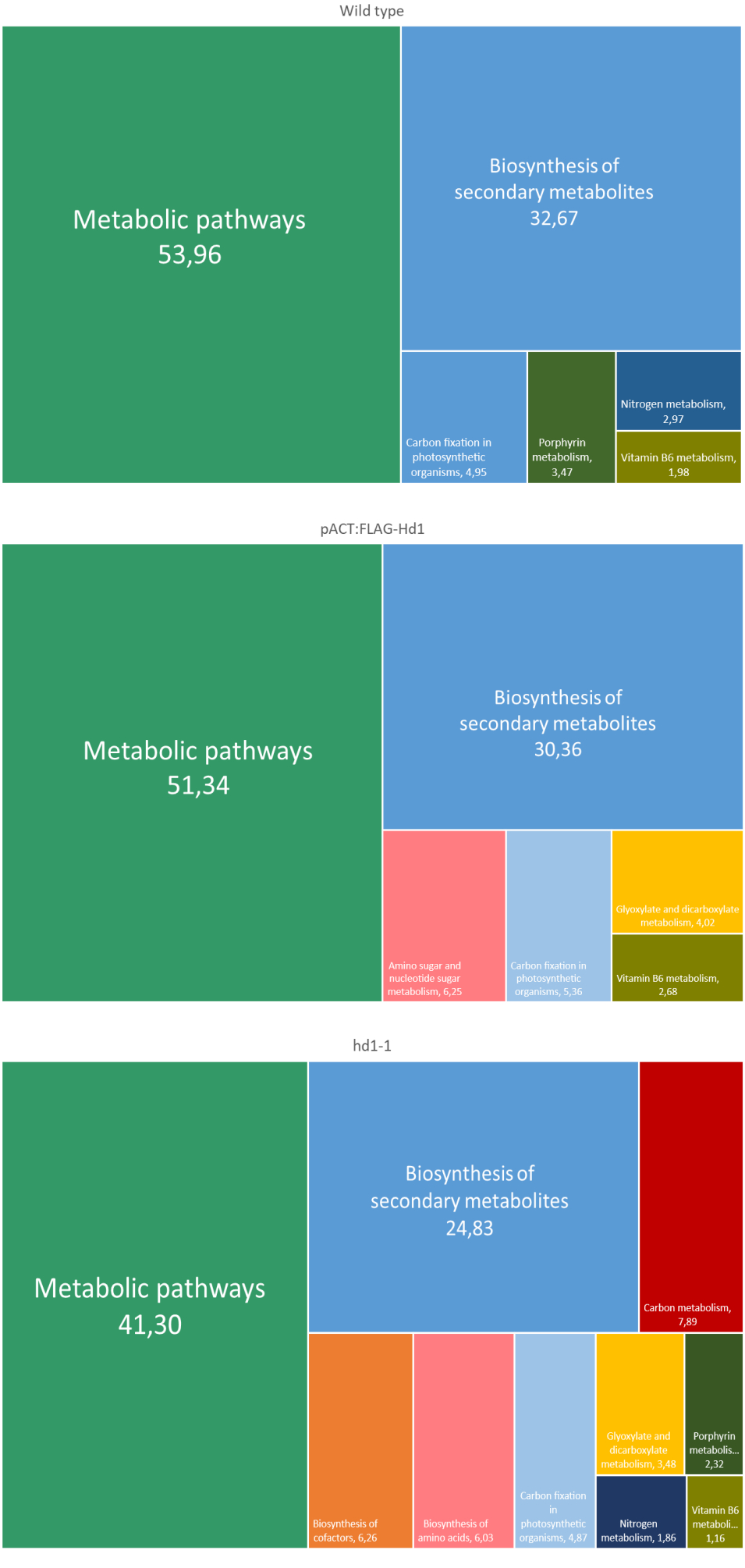

Figure 13. **Rectangle chart on the biosynthetic pathways that are enriched during the photoperiodic shift in wild type, pACT:FLAG-Hd1 and *hd1-1* plants.** The graph shows the KEGG categories that are represented by hit and candidate proteins when comparing LD and SD leaf proteome inside of the same genotype. The dimension of each rectangle is proportional to the percentual coverage of the KEGG categories on the total number of enriched proteins. Colours are indicative.

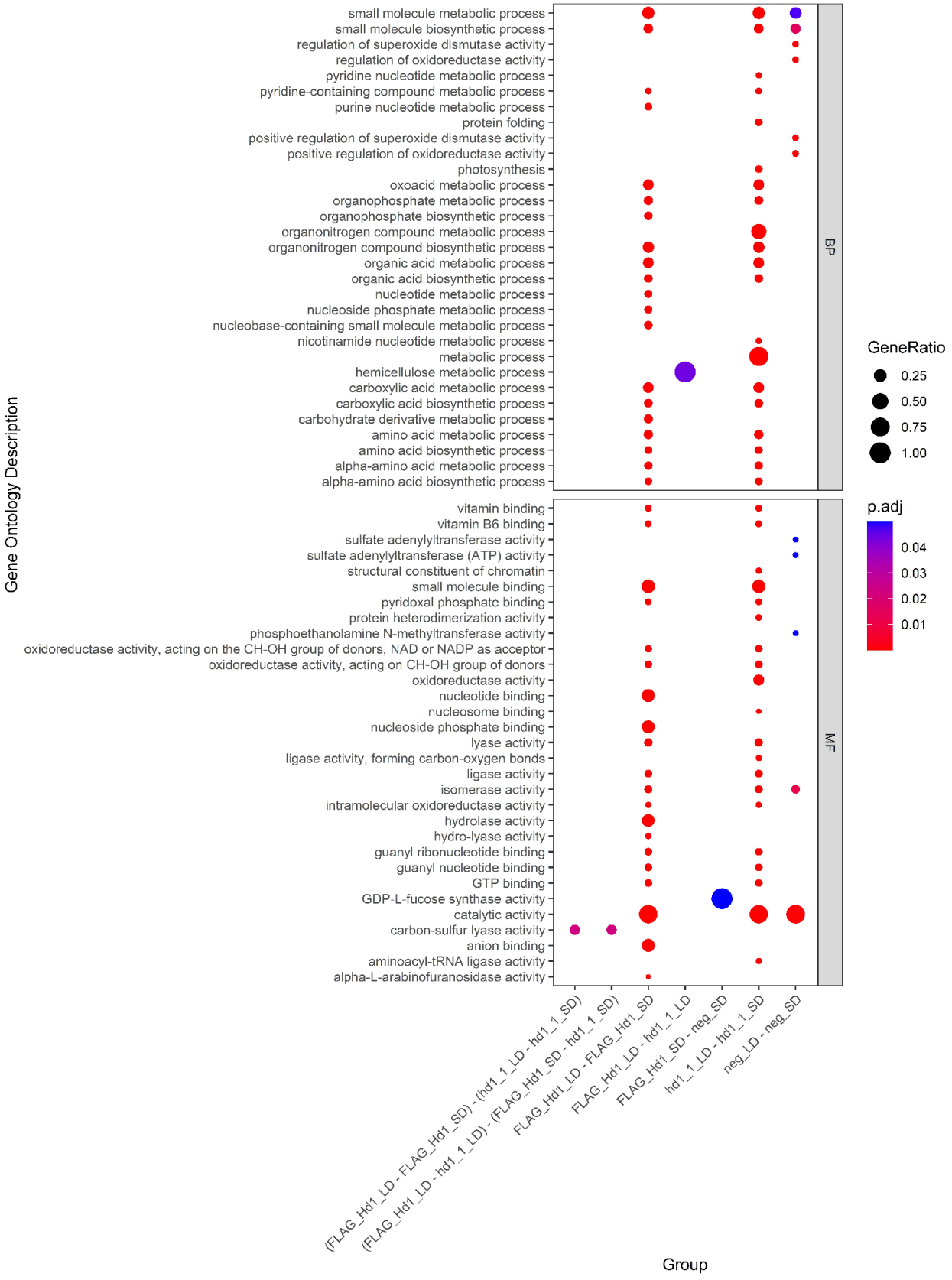

Figure 14. **Dot plot on GO biological process and molecular function categories for leaf proteomic analysis.** Enrichment for specific GO categories (y axis) in each genotype-*vs*-photoperiod comparison (x axis) is represented by dots. Dot dimension is proportional to differential protein abundance, while the colour is related to level of significance.

Overall, the interpretation of proteomic results clearly suggests, for the first time, an involvement of day length in the control of cell wall formation and/or composition, in a way that still needs to be clarified. A deeper bioinformatic analysis of transcriptomic and proteomic data correspondence is being performed to corroborate this finding.

2. Traditional approaches to test FTL1 interactors

Yeast two hybrid

Before the start of my PhD, bimolecular fluorescence complementation (BiFC) and FRET-FLIM assays had demonstrated the capacity of FTL1 to interact with the transcription factors OsFD1/OsbZIP77, OsFD4/OsbZIP69 and OsFD7/OsbZIP62, as well as with 14-3-3 proteins to form florigen activation complexes [47]. Kaur et al., 2021 [57] had previously shown that the interaction with OsFD7 is strongest than with Hd3a/RFT1, and that it can take place also with no intermediary proteins. In my PhD, I screened 3 additional rice OsbZIP proteins for interaction with FTL1 using a yeast-two hybrid (Y2H) assay. In the past, these transcription factors - namely OsbZIP24/OsFD3, OsbZIP42/HBF1, and OsbZIP9/HBF2 - had been tested in a similar way for the ability to form complexes with the main rice florigens Hd3a and RFT1, resulting in various combinations of interactions [54–58] . To further study their potential role in flowering, FTL1 was cloned into both activation domain (AD) and DNA-binding domain (BD) vectors and interactions were tested in both senses in every case.

A positive interaction was observed between BD FTL1 and AD bZIP24/OsFD3, but not in the other way. Also, FTL1 was able to interact with himself, and a slight interaction between AD FTL1 and BD Hd3a was also observed (Fig. 15).

It must be noted that florigen homo- and hetero-dimerization is compatible with the hypothesis of hybrid florigen activation complexes. Notably, no growth was observed in yeast colonies expressing FTL1 and the transcription factors bZIP62/OsFD7 and bZIP69/OsFD4, in any of the AD/BD combinations tested (Fig. 15).

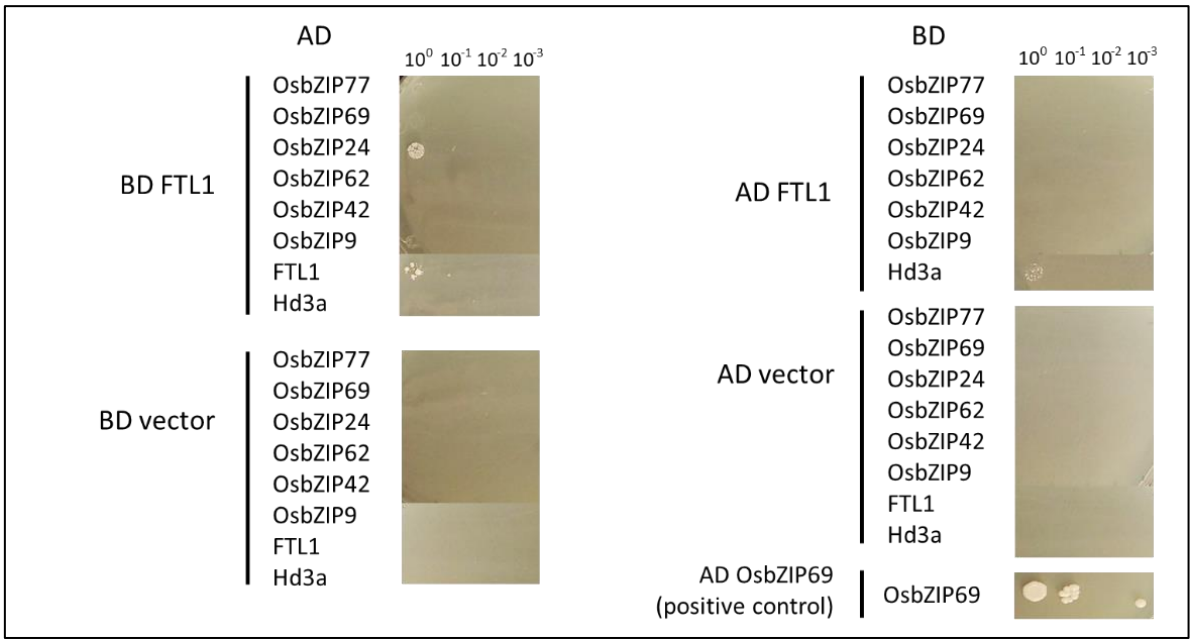

Figure 15. **Yeast-two Hybrid on FTL1 and a group of OsbZIPs**. Yeast colonies are grown on selective media containing 5 mM 3AT and observed 4 days after plating. Each interaction is tested in both BD and AD senses. Positive interactions are observed between BD FTL1 and AD OsbZIP24, and between AD FTL1 and BD Hd3a. A slight tendency to homodimerization in BD-AD FTL1 is also observed. Self-interacting AD-BD OsbZIP69 is used as positive control, as from Brambilla et al., 2017 [67].

OsbZIP24/OsFD3 is phylogenetically close to OsbZIP77/OsFD1 [56]. To validate the potential interaction between FTL1 and bZIP24/OsFD3, a coimmunoprecipitation (co-IP) assay in agroinfiltrated *N. benthamiana* leaves, using constructs expressing FTL1-YFP and bZIP24-RFP, is being performed in the next month.

If FTL1 and bZIP24 form a transcriptionally active complex in rice flowering meristems, a phenotypic effect of bZIP24 loss of function could resemble that of *ftl1* mutants. *bZIP24* CRISPR NB rice lines were available in our laboratory and had already been studied for anomalies in flowering time, showing no difference with the wild type [55]. Since FTL1 also control meristem differentiation and panicle branching, it is still possible that a phenotypic effect in panicle architecture can be observed. For this reason, *bZIP24* CRISPR lines are now being propagated to be used for phenotypic analysis.

3. Preliminary tests of Hd1-TbID and FTL-TbID in *N. benthamiana* and rice protoplasts

The main requirement to exploit the PL approach is the generation of transgenic plant lines expressing the proteins of interest fused to the engineered biotin ligase Turbo-ID (TbID). Before starting the time-consuming *in vitro* generation of rice transgenic lines, the feasibility of the system was initially tested in transient assays, to check the stability of fusion proteins and their biotinylation efficacy.

At first, FTL1 and Hd1 stable expression was tested in Hd1-YFP and FTL1-YFP *Nicotiana benthamiana* agroinfiltrated leaves. The choice of the C-terminal tag was inspired by previous research experience and studies on protein structure and was considered as proxy for biotin-ligase cloning.

N. benthamiana leaves agroinfiltrated with p35S:FTL1-YFP and p35S:Hd1-YFP were observed under confocal microscopy to check the constructs expression. Both proteins were visible and localized to nuclei as expected (Fig. 16). To quantitatively test protein stability over time, western blots were performed on protein extracts, obtained from leaves sampled 3, 4 and 5 days after agroinfiltration, using an anti-YFP antibody. WBs showed that FTL1-YFP was abundant and easily detectable since day 3 after agroinfiltration. Hd1 stability, instead, was comparably lower, and the protein was detectable only at 4 days after agroinfiltration (Fig. 16). This atypical accumulation pattern could result from the coincidence of insufficient synthesis (day 3) and fast degradation (day 5) processes on the exogenous protein. Further protein losses during and after extraction, due to any Hd1-specific stability property, cannot be excluded.

Interestingly, on day 5 Hd1-YFP agroinfiltrated leaves started to present necrotic symptoms in the infiltrated area, and this effect was observed several timesalso for other Hd1-contanining constructs.

Based on these experiments, day 3 and 4 after agroinfiltration were established as the optimal time points for sampling leaves agroinfiltrated with FTL1- and Hd1-expressing constructs, respectively.

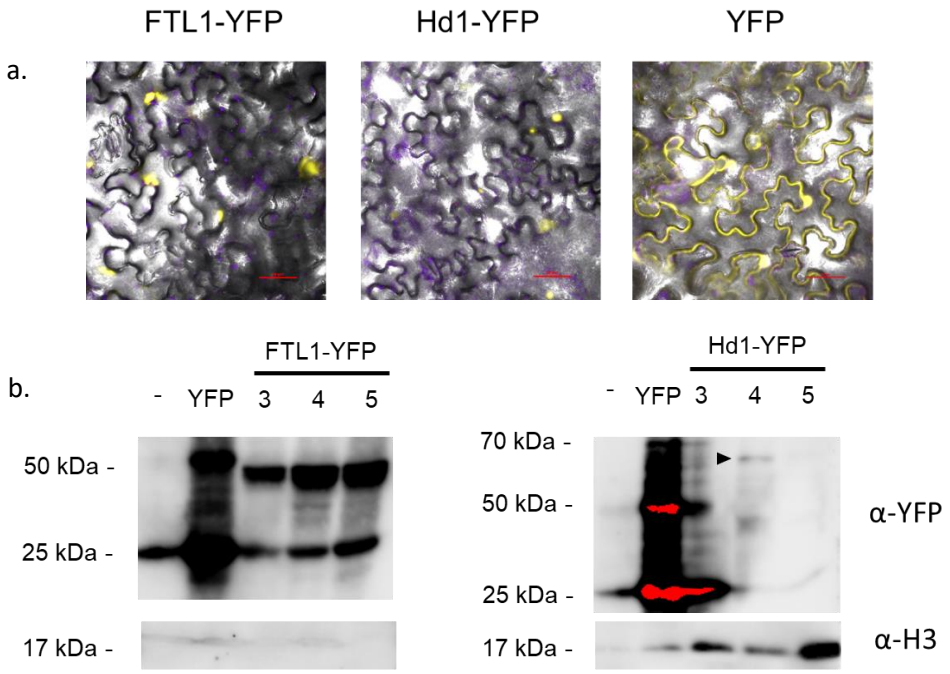

Figure 16. **Hd1-YFP and FTL1-YFP stability and cell localization.** YFP fusion proteins, under the control of 35S promoter, were agroinfiltrated in *N. benthamiana* leaves and observed under confocal microscopy. Hd1-YFP and FTL1-YFP localize to the nuclei, whereas the YFP control is ubiquitous. Scale bar is 50 µm (a). Protein accumulation through time was determined by western blot. FTL1-YFP is easily detectable, starting from day 3 until day 5 after agroinfiltration. Hd1-YFP is less abundant and detectable only at day 4 after agroinfiltration (b).

For PL experiments and preliminary biochemical analyses, FTL1 and Hd1 sequences were cloned N-terminally fused to the TbID biotin ligase. To facilitate protein synthesis in transgenic rice lines, the sequence of the enzyme was optimized according to rice codon usage. It is important to mention that an HA tag is also included in the TbID tag, before the stop codon, to allow immunological detection of the fusion proteins (Fig. 17).

Hd1 and FTL1 coding sequences were separated from the TbID tag by a disordered linker sequence made up by a repetition of the GGGGS sequence. To fully test PL technology efficiency, each protein was designed in two slightly different versions, one with a short linker (2xGGGGS)[68] and another one with a long linker (12XGGGGS) (unpublished).

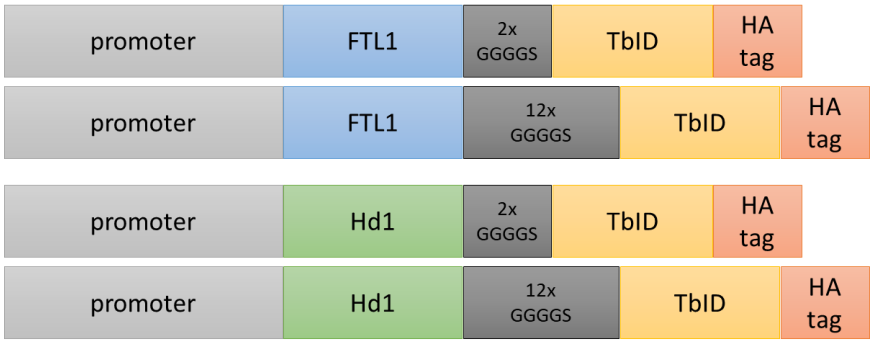

Figure 17. **Schematic fusion protein structure.** FTL1 and Hd1 are N-terminally fused to TbID with a short (2XGGGGS) or a long (12xGGGGS) linker. In the TbID, an HA tag is included at the C terminus of the enzymatic moiety. Promoters are adapted to the experimental system: throughout our experiments, CaMV 35S, maize ubiquitin, GOS2 and FTL1 promoters were used.

The intention behind this approach is to test whether a wider physical separation between the protein of interest and the TbID tag could eventually influence the function of the protein of interest. Moreover, since the cloning strategy employed a Gateway system, an additional *attB* recombination site is included at the C-terminus of the gene coding sequence, in frame with the linker and the *TbID* coding sequences (Fig. 17).

The choice of promoters was adjusted to each experiment and to the employed plant model. Protein stability studies of TbID fusion proteins were carried out by *N. benthamiana* agroinfiltration, cloning the fusion proteins under the control of 35S promoter. Western blots were performed on protein extracts using an anti-HA antibody, which confirmed that FTL1-TbID and Hd1-TbID were present at the expected molecular weight in transiently transformed leaves (Fig. 18).

In a different kind of experiment, exogenous biotin was provided to agroinfiltrated *N. benthamiana* leaves, and total biotinylated proteins in leaves extracts were observed in WBs using streptavidin-HRP to determine the efficacy of total biotinylation.

To identify the optimal conditions for a PL assay, two different biotin concentrations (50 and 100 µM) and two different exposition times (biotin given for 1 and 3 hours before sampling) were tested.

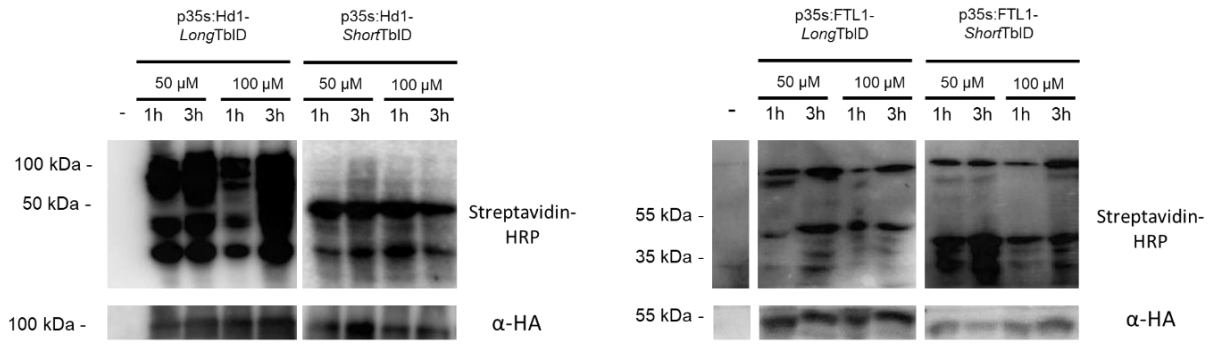

Figure 18. **Stability and biotiniylation rate of TurboID fusion proteins in agroinfiltrated *N. benthamiana* leaves.** Anti-HA antibody is used to visualize Hd1- and FTL1-TbID, which are easily detectable in *N. benthamiana* leaves . Streptavidin-HRP is used to visualize total biotinylated proteins under different biotin concentrations (50 uM and 100 uM) and exposition times (biotin provided for1 and 3 hours before sampling). Biotinylation rate varies according to protein type (Hd1 or FTL1), linker length and conditions of biotin infiltration.

Selected biotin concentrations are significantly higher than those of most plant cells, which have been assessed to be <13 µM, depending on cell type and cellular compartment [69–71].

The Km of BioID (the initial, less efficient version of *E. coli* biotin ligase used for PL) for biotin is 0.3 µM, so that endogenous biotin levels are theoretically sufficient for effective protein biotinylation [68]. Considerable exogenous biotin supplementation, used in our experiments, is to be taken as an adjustment to *N. benthamiana* agroinfiltration assays (heterologous protein overexpression, low exposition times, abnormal protein turnover and accumulation patterns).

For all the conditions in our experiments, the amount of total biotinilyated proteins is visibly higher than in the non-infiltrated control. A general tendency to higher biotinylation rate is observed when the exposition time is increased. Also, the linker length seems to influence the final result, given that the amount of biotinylated proteins is equal or higher in the long-linker protein versions when compared to the short-linker ones. This observation is in agreement with the fact that a long linker allows the TurboID moiety to reach more proximal proteins. However, Hd1-TbID biotinylation efficiency was particularly affected by the above-mentioned parameters, whereas FTL1-TbID protein seemed to have a more stable biotinylation activity (Fig. 18).

The results of preliminary tests on TbID fusion proteins in *N. benthamiana* agroinfiltrated leaves indicate that, despite rice codon usage, the expressed proteins are synthesized and stable in this overexpression system, and the output is particularly improved for Hd1-TbID variants, if compared to the results on Hd1-YFP mentioned in the previous paragraph. Also, these experiments confirm that the fusion proteins are enzymatically active and that the biotinylation rate responds to external conditions, depending on the nature of the considered protein.

Along with *N. benthamiana*, also rice protoplast cultures were partially tested for transient expression of TbID fusion proteins in our laboratory. Though artificial, rice protoplasts transformation system can be highly informative to transiently test the expression and interactions of proteins belonging to the same species. For protoplast isolation and transfection, the efficiency of two different protocols was tested [72,73]. The main difference between the two methods tested is the use of etiolated or non-etiolated seedlings for cell isolation, respectively. To validate each protocol pipeline, protoplasts were firstly transfected with p35S:YFP and observed under a fluorescence microscope (Fig. 19).

We were able to achieve successful protoplast isolation and transfection only by using the first protocol (i.e. using etiolated seedlings). The failure of the non-etiolated seedlings protocol may be attributed to a higher difficulty in cell isolation and maintenance in presence of a more developed cell wall.

Protoplasts were transfected with constructs expressing either the TbID fusion proteins, or the TbID alone, under the control of different promoters (pGOS2, pUBQ and p35S). Based on protein detection, maize ubiquitin promoter resulted to be the most appropriate for rice protoplasts expression, compared to GOS2 and 35S. All TbID fusion proteins, as well as the TbID only, were detectable in transfected rice protoplasts. Hd1-TbID proteins were visibly less abundant, as previously observed in *N. benthamiana* Hd1-YFP agroinfiltrated leaves. This may indicate that synthesis of Hd1 C-terminal fusion proteins has the tendency to be limited, or that the proteins themselves are less stable, depending on the context (Fig. 19).

As for *N. benthamiana* experiments, biotinylation tests were performed in transfected rice protoplasts, which were given exogenous biotin and sampled to detect total biotinylated proteins.

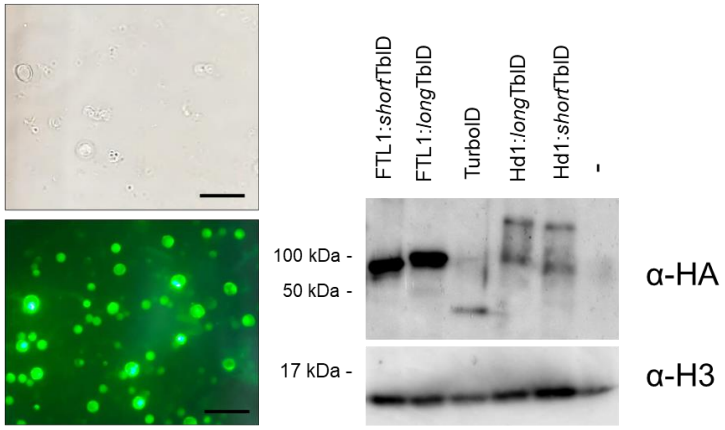

Figure 19. **Protoplast transfection with TbID fusion proteins**. Successful rice protoplast isolation and transfection with p35S:YFP are confirmed by optical and fluorescence microscopy (Scale bar = 40 µM). Expression and stability of TbID fusion proteins, under the control of maize UBQ promoter, is observed through WB using an anti-HA antibody: in both linker variants, FTL1-TbID proteins are the most stable, similarly to FTL1-YFP in *N. benthamiana* agroinfiltration experiments.

As for *N. benthamiana*, two different biotin concentrations (50 and 100 µM) and two different exposition times (biotin given 1 and 3 hours before sampling) were established as test parameters. Despite several attempts, there was no chance to visualize biotinylated proteins in any protoplast extract.

This may be due to a lack of ATP in dark-grown cells, which would not allow the TbID enzymatic reaction of biotinylation. Also, total protein concentration in protoplast extracts, measured with Bradford solution, was in every case below the threshold of protein detectability. This leads to think that the abundance of the heterologous protein – even if immunologically detectable – was not sufficient to cause any changes in biotinylation patterns.

The experiments in rice protoplasts cultures show that the expression of heterologous constructs is particularly sensitive to the promoter choice, and that TbID fusion proteins are generally stable and detectable in rice cells, also at very low total protein concentrations. Higher signal intensities observed for the bands corresponding to FTL1-TbID variants in WBs could be attributed to FTL1-specific properties, such as higher protein hemi-life, compared to TbID and Hd1-TbID.

4. Transient application of proximity labelling in *N. benthamiana*

Standing on the results of preliminary tests, *N. benthamiana* was chosen as the most appropriate system for a straightforward transient trial of the PL protocol in our laboratory. This choice was dictated by two reasons. The first was to set up a rapid system that would allow for completion of the biotin labelling, immunoprecipitation, and protein sequencing experiments, also given the technical challenges of using rice protoplasts. The second reason was to profitably use the time of vectors transformation into rice calli. The generation of stable transgenic rice expressing TurboID-tagged proteins, required a considerable part of my PhD. Considering native proteins localization, FTL1-TbID protein variants were excluded from these experiments because the protein is not itself expressed in leaves. Therefore, on top of the limitations imposed by the heterologous system, we were uncertain about the presence of potential interactors. On the other hand, though Hd1 is localized in leaves and is homologous to *N. tabacum* CONSTANS homologue (LOC107785832), the limitations on the biological relevance of the experiment were taken into account. Rice is a monocot, short-day flowering plant and is phylogenetically distant from *N. benthamiana*. As a premise to this PL experiment, it was intended that any potential interactor candidate for Hd1 resulting from a successful outcome must be validated in a more reliable system.

At the moment of leaf agroinfiltration and biotin treatment , the following parameters were selected:

- The long-linker version of Hd1-TbID, as well as the TbID alone, were agroinfiltrated in *N. benthamiana*. The choice over the linker length of Hd1 was made after comparisons of precipitation enrichment through WB and silver staining, as shown in Fig. 20.
- According to the accumulation pattern of Hd1 fusion proteins, all samples were collected 4 days after agroinfiltration.
- Based on preliminary biotinylation tests, 100 µM exogenous biotin was provided to the leaves, with an exposition time of 3 hours from biotin infiltration to sampling.

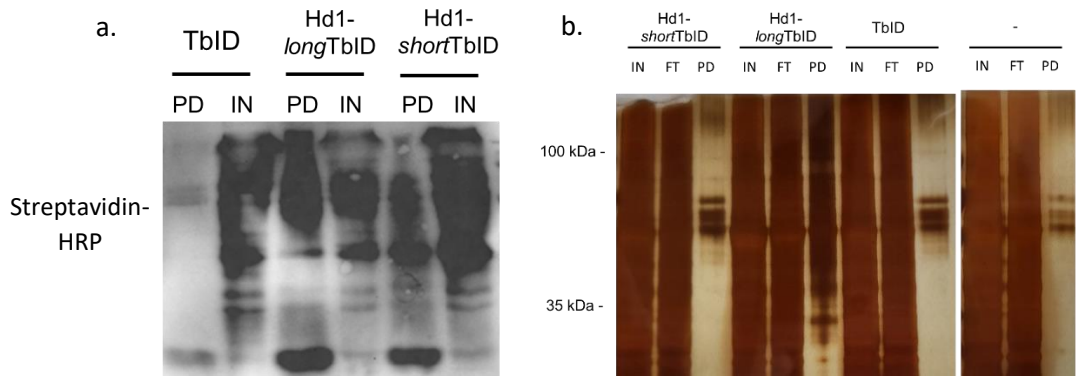

Figure 20. **Quantitative comparison of immunoprecipitated bioinylated proteins in *N. benthamiana* TurboID, Hd1-*shortlinker*TbID and Hd1-*longlinker*TbID agroinfiltrated leaves.** Immunoprecipitation input (IN), flow-through (FT) and pull-down (PD) are analysed. Streptavidin-HRP is used for western blot visualization of biotinylated proteins in *N. benthamiana* agroinfiltrated leaves. Signal intensity is higher in Hd1-*longlinker*TbID PD, compared to those of Hd1-*shortlinker*TbID and the TbID control (a). The silver staining confirms that the total protein content is highest in Hd1-*longlinker*TbID PD (b).

To maximally reduce protein degradation and consequent negative artifacts, protein extraction was performed immediately after sampling, and all the processing steps were done consecutively.

The application of PL approach on plant protein extracts requires the execution of two important steps, which are added to common protein extraction procedures:

- Desalting

This step is fundamental to remove free biotin and avoid immunological saturation of streptavidin-coated beads in the next step. Several trials with different desalting columns (PD-10 and Zeba Spin) were performed. The PD10 fitted best with high extract volumes (> 3 mL) which were not compatible with our extraction protocol. In fact, the dilution of protein extract, needed to reach the minimum volume to be filtered, resulted in great protein losses, and, as a consequence, in loss of biotinylated proteins enrichment. Zeba Spin columns, instead, can be used to process lower volumes (<300 uL) and were the best option to us, delivering high-quality filtered protein extracts without any contaminating compound with a mass lower than 7 kDa.

- Biotinylated proteins precipitation

Incubation of the desalted protein extract with C1 (Thermofisher®) streptavidin-conjugated magnetic beads allows the efficient recovery of biotinylated proteins from the desalted leaf extract. Though the producer advice is 1 hour incubation, we tested 4 hours and overnight incubation times to optimize the procedure. The latter condition was then used for all the further protocol applications to maximize protein enrichment. After incubation, thebeads and attached proteins were washed with a non-denaturing solution to remove any non-specific protein excess. The proteins were then detached from the beads, after boiling the mixture in sample buffer, to undergo mass spectrometry and/or protein enrichment tests.

In fact, before MS analyses, pull downs are required to be checked for protein content by silver staining and WB with streptavidin-HRP. As the results suggest, the pull down of Hd1-*longlinker*TbID was more enriched in biotinylated proteins than the TbID and the mock non-agroinfiltrated leaf controls (Fig. 20).

Mass spectrometry analyses on Hd1-*longlinker*TbID and TbID as a negative control was successful and the output was blasted onto *N. benthamiana* and *N. tabacum* protein datasets to optimize peptides assignment. This resulted in 97 *N. benthamiana* proteins, 16 of which were enriched in the Hd1-*longlinker*TbID samples, and 158 *N. tabacum* proteins, 41 of which were enriched in Hd1-*longlinker*TbID (Supplementary table 2).

Parameters for the definition of differentially abundant proteins are the same mentioned for previous proteomic analyses.

Many of the enriched proteins in the Hd1-*longlinker*TbID samples are related to general metabolic processes or to protein translation and degradation, which seem not to be specifically linked to any known function of Hd1 or its homologues (Fig. 21).

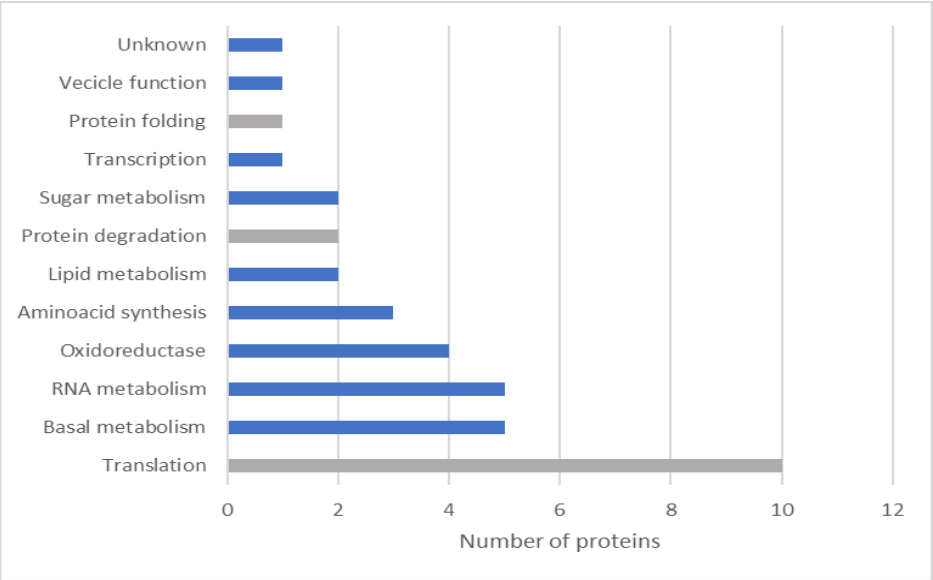

Figure 21. **Hd1-TbID proximal proteome in *N. tabacum* by GO categories**. Gene ontology (GO) categories for the list of significantly enriched proteins in N. benthamiana transient PL application are shown. Grey columns indicate protein functions that are related to TbID processing and degradation. Among specific metabolic processes, RNA metabolism and aminoacid biosynthesis are the most represented. The transcription category contains only *N. tabacum* as a candidate Hd1-TbID interactor.

The potential interactors list also contains *N. tabacum* FRIGIDA-LIKE (FRL) protein. FRIGIDA is a vernalization and flowering regulator in dicotyledons, and FRL proteins can be also found in monocotyledons. Even if rice is not a vernalization requiring cereal, its genome contains 8 FRL genes, whose role in flowering induction has not been elucidated yet. The proteins encoded by 3 of these genes show >90% homology to *N. tabacum* FRL and have been selected for further cloning into destination vectors to validate their possible interaction with Hd1.

Even though rice is a non-vernalizing species, its flowering induction pathway presents similarities to cold-climate cereals. If confirmed, the ability of Hd1 to interact with FRL proteins could help discover new aspects of the rice flowering induction pathway and would provide new knowledge on the interaction properties of CONSTANS-like protein. Independently of the biological interpretation of the generated proteomic dataset, the application of the PL approach in *N. benthamiana* was technically successful and represented a milestone to start interactomic studies on stably transformed rice lines.

5. Preliminary tests on transgenic rice lines expressing Hd1-TbID and FTL-TbID

In vivo PL proteomics implies the use of transgenic plants expressing the TbID fusion proteins preserving localization of the native protein of interest. In rice, the most common protocol for the generation of transgenic plants is based on *in vitro* calli culture and transformation. In our case, rice calli were transformed with both linker variants of Hd1- and FTL1-TbID, as well as with the TbID alone. For all the transgenic lines, the NB variety was used, in agreement with most of the rice scientific community. NB has the best annotated genome among sequenced rice varieties and its transformation efficiency is high.

pGOS2:Hd1-TbID lines were generated in the loss-of-function mutant *hd1-1* background. The GOS2 promoter activity is higher, yet comparable, to that of the Hd1 endogenous promoter, but lower than a strong promoter as the maize ubiquitin one. GOS2 promoter has been preferred over *Hd1* endogenous promoter because it ensures constant and homogeneous gene expression during the whole plant life cycle, presumably facilitating protein studies. pFTL1:FTL1-TbID lines were generated using the wild type, because at the time of transformation, Nipponbare FTL1 loss-of-function mutants were not available yet. For experimental convenience, pUBQ:TbID transformation was set only in the wild type Nipponbare background, to be used as negative control for both FTL1 and Hd1 PL experiments.

Transformation of rice calli is a time-consuming process and its success rates are highly variable. At the moment of writing, no rice plants expressing Hd1-*shortlinker*TbID are available yet.

Several independent Hd1-*longlinker*TbID had been obtained; transgene expression was confirmed by qPCR, but, unfortunately, all lines were eventually lost due to extremely late flowering and seed sterility phenotypes. Though attempts of vegetative propagation were done, this practice has little success in *in vitro*-generated plants, which tend to be less vigorous than sown plants and are more susceptible to pathogen infection.

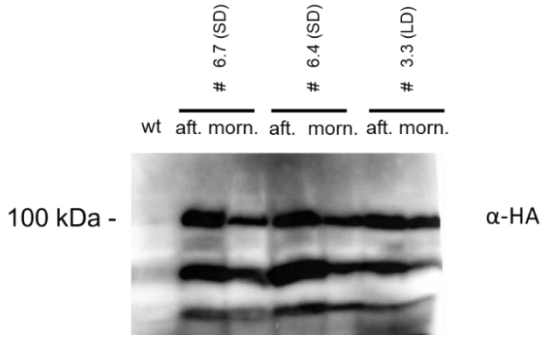

Figure 22. **Hd1-*longlinker*TbID protein in rice transgenic lines.** This western blot shows Hd1-*longlinker*TbID stability in three independent T0 rice lines under SD and LD conditions. Morning samples are recollected 1 hour after reference dawn, and afternoon samples 5 hours later. An anti-HA antibody was used for immunological detection. Protein detectability is comparable in all samples.

Even if no statistically relevant data is available on the phenotype of Hd1-*longlinker*TbID lines, there are reasons to think that the fusion protein preserved the native Hd1 function and that mutant complementation had been obtained. Escape transformants (i.e. plants that are regenerated by mistake and do not contain the desired construct) flowered under SD conditions in <2 months from transfer to soil, compatibly with the absence of Hd1 flowering repressing function in a *hd1-1* mutant background. Conversely, the positive Hd1-*longlinker*TbID plants did flower under SD conditions, similarly to the wild type. Also, the extremely late flowering phenotype under LD conditions can be explained by Hd1-*longlinker*TurboID overabundance and preservation of Hd1 flowering-repressing function under non-inductive conditions, as reported by Eguen et al., 2019 [74].

Western blots performed on leaves extracts from 3 independent Hd1-*longlinker*TurboID lines showed that the transgenic protein was stable and abundant in all lines. A slight increase of protein abundance was observed in afternoon-sampled leaves (Fig. 22).

Conservation of flowering-time-related native function was also observed in FTL1-TbID lines, which were successfully generated for both of the linker variants. All the lines showed an early flowering phenotype under LD conditions, which is coherent with a dose-dependent effect of heterologous FTL1-TbID. Notably, the phenotype was more extreme in pFTL1:FTL1-*longlinker*TbID: T_0 transformants were dwarf, showed an early flowering phenotype, flowering less than one month after soil transfer, and produced abnormal and sterile panicles (Fig. 23). Similarly to what happened for Hd1-*longlinker*TurboID, it was not possible to obtain fertile seeds, due to the loss of all pFTL1:FTL1-*longlinker*TbID transformants.

Figure 23. **Flowering and panicle phenotype of pFTL1:FTL1-*longlinker*TbID plants.** From left to right, the size of wild-type, escape transformant and pFTL1:FTL1-*longlinker*TbID single plants are compared (a). Extremely early formed panicles are abnormally conformed and present sterile seeds (b).

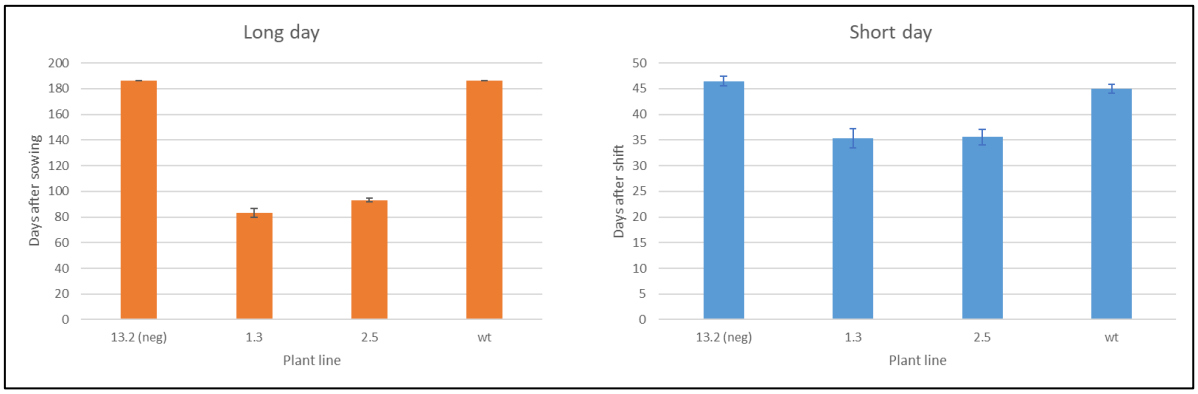

Figure 24. **Flowering time of pFTL1:FTL1-*shortlinker*TbID lines under long and short day conditions.** Line 1.3 and 2.5, expressing FTL1-*shortlinker*TbID under the control of FTL1 endogenous promoter, flower earlier than the wild type and the negative control line 13.2 (escape transformant) both under long and short days. Values of wild type and 13.2 under LD conditions are indicative: none of the plants has flowered 186 after sowing, when the experiment was concluded.

Plant size and seed fertility of FTL1-*shortlinker*TbID plants is, instead, comparable to the wild type. The flowering time experiment shown in Fig. 24 has been carried out on two independent lines (1.3 and 2.5) under both SD and LD conditions and is a statistically significant confirmation of the above-mentioned early-flowering phenotype.

This conclusion, along with the results of biotinylation tests on *N. benthamiana*, indicate that the short linker length is not pernicious to FTL1 native function and does not reduce the efficiency of the biotinylation activity of the TbID.

For these reasons, the two FTL1-*shortlinker*TbID independent lines 1.3 and 2.5 were chosen for protein stability assessments and PL application on rice meristems.

The TbID control transformation has given one independent line which is currently in the seed propagation stage and cannot be used for destructive meristems sampling. Therefore, due to momentaneous unavailability of this control, NB wild type plants have been employed as negative control in the following experiments.

As a starting point, protein extraction and WB analyses were conducted on three pools of FTL1-*shortlinker*TbID flower meristems, approximately consisting of three subsequent inflorescence developmental stages (Fig. 25). Since meristems are internal organs of the plant and any tissue handling before sampling would invalidate *in vivo* proteomic analyses, we decided not to provide external biotin to the samples. In this way, we also tested the ability of the TbID to efficiently work at endogenous biotin concentrations.

In both 1.3 and 2.5 lines, FTL1-*shortlinker*TbID (here after FTL1-TbID) fusion protein was stable and easily detectable using an anti-HA antibody.

Because of high protein amount and excessive overexposure during WB visualization, the band corresponding to FTL1-TbID is bleached. Another WB for lines 1.3 and 2.5 will be repeated on newly sampled flowering meristems.

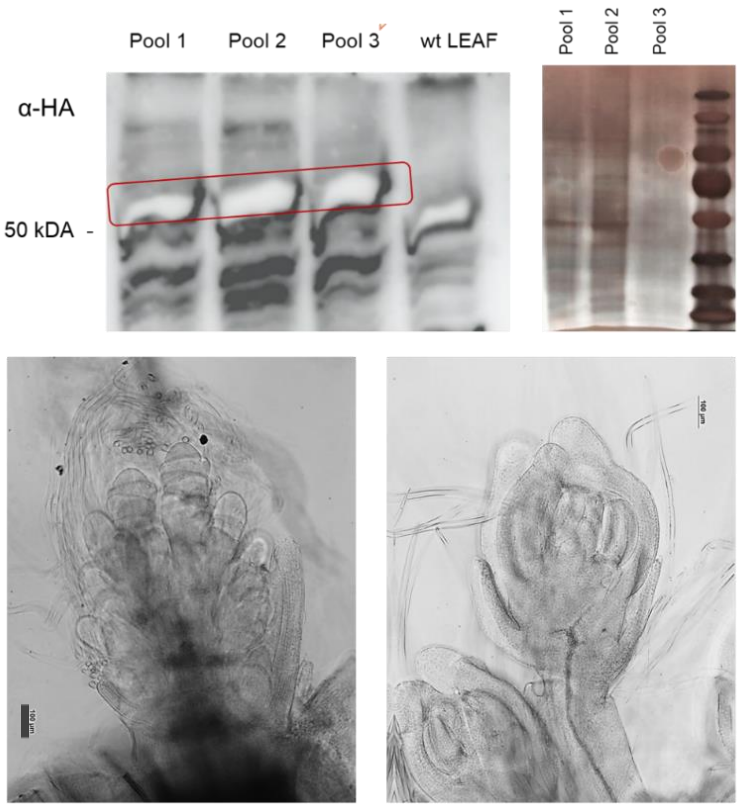

Figure 25. **Assessments of FTL1-TbID stability and success of biotinylated proteins precipitation in rice developing inflorescences.** Anti-HA antibody shows FTL1-TbID presence in pool 1 (inflorescence to spikelet meristems), pool 2 (spikelet meristem to floral organs development) and pool 3 (bolting panicle) protein extracts. Silver staining on precipitated biotinylated proteins shows high protein amount in Pool 2 and pool 1 pull downs. The pictures represent spikelet meristems and nascent floral organs as contained in pool 2 (scale bar is 100 µm).

Silver staining on protein pull down using streptavidin-conjugated beads indicates that the content of precipitated biotinylated proteins is detectable in pool 1 and 2, but not in pool 3 (Fig. 25). Protein detectability through silver staining is a prerequisite for successful MS analyses. In our experiments, it also indicates that endogenous biotin amount are sufficient to produce effective biotinylation of FTL1-TbID interactome.

Pool 3 was made up by already developed bolting panicles, in which meristematic identity is lost due to completion of organogenesis. The absence of consistent biotinylation in this stage, even if FTL1-TbID protein is strongly detectable, might indicate that FTL1 activity is no longer required after floral organs have developed.

The first proteomic analyses were conducted on meristems in a developmental phase that goes from spikelet initiation to floral organs differentiation, as in Pool 2. Analyses on earlier stages, corresponding to pool 1, will be carried out in the next months.

For PL, the same protocol, developed in *N. benthamiana* for Hd1-*longlinker*TbID, was employed after samples recollection. An average of 15 meristematic panicles have been used for each replicate.

6. Proximity labelling on rice meristems expressing FTL1-TbID

PL on meristems from pool 2 has delivered a list of >3000 proteins. Among them, 102 are commonly enriched in 1.3 and 2.5 independent lines, consisting of around 3% of the total detected proteome. Parameters of statistical significance are the same of proteomic analyses previously presented here. In the interpretation of the results, the distinction between hit and candidate proteins has been ignored for simplicity. Also, due to the dimension and complexity of the database, the proteins that are enriched in the wild-type control, rather than in the FTL1-TbID lines, have been excluded from the analysis.

This experiment is the first case of successful PL application in rice. Also, it demonstrates the possibility to obtain complex proteomic data from delicate tissues, difficult to collect, such as rice flowering meristems. The 102 proteins that are enriched in the two lines have been screened

individually and through GO analyses. A representation of curated GO categories is shown in Fig. 26.

RNA metabolism is the most represented category (18 interactors involved in transcription, RNA processing and transport), followed by unknown function (13 interactors) and chromatin (11 interactors involved in different aspects of chromatin metabolism). The number of interactors involved in protein translation, folding and degradation is also consistent. Since similar categories were identified also among *N. tabacum* pulled down proteins, we interpret them as the result of biotinylation of the translation and degradation machineries, resulting from translation and degradation of TbID. These categories, even if not exclusive of FTL1 function, are indicative of efficient biotinylation of the proximal TbID proteome.

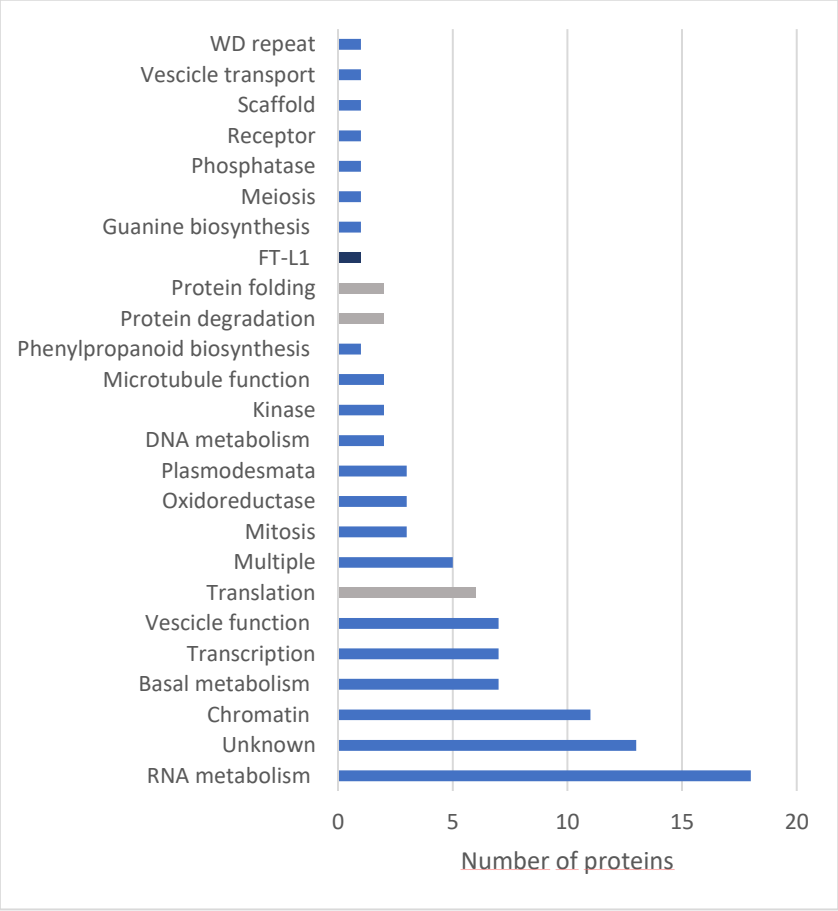

Figure 26. **Candidate interactors of FTL1-TbID by GO categories.** Gene ontology (GO) categories for the list of significantly enriched proteins in the two FTL1-TbID lines are shown. FT-L1 protein has been reported in a separate own category and coloured in black. It represents an expected candidate, because of auto-biotinilation of FTL1-TbID. Grey columns indicate protein functions that are related to TbID processing and degradation. The most represented category is RNA metabolism, followed by proteins of unknown function and chromatin.

Interestingly, both in *N. benthamiana* and rice PL datasets, the ratio between the number of proteins involved in folding/degradation and translation is less than 1:2, suggesting that we might be looking at a common phenomenon.

Comparation of the two datasets also highlights that proteins belonging to the RNA metabolism is notably enriched in both: the elements belonging to this category are 12% and 18% of total enriched proteins for Hd1-TbID and FTL1-Tb PL datasets, respectively. The slightly higher enrichment of this category in FTL1-TbID interactome may be case-specific and dependent on a peculiar biological function of FTL1.

The abundance of elements belonging to the chromatin category is consistent with known FTL1 localization to the nucleus and with the ability to form DNA-binding complexes. On the other hand, no clear connection between FTL1 and proteins involved in RNA metabolism was reported yet.

Remarkably, a few underrepresented categories include some of the elements that are the mostly enriched in both studied lines: microtubule function, plasmodesmata and vesicle function (Fig. 26 and 27). The reduced list of 15 most-enriched proteins shown in Fig. 27 heatmap can be intended as the most valuable data in our collection, since their high abundance and significance level make them the first potential FTL1-TbID interactors *in vivo.*

Two of the three proteins that are included in the plasmodesmata category, namely germin-like protein 5-1 and the microtubule-binding protein encoded by Os06g0284800, are in this list. It is a striking result, given that no mention of the interaction of FT-like proteins and plasmodesmata elements has ever been done before. The 15-proteins list also contains FTL1-TbID itself, an oxidoreductase (encoded by Os03g0815200), two transcription factors (HSFA1, OsDLN95) and two enzymes of the phenylpropanoid pathway which are localized to the cell membrane (LOC_Os10g34360, 4CLL9).

Excluding transcription factors, this list of proteins is suggesting a localization of FTL1 in proximity of the plasmodesmata and in contact with vesicle transport elements and the plasma membrane. On the other hand, HSFA1 and OsDLN95 have no characterized role in rice.

Outside of this preferential list of proteins, particular mention is needed for a set of proteins which may provide additional information on FTL1 role in

meristematic rice cells. Thioredoxin H is enriched in both the studied lines. This enzyme is involved in the regulation of plasmodesmata conductance, compatibly with the hypothesis that FTL1 localizes close to this cell structures [75]. The list of enriched proteins also includes OsFCA and OsFY, whose Arabidopsis homologues are classical flowering regulators upstream of FLOWERING LOCUS C and assemble in a complex required for alternative polyadenylation of an FLC antisense transcript [76,77]. Remarkably, the bidirectional sugar transporter OsSWEET1A is a candidate FTL1 interactor according to our proteomic analyses.

No OsbZIP transcription factors, such as OsFDs, have been found in our meristematic samples. However, the presence of SQUAMOSA Promoter-binding protein-like (SPL) 13 and 14 clearly sets the timing of our PL analysis in advanced stages of flowering meristem differentiation.

SPL genes are major regulators of inflorescence architecture, expressed during branch and spikelet meristematic stages [78]. Their mode of action is closely related to FTL1, and SPL14 represses FTL1 transcription by directly binding to its promoter [47].

Considering the dimension of the protein list, further analyses and interpretations will be needed to get a full understanding of the obtained information. In the next section, the biological meaningfulness of the above-mentioned proteins and their possible relation with FTL1 will be discussed.

Figure 27. **Heatmap listing the first 15 most enriched proteins from PL on line 2.5 and line 1.3.** Proteins are ordered for the highest log2fold and lowest fdr values. Loci and protein names are reported on the left on the heatmap, while brief proteins descriptions are reported on the right.

DISCUSSION

1. Hd1 as a photoperiodic and metabolic hub in rice leaves

Hd1 has been defined as a powerful photoperiodic regulator with multiple DNA and protein-binding properties in rice. Its function is modulated through photoperiod and interaction with the protein context [21,33,34,43]. Here we show that Hd1 overexpression leads to constant protein presence in the rice leaf throughout the day, suggesting that its abundance is not regulated through protein degradation, but rather transcriptionally. Indeed, Hd1 transcription is rhythmically regulated during the day in adult rice leaves (Fig. 9). In the plant life cycle, the highest Hd1 expression levels are reached after the second month of growth [17,32,79]. Combination of circadian transcript fluctuation and exposition to varying daytime duration is believed to be at the origin of Hd1 functional switch between LD and SD conditions. Under LD conditions, the transcript maximum expression can be measured at the end of the reference afternoon, and the interaction with activated phytochromes leads to a repressing effect on florigen promoters [32]. Flowering repressing effect is achieved in cooperation with Ghd7, which is also stabilized by phytochromes under LD conditions [16,36]. Under SD conditions, Hd1 transcript maximum entirely coincides with reference nighttime, where Hd1 would work as a flowering promoter on its own, in the absence of Ghd7 [32,37,80].

Hd1 contains two functionally relevant domains at its extremities: a BBOX region at the N terminus and a CCT domain at the C terminus. Both regions are believed to mediate protein-protein interactions [34,41]. In plants and animals, BBOX domains are presumed to mediate homo- and hetero-dimerization also with proteins that do not belong to the zinc finger family [81]. Specifically, Hd1, like Arabidopsis CO, contains two BBOX domains at its N terminus of about 40 aminoacids each [82].

At the C-terminus, the CCT domain is named after the CONSTANS, CO-LIKE AND TOC1 (CCT) family of transcription factors, which includes Arabidopsis CO and rice Hd1, and is characterized by a highly conserved sequence of 41-43 amino acids [82,83]. The C-terminal CCT domain is fundamental for NF-Y complex formation and for the recognition of *CO RESPONSE ELEMENT* (*CORE*) sites in the target genes promoters [41,67]. The standard CORE domain

recognized by Arabidopsis CO was initially defined as *TGTG(N2-3)ATG* [84]. Subsequent studies narrowed down the CORE to *TGTGGT* (for potato StCOL1 [85]) and *TGTGG* (for Arabidopsis CO and rice Hd1 [17,40]). Finally, crystal structures of CO and Hd1, in complex with NF-YB/C subunits and DNA, indicated that essential contacts are made with the *TGTG* motif only [41,86].

Our experiments with pACT:FLAG-Hd1 rice lines indicate that the overexpressed protein accumulates at all timepoints during the day, both in light (ZT0-12) and dark (ZT16) conditions. Western blots on FLAG-Hd1 seems also to indicate that the protein might be subjected to post-translational modifications. Hd1 was reported to be phosphorylated by of the HDR1-OsK4 complex, similarly to what happens for Arabidopsis CONSTANS [87,88]. The differences in band height observed in our estern blots suggest that we may be looking at a phosphorylated and a dephosphorylated version of the protein, simultaneously present in the leaf under both SD and LD conditions. However, while CO phosphorylated form is a substrate for COP1-mediated degradation following a circadian pattern, FLAG-Hd1 presumed phosphorylation seems not to interfere with its diurnal accumulation [88].

A post-translational modification on Hd1 may be needed to control its complex-forming capacity. CO is phosphorylated in its C-terminal region, containing the CCT domain [88]. Knockout mutations of *HDR1* and *OsK4* genes cause early flowering under LD conditions in rice, meaning that Hd1-mediated flowering repression may depend on phosphorylation, which could enable specific protein interactional properties [87]. In depth analysis of the Hd1 structure and protein-protein interface, as well as phosphoproteomic analyses, could help to confirm this hypothesis.

Previous research have reported that overexpression of Hd1, containing a C-terminal tag, generates a late flowering phenotype under SD conditions [32]. Since the protein is not subject to light- or daylength-dependent degradation, its accumulation pattern under SD conditions in Hd1-overexpressing plants resembles the one that is commonly observed under LD conditions in wild type plants [80] (Fig. 4). In this case, Hd1 could maintain a partial flowering-repressing function, which would be presumably carried out by interaction with minimum concentrations of Ghd7. The formation of a low number of Hd1-Ghd7 repressive complexes during a few hours of the day would not be sufficient to block the entire flowering induction

machinery, but could antagonize the flowering-activating function of Hd1 during nighttime, causing a delay in the floral transition.

In our experiments, pGOS2:Hd1-*longlinker*TbID can rescue the mutant phenotype under LD conditions and shows late flowering under inductive SD conditions. Hd1-TbID protein is equally abundant in morning- and afternoon-collected leaf samples, independently of the photoperiod (Fig. 22). Standing on these observations and on the external coincidence model, Hd1-TbID preserves the original protein properties of Hd1. Indeed, the phenotypic behaviour of pGOS2:Hd1-*longlinker*TbID plants suggests that C-terminally-fused Hd1 does not lose any of its interactional abilities during plant development. Based on protein primary structure, the TbID moiety at the C-terminus seems not to affect the functionality of the CCT domain to bind the DNA CORE regions inside of the NF-Y trimer, possibly because of the spatial distance provided by the long linker sequence. Preservation of interaction domains can also be attributed to the unmodified BBOX at the Hd1 N-terminus. This domain is thought to be needed for CO homodimerization in Arabidopsis to properly induce FT transcription [89]. The importance of alleged Hd1 homodimers in rice has not been clarified yet, but studies with microproteins have demonstrated that the BBOX region is needed for Hd1-mediated floral repression under LD conditions [74].

The pACT:FLAG-Hd1 lines we used in our experiments flower later than the wild type under LD conditions, but not under SD conditions [74]. The lack of this phenotypic change under SD conditions, which is instead observed in C-terminally tagged Hd1 lines, may indicate that the disruption of the N terminus is invalidating the arrangement of transcriptional repressive complexes, which would be fundamental to hinder flowering under SD conditions. Therefore, the integrity of the BBOX terminal region looks as important as that of the CCT domain to ensure proper flowering repression, and this function may depend on the ability to associate with different proteins in different photoperiodic conditions. The BBOX domain of Hd1 is important for complex formation with Ghd7 to downregulate *Ehd1*, and this interaction would take place at low levels during Hd1 overexpression under SD conditions [33]. The presence of a N-terminal tag could be detrimental to this interaction, both under SD and LD conditions, yet might not interfere with the formation of NF-Y complexes, ensuring flowering repression through direct inactivation of florigenic genes under LD conditions. If phosphorylation of the C terminus was needed to ensure the DNA-binding

and protein-binding features of the CCT domain, as speculated above, the absence of a late-flowering phenotype in *hdr1* and *osk4* plants, reported by Sun et al. [87], could be evidencing that the flowering-repressing function of Hd1 results from a balance of its ability to form either C-terminally- or N-terminally-mediated complexes, and that the formation of NF-Y trimers is essential to repress flowering only under LD conditions.

Conversely, it is still unknown whether the presence of a C-terminal tag influences the ability of Hd1 to bind gene promoters on its own, inducing flowering in the lack of other flowering repressors.

Our ChIP PCR experiment demonstrates that Hd1 can recognize promoter regions other than those of florigen genes, specifically that of *PHENYLALANINE AMMONIA LYASE 4* (*PAL4*). The promoter of *PAL4* contains several TGTG/CACA sequences, that could be recognized by Hd1. A ChIP-seq experiment has been planned to identified other possible DNA regions bound by Hd1.

PAL4 encodes for a fundamental enzyme of the phenylpropanoid biosynthetic pathway, and its transcription is differentially regulated when rice plants are exposed to different types of stress [90–92]. Transcriptomic analyses preceding this PhD project had reported that also the CINNAMYL ALCOHOL DEHYDROGENASE (CAD) gene, encoding another enzyme of the same pathway, is downstream of Hd1. ChIP-PCR failed to prove that Hd1 can bind this gene promoter, and this result reflects the one of the qPCR time course analysis, which shows that diurnal CAD transcript fluctuation is far less affected than that of PAL4 in the knockout mutant *hd1-1*.

The phenylpropanoid pathway originates from aromatic amino acids through shikimate to lead to the generation of several types of compounds, involved in all kinds of stress response [93–95]. Though it is usually classified as a secondary metabolism pathway, phenylpropanoid biosynthesis allows to produce substances that are fundamental to the plant cell, like lignin and tannin polymers, which contribute to cell wall robustness. Previous experiments in our laboratory have shown that Hd1 influences the biochemical composition of leaf cell walls and the plant salt tolerance. The composition of cell walls is strictly related to the ability of plants to respond to osmotic stress [96,97]. Interaction between abiotic stress and flowering time are complex and largely reported in plants [31,98]. A cross-link between these two phenomena exists, since stress events can modify flowering time, and

the switch from vegetative to reproductive development can itself influence the plant sensitivity and responsiveness to stresses. Adjustment of leaf cell wall composition in mesophyll and vasculature cells can be interpreted as a plant adaptive strategy when entering the reproductive phase [99,100]. On the other hand, deposition and degradation of cellulose, hemicelluloses and pectins has been demonstrated to be diurnally regulated [101,102].

Our theory is that Hd1 integrates photoperiodic signals into the flowering induction pathway, while promoting structural metabolic changes that prime the plant to stress tolerance during the reproductive phase (Fig. 28).

The results of our leaf proteomic analyses furtherly show the range of Hd1 effect on leaf metabolic networks. Our experiment is the first to show which biosynthetic pathways are influenced by photoperiodic shift in rice plants. Under flowering inductive conditions, changes in leaf primary and secondary metabolism occur, presumably to prepare the plant for reproductive development and seed filling. Changes in nitrogen metabolism can be intended as a way to regulate the incorporation of nitrogenated compounds into the principal synthetic pathways in different plant tissues, such as the newly forming inflorescence. In Arabidopsis, reproductive organs regulate leaf nitrogen metabolism through hormone signalling [103]. Changes in porphyrin and B vitamin metabolism reflect a photosynthetic adjustment when entering the reproductive phase, since both pathways are upstream of chlorophyll production, and the synthesis of these nitrogenated aromatic compounds is intimately connected to nitrogen availability [104–106]. During reproductive transition, the higher sugar demand is associated with increase in photosynthetic rate, which generally decreases during grain filling, when photosynthetic apparatuses are dismantled to provide nitrogenated compounds [107,108].

Interestingly, Hd1 overexpression or absence causes small but visible changes in the photoperiodic-shift-mediated metabolic pathways. As mentioned above, the highest differences in KEGG categories composition are observed in the *hd1-1* mutant rather than in pACT:FLAG-Hd1. From this point of view, Hd1 overexpression can be considered closer to wild type conditions, if compared to complete *Hd1* knockout, in spite of above-described differences in the flowering phenotype.

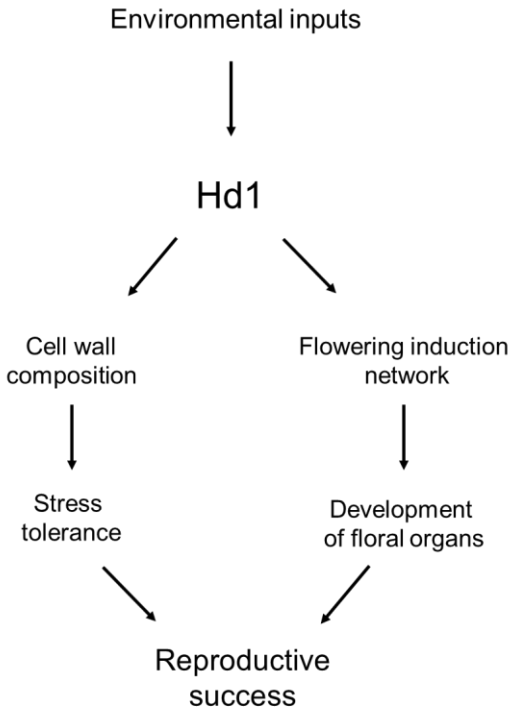

Figure 28. **Proposed model of Hd1 centrality in flowering-associated stress priming.** Hd1 integrates photoperiodic signals into the leaf flowering induction network to regulate the reproductive transition, while controlling phenylpropanoid biosynthetic pathways to ensure proper stress tolerance during flower and seed development.

Since the phenylpropanoid biosynthetic pathway draws in from the cellular pool of aromatic amino acids, modifications in the aminoacidic biosynthetic category in the *hd1-1* mutant could be explained by an altered request of phenylpropanoid compounds. Also, the appearance of enzymes belonging to glyoxylate and dicarboxylate metabolism among the differentially abundant proteins in the *hd1-1* mutant can be reconducted to modified cell wall requirements. The glyoxylate cycle can contribute to increase glucose availability in the plant cell when carbohydrates demand overcomes photosynthetic capacity. For example, when the synthesis of cellulose and cell glucans is increased, glyoxylate and dicarboxylate metabolism allows a rapid conversion of lipids into glucose via the acetate that is generated by fatty acid β-oxidation [109,110].

Differences in the metabolism of hemicellulose and GDP-L fucose (a component of cell wall glycans) in pACT:FLAG-Hd1, *hd1-1* and wild type plants, emerging from GO analyses, converge with KEGG analyses and

transcriptomic results towards a clear involvement of Hd1 in determining cell wall composition during the photoperiodic shift in rice plants.

To this date, thanks to RT-PCR and ChIP-PCR experiments, only the effect of Hd1 on *PAL4* and *CAD* genes of the phenylpropanoid pathway is precisely known. Further analysis of our proteomic dataset will clarify which proteins, belonging to the above-mentioned most relevant biosynthetic pathways, are exactly up- and/or down-regulated by Hd1 in the two photoperiodic conditions.

Given that the protein primary function on flowering time relies on complex formation, it is possible that also cell wall-related biosynthetic pathways are similarly regulated. Hd1 could therefore stand as a wide-range, interconnection hub between photoperiod-dependent control of flowering time and central biosynthetic pathways in rice plant cells (Fig. 28).

At the moment of writing, ChIP-seq analyses on FLAG-Hd1 lines are being carried out to unveil any other DNA binding sites for Hd1 in the rice genome. Future PL experiments, instead, are expected to provide growing information on Hd1 interactome.

2. Developing a functional proximity labelling protocol

During my PhD, the generation of rice transgenic lines for Hd1 PL has encountered a series of problems that have been previously described. These difficulties are intrinsically linked to the rice calli transformation and regeneration process, which is being continued in our laboratory in order to obtain Hd1-TbID-expressing plants in the next future.

The only PL data that we obtained on Hd1 are derived from PL application in *N. benthamiana*. As said before, this experiment mainly served to implement a powerful pipeline for this technique, to be then used in our laboratory independently of the original plant system.

Protocols for PL in *N. benthamiana* have already been published at the time our project started [60,61,68] . However, a number of technical variants existed, and optimal working conditions needed to be established. Above all, the preliminary tests that preceded MS analyses on Hd1-TbID injected leaves

allowed us to confirm that our fusion proteins were stable and had a verifiable biotinylation efficiency (Fig. 18).

Also, these experiments let us immediately observe which was the effect of two different linker sequences on the number of biotinylated proteins in *N. benthamiana* agroinfiltrated leaves, which was never tested before. Hd1-*longlinker*TbID performed better than the shorter version in biotinylation and immunoprecipitation tests (Fig. 20). This can be explained by a higher steric freedom of the TbID moiety inside of the fusion protein. A longer distance between the protein of interest and the biotin ligase can increase the number of possible conformations for the entire fusion protein, due to the intrinsic disordered nature of the linker sequence. This would improve the ability of the biotin ligase to intercept surrounding proximal proteins.

Also, an effect of linker length on the native protein function can be extrapolated by the phenotype of pFTL1:FTL1-TbID transgenic lines (Fig. 23). Rice lines expressing both versions of FTL1-TbID have an early-flowering phenotype, which is particularly extreme in FTL1-*longlinker*TbID.

This indicates: 1) that the early flowering-promoting function of FTL1 is preserved even in presence of the C-terminally fused TbID and 2) that the dose-dependent expression effect of FTL1-TbID, which is under the control of its endogenous promoter, is higher when the linker length increases. A possible explanation to such strong phenotypic differences may be attributed to a steric freedom of the FTL1 portion of the fusion protein, which would be higher when increasing the distance from the TbID counterpart, as for TbID biotinylation efficiency. The early flowering phenotype observed in the transgenic lines of both FTL1-TbID linker versions is to be attributed to an augmented protein expression: indeed, even if the fusion protein is under the control of *FTL1* promoter, these transgenic lines have been obtained by transformation of a wild type background line, so that an addictive effect on endogenous FTL1 expression could be expected.

Similar conclusions cannot be drawn on the two Hd1-TbID linker versions, since no rice transgenic line expressing Hd1-*shortlinker*TbID has been obtained so far and no phenotypical or protein properties comparison can be made with the Hd1-*longlinker*TbID lines.

Proteomic analyses on *N. benthamiana* agroinfiltrated leaves have delivered a transient PL interactors dataset, which is consistently reduced if compared to those of other proteomic analyses reported in this text. PL Mass spectrometry datasets obtained from PL application in *N. benthamiana* are usually short [111,112]. This is probably because *N. benthamiana* is a paleotetraploid and draft genome assembly is highly fragmented, often containing incorrect gene models [113].

Referring to the content of the dataset, most of the enriched biotinylated proteins in Hd1-TbID-agroinfiltrated leaves is not related to any Hd1 or Arabidopsis CO known interactor. A consistent part of the dataset is ascribable to protein processing and degradation. In fact, it is expected that the TbID enzyme starts to biotinylate surrounding proteins since its translation and throughout all protein lifetime until degradation. Other proteins belong to general cellular processes such as primary or RNA metabolism: enrichment in these categories may be intended as a metabolic consequence of the agroinfiltration of Hd1-TbID construct itself, rather than to PL, which caused accelerated necrotic events on *N. benthamiana* leaves that were not observed in the control TbID experiment (Results section n. 3). Only one transcription factor (FRL), consistent with Hd1 function and localization, was found in the list of potential interactors, and speculations can be made about the plausibility of this interaction. FRIGIDA (FRI) is a vernalization regulator in Arabidopsis that binds the FT repressor FLC to activate its transcription and inhibit flowering [114]. The FRI-FLC regulatory pathway is not present in monocots, but FRIGIDA-LIKE genes are present in their genomes. Quantitative loci analyses have shown the possible implication of these transcription factors in vernalization and flowering control also in winter cereals [115–117].

Rice is a non-vernalizing plant but its flowering induction pathway shares common element with that of temperate cereals [115]. As a proof of this, the presence of FRL genes in rice genome indicates that they may play some transcriptional function. In up-to-date literature, there is no hint to a possible interaction between FRL and CO-like proteins. In fertilized flowers, the NF-YA protein LEAFY COTYLEDON 1 (LEC1) targets FLC promoter to re-initiate its transcription in developing seeds [118] . The recognized motif (CCAAT) is not a CORE, so that it cannot be recognized by CCT transcription factors. We cannot exclude that some kind of interaction between Hd1 and

FRLs takes place in rice, possibly through mediation of other DNA-binding proteins.

Transcription factors are synthesized at lower concentration in cells if compared to other proteins, like metabolic enzymes. The probability that this FRL protein is randomly present in the potential Hd1-TbID interactome of *N. benthamiana* is low, especially considering that FRI and Hd1-homologue CO belong to closely related regulation pathways [119].

If future interaction tests validate the interaction of Hd1 with rice FRL proteins, this would disclose new aspects of the rice flowering induction pathway and would provide new knowledge on the interaction properties of CONSTANS-like proteins in flowering plants.

3. FTL1 protein has diversified regulatory functions in spikelet and floret meristems

Florigens play a key role in initiating flowering and regulating the acquisition of floral identity in plant shoot apical meristems. FT-like proteins are also essential after floral organs differentiation, contributing to ovule maturation, pollen development and seed maturation [120,121].

To our knowledge, FTL1 is expressed in rice shoot apical meristems immediately upon photoperiodic flowering induction and until late stages of meristem differentiation. A specifically fundamental role is played in controlling the transition from branch to spikelet meristem [47].

As mentioned before, FTL1-*longlinker*TbID lines were not suitable for proteomic sampling and could not be propagated due to their extreme phenotype - early and continuous flowering, abnormal panicles, sterile seeds (Fig. 23). Two independent FTL1-*shortlinker*TbID (from now on, just FTL1-TbID) lines, instead, were selected and used for proteomic analyses. In these lines, FTL1-TbID was visible on western blots at the expected molecular weight and was stable in all the flower developmental stages considered (Fig. 25). Notably, the protein was equally detectable both in meristematic stages (pool 1 and 2) and in non-meristematic stages (pool 3). Since the protein is expressed under FTL1 endogenous promoter, it could be hypothesized that the protein is also involved in floral organs development.

Indeed, FTL1 mutants have been shown not only to produce more panicle branches than a wild type plant, but also to have a higher proportion of sterile flowers [47]. Experiments in our laboratory are being carried out to clarify the potential participation of FTL1 in ovaries and anthers formation, as well as in fertilization processes.

Even if FTL1-TbID protein concentration is equal at all considered stages, higher immunoprecipitated content in pools 1 and 2 suggests that FTL1-TurboID is in direct contact with a higher number of proteins during meristematic differentiation than in already committed flowers (Fig. 25). Such implication seems more trustable if we consider that the tissues were not supplemented with exogenous biotin, and this increases the specificity of enzymatic labelling. The difference in total biotinylated proteins between the pools could be explained by FTL1 being more mobile or having a more diversified function in the former stages. However, technical reasons due to transgenesis cannot be excluded: the protein could still be synthesized after acquisition of floral identity because of the lack of repressive regulatory elements in the promoter segment used.

Proteomic analysis on rice flowering meristems was considered a priority for our first PL application in rice. A successful mass spectrometry output on late meristematic and floral developmental stages (pool 2), followed by GO and manual analyses of enriched proteins, has provided a set of new potential FTL1 interactors (Supplementary table 3; Fig. 26). If verified, these results would enormously expand our knowledge on protein-binding properties, cell localization and general function of rice FTL1 and other FT-like proteins.

As briefly mentioned in the results section, candidate interactors involved in translation, protein folding and protein degradation are considered as nonspecific, as for transient PL on Hd1-TbID in *N. benthamiana*. Self biotinylation, demonstrated by the fact the FTL1 is one of the most enriched proteins in the dataset, reinforces this hypothesis. Therefore, the proteins belonging to these categories have been excluded from further research, while waiting for pUBQ:TbID control data.

Until now, florigens have been studied mainly for their ability to regulate the expression of downstream genes involved in flowering meristem determination, by interaction with transcription factors [122,123]. In our dataset, 9% of significantly enriched proteins (9 of 102) are transcription

factors, whereas a similar number of proteins are classifiable as chromatin-remodelling factors (Supplementary table 3).

Due to the conceptual complexity of the dataset, we made a list of 15 most significantly abundant proteins (highest logfold value, lowest false discovery rate value, see Methods) which very probably represent the most proximal elements to FTL1-TbID in rice meristematic cells. The number of proteins to be included in this list was established arbitrarily and include most of FTL1 candidate interactors discussed in this text.

HEAT SHOCK TRASCRIPTION FACTOR A1 (HSFA1) I is the first mostly enriched transcription factor in our dataset. HSFs are transcription factors that regulate the expression of heat shock proteins (HSPs), which are involved in temperature-dependent development and thermic stress response [124]. *HSFA1* is expressed at distinct levels throughout the whole plant life cycle. In particular, the gene is active during the development of the inflorescence and of female and male flower organs (https://ricexpro.dna.affrc.go.jp/).

A number of HSFs play important roles in Arabidopsis leaves during temperature-dependent flowering and vernalization [125]. For instance, HSFB2b represses the transcription of the circadian clock gene PRR7 and integrates stress signals into the control of flowering time [126]. Concurrently, HSP101 and HSP90 act as molecular scaffolds, recruiting crucial flowering transcriptional regulators to coordinate reproductive development [127,128]. In rice, HSF9, also named DELAYED HEADING DATE 3 (DHD3), is a flowering repressor and its transcription is enhanced by cold treatment [129]. In wheat, HSFA2 is a candidate gene for controlling anther development [130]. Rice HSFA1 has not been studied yet, and it may be involved in temperature-dependent control of reproductive development. Temperature is one of the main factors influencing the formation of floral organs [131,132]. Extreme temperatures are often associated with abnormal male and female organs, and many transcription factors have been found to regulate their development in a temperature-dependent manner [133–135]. By interacting with HSFA1, FTL1 could integrate temperature sensing into spikelet and flower meristem determinacy.

The second mostly enriched transcription factor in our dataset is OsDLN95/ONAC6. The NAM/ATAF1/CUC2 (NAC) family of transcription factors has been widely studied in plants and groups genes that are involved in several developmental processes [136–138]. In rice, some of these genes have

been shown to take part to organ boundary formation in different kind of tissues, as well as to stress response [139,140]. OsDLN95/ONAC6 has been associated to both biotic and abiotic stress response. In particular, its transcription is upregulated in leaf by cold treatment [141,142]. The gene is also expressed at early stages of ovary formation and its overexpression causes reduced reproductive yield [141]. Therefore, as hypothesized for HSFA1, the interaction of FTL1 with OsDLN95/ONAC6 could be a link between temperature response and cell differentiation in rice flowering meristems and forming floral organs.

Arabidopsis FT has been recently displayed to have a temperature-dependent behaviour, related to its ability of being incorporated into the plasma membrane at temperatures lower than the optimal flowering ones [143]. In this way, the mobilization of the florigen signal from the leaves to the shoot apical meristem would be thermoregulated. The list of top most-enriched proteins in our PL database clearly illustrates that FTL1 localizes close to lipid membranes and cell wall (Fig. 27). The hydrophobic properties of cell membranes make them fine sensors of temperature variations, and some transcription factors of the HSF and NAC families directly localize to the nuclear membrane [144,145]. However, protein structure prediction is not available for OsDLN95/ONAC6, while HSFA1 exhibits a hydrophobic peptidic region, that could serve for protein oligomerization (https://www.uniprot.org/uniprotkb/Q84T61/entry). Based on existing literature and on the high number of chromatin-associated proteins in our dataset, a constant presence of FTL1 close to the DNA must not be excluded. Anyway, independently from FTL1 localization to the nuclear matrix or to the nuclear membrane, it can be assumed that it takes part to temperature-dependent control of gene transcription in late flowering meristems and forming floral organs, transiently or stably interacting with the above-identified transcription factors. In Arabidopsis leaves, FT is exported from phloem companion cells to the sieve elements in vesicles through the endosomal trafficking. This process is mediated by the MCTP-SNARE complex [146]. In our database, a SNARE-type protein, encoded by Os05g0553700, is enriched only in line 1.3. However, the presence of microtubule- and plasmodesmata-associated proteins amongst the most enriched proteins, suggests that FTL1 might take part to intracellular and intercellular protein trafficking. Since FTL1 is synthesized in meristems committed to flowering, and does not move into the phloem, a cell-to-cell

migration through plasmodesmata in these cells is plausible. Recent data document that germin-like proteins can act as intracellular receptors to modulate plasmodesmata opening in response to specific protein binding [147]. Rice contains 39 germin-like proteins and our mass spectrometry analysis has identified only one of them in meristematic biotinylated proteins. To corroborate this hypothesis, oxidoreductases and RNA-binding elements, the most enriched in our dataset, are compatible with protein sensing at the plasma membrane and modulation of plasmodesmata size exclusion limit [75].

Germin-like protein 5-1 is a powerful candidate sensor of FTL1 to operate its relocation to adjacent cells. If this hypothesis is true, a new mobility property of our florigen protein would have been discovered. To this date, there are no hints at a non-cell-autonomous function of FTL1, and meristem areas of gene transcription and protein localization coincide[47]. However, short-distance movement of FTL1 protein between cells is yet to be investigated. Finally, we cannot discard the idea that FTL1 may regulate trafficking of other proteins through plasmodesmata.

Though some RNA-binding proteins may localize to the plasma membrane, as mentioned above, several elements of the RNA metabolism category, which is one of the most represented in our dataset, can be rather associated to the genetic splicing machinery. This ontological groups includes splicing factors, polyadenylation factors and general RNA-binding proteins which would locate FTL1 in an intermediate position between transcriptional regulation and protein translation (Fig. 26; Supplementary Table 3).

A comparation with *N. benthamiana* PL daset, which also contains a considerable percentage of proteins that contribute to this category, is obliged, and leads to thinking that only part of these proteins is related to an actual FTL1 function in these cellular processes. To sustain FTL1 specific involvement in transcript splicing, the OsFCA protein is enriched in both studied FTL1-TbID lines. In Arabidopsis, FCA belongs to the autonomous flowering pathway, and has been largely shown to modulate target gene expression through RNA processing [76,148]. Also, OsFY, whose Arabidopsis homologue forms a complex with FCA, was enriched in one of the two lines used for our proteomic analysis [149].

Arabidopsis FCA contains several RNA-binding domains, and its functions are multiple and depend on different external inputs. One of these is the processing of an antisense transcript, called COOLAIR, that stems from the 3' end of FLC and contributes to stable silencing of FLC gene by deposition of repressive histone marks [77,150]. Therefore, FCA promotes flowering by creating a chromatin repressive environment on FLC promoter. Since FLC is activated by the vernalization gene FRI, the balance between FRI and FCA proteins determines the final success of the reproductive transition in Arabidopsis plants [151]. Other functions of FCA include the processing of miRNA172 primary transcripts at warm temperatures to promote FT expression [152]. Self-cleavage has also been reported as an autoregulation strategy to control FCA own expression [153]. RNA processing during FLC- and auto-regulation is carried out in cooperation with a number of other proteins, including previously mentioned FY [150,153,154].

The FCA-based flowering autonomous pathway is partially conserved in monocots and multiple RNA-processing properties have been attributed to OsFCA and OsFY [155,156].

Information on any direct protein interaction between FT and FCA or between OsFCA and rice Hd3a/RFT1 is lacking. However, our proteomic data on FTL1-TbID are describing a context that is very similar to the one in which FCA acts, implying that FTL1 may take part to RNA-based regulation processes of target genes, or of its own transcript. However, further data are needed to prove this hypothesis.

Finally, interaction with the bidirectional sugar transporter SWEET1A emerges from our analysis. Transmembrane SWEET transporters have already been related to florigen-like proteins in other plant systems. The tuberization process of potato shares conserved elements with the floral induction pathway and is induced by photoperiod [80,157]. The potato FT-like protein StSP6A physically interact with StSWEET11 [158]. Such interaction results into the inactivation of this efflux transporter in tubers, to promote symplastic sugar transport.

Sucrose has an essential role also during flowering. In Arabidopsis, *SWEET10* is downstream of FT [159]. Its transcription is indirectly activated by FT in mesophyll cells and is believed to facilitate sugar accumulation in the phloem and prepare the plant to reproductive development [159]. Increase in foliar sugar has been reported to accelerate flowering and an analogous

phenotype is observed in SWEET10 overexpressing lines, indicating that sucrose levels also have a regulatory effect on flowering regulation components [160]. Maintenance or modification of sugar transport may be fundamental for proper flower meristem development, and an interaction pattern analogous to the one observed in potato tubers could be transposed on FTL1 and SWEET1A.

At first glance, our PL dataset seems to suggest that FTL1: 1) localizes in different compartments of rice SAM cells, 2) that it may regulate downstream gene expression by integrating temperature signals, 3) that it may take part to transcripts processing, and 4) that it may move or mediate the movement of other proteins between cells. In the last paragraph, a possible involvement in the control of cell sugar content by direct interaction with membrane transporter has also been discussed.

Interestingly, no FAC components have been detected in our proteomic analyses. Indeed, FT and its rice homologues are mainly known to control meristem determinacy through interaction with FD-like transcription factors, with or without mediation of 14-3-3 proteins [54,55,58].

Recently, *N. benthamiana* BiFC and FRET-FLIM assays reported that FTL1 interacts with OsFD1, 4 and 7 [47], even if this was not replicable in our yeast-two hybrid assays, which rather indicates OsbZIP24/OsFD3 as a new possible direct partner of FTL1. Only a weak interaction of this transcription factor with the RFT1 florigen is reported in literature, along with the ability to bind Gf14 proteins [56,161]. The validity of these results and any phenotypic similarities between *osfd3* and *ftl1* mutants will be verified by future experiments.

All the above-cited OsFDs have been reported to be expressed in different moments of inflorescence and floral organ development in rice. Their absence in our proteomic dataset indicates that they are not biotinylated, nor in the wildtype or in the FTL1-TbID lines. We can speculate that FTL1-OsFD complexes may not form in all stages of meristem differentiation, and rather take place during early flowering commitment. This will be confirmed by our next on-going MS analysis on FTL1-TurboID meristems at an earlier timepoint.

CONCLUSIONS

The study of proteins and protein-protein interactions poses a few difficulties that are not usually encountered in genetics research. Indeed, proteins are easily degradable during sample extraction and processing, and their presence and detectability may depend on the context. Also, most protein complexes are unstable and cannot be isolated through traditional co-immunoprecipitation, while other techniques, such as yeast-two hybrid, easily produce artifacts due to the intrinsic nature of the heterologous system and the impossibility to determine the exact influence of the host protein background on the final result.

Notwithstanding, studying proteins is fundamental to explain gene function and to uncover post-translational connections between genes which complement genetic research.

Our proteomic analysis on rice leaves in the Hd1 project has shown for the first time which proteomic changes take place during the photoperiodic shift in leaves, and has unravelled the long-range effect of Hd1 on leaf biosynthetic pathways.

Modern proteomic techniques, like PL, have been precisely thought to overcome the technical barriers of traditional methods of protein and proteomic analyses, and can be used for *de novo* discovery of protein networks.

During my PhD, the implementation of a working PL protocol in *N. benthamiana* has delivered stimulating information about possible Hd1 new interactors. The successful pipeline application of this technique in rice transgenic rice lines, which is reported here for the first time, has demonstrated the great potential of proteomic analyses in rice meristems and has uncovered a list of possible new functions of FTL1 and its homologues also in other species.

The protein dataset is still to be fully investigated, and new proteomic informations on different meristematic stages of FTL1-TbID lines are expected in the next months. Biochemical and microscopy-based experiments will be set up to validate new intriguing FTL1 roles through protein-protein interactions.

Throughout history, the variability in genes that regulate flowering has played a crucial role in enhancing yield and adapting rice cultivation to diverse latitudes. In the last millennia, rice has spread from its Asian homeland westwards to the current cultivation regions of the Mediterranean area. Nowadays, climate change poses a serious risk to the European rice production.

The intricate molecular network governing rice floral induction and panicle development is characterized by a complex interplay of transcriptional, epigenetic, and post-translational interactions. Thanks to PL, the research on newly discovered flowering co-regulators is expected to shed light on their roles, potentially providing selectable genetic markers and gene-editing targets for future agricultural innovation. Our studies hold therefore the promise of establishing a path towards yield improvement and environmental adaptability of cultivated rice varieties.

METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

All the rice plants used in the here-reported experiments belong to the *O. sativa sb. japonica* Nipponbare cultivar.

Plants were grown at an average temperature of 28°C, both in growth chambers and in the greenhouse, at two photoperiodic conditions, according to the experimental needs:

- 10 h of light and 14 h of dark for SD conditions
- 14.5 h of light and 9.5 h of dark for LD conditions

Note: 16 h of light and 8 h of dark were used as LD conditions in the flowering-time experiment on FTL1-*shortlinker*TbID lines 1.3 and 2.5.

N. benthamiana plants employed in transient assays were grown at an average temperature of 20°C under SD conditions (8 h of light and 16 h of dark). Adult young leaves were infiltrated approximately at 1-1,5 months from sowing.

For the research on Hd1 in rice plants, the transposon mutant *hd1-1* was used as a knock-out line, in which Hd1 coding sequence is completely disrupted [162]. pACT:FLAG-Hd1 lines, used as protein overexpressing lines, are as in Eguen et. al, 2020 [74].

VECTORS CONSTRUCTION

For all the other experiments, the constructs were cloned with the Gateway® technology, using DB3.1 (chloramphenicol resistant) and DH5α *Escherichia coli* strains [163].

For YFP- and RFP-fused proteins, employed in microscopy and immunological assays, gene coding sequences were first cloned into pDONOR207 P1-P2 by BP reaction, and then into pB7YWG2 (p35S::GW:YFP) or pUBC-RFP (pUBQ::GW:RFP) destination vectors by LR reaction [164,165].

For the generation of PL fusion proteins, the MultiSite Gateway technology was employed. Genes and promoters were amplified adding appropriate AttB extensions at their extremities and cloned by BP reaction into pDONOR221 P1-P2 and pDONOR221 P4-P1r, respectively. The short-linker and long-linker TbID sequences were artificially synthesized through the GeneWiz synthesis service (https://www.genewiz.com), and inserted into pDONOR221 P2r-P3 . The TbID sequences were optimized for rice, according to the Codon Usage Database of Kazusa DNA Research Centre, Japan (https://www.kazusa.or.jp/codon).

To obtain each final construct, three donor plasmids containing one promoter, one gene coding sequence and one TbID version were recombined into pDEST pH7m34GW through LR reaction.

The control TbID constructs were obtained by traditional Gateway. Recombination sites at the extremity of TbID sequences were modified through PCR, and the enzyme sequence was firstly cloned by BP reaction into a pDONOR207 P1-P2, and then into pYPQ203 (pUBQ::GW) or pB2GW7 (p35S::GW) by LR reaction.

The constructs were transformed into EHA105 agrobacterium cells for *N. benthamiana* agroinfiltrations and rice calli transformations. For protoplasts transfection, pure plasmids were isolated from *E. coli* cells.

All primers used for cloning are reported in the primers list at the end of this section.

PROTOCOLS OF PLANT TRANSFORMATION

N. benthamiana leaves were agroinfiltrated with OD_{600}= 0,4 Agrobacterium for all constructs, except for Hd1, where OD_{600}= 0,3. P19 silencing inhibitor was included in each agroinfiltration event. As reported in the result section, samples for FTL1-fused proteins were recollected 3 days after infiltration. Hd1-fused proteins and the TbID employed for transient PL assay were recollected at 4 days after infiltration.

For protoplasts isolation and transfection, we followed the protocol described by He e. al, 2016 [72], which is based on dark grown rice seedlings. Another protocol was tested, starting from light grown seedlings, giving no positive outcome [73].

The generation of rice transgenic lines was carried out *in vitro* on seed-derived calli. The calli transformation protocol includes subsequent steps of Agrobacterium co-cultivation, antibiotic selection, and plant regeneration, as in Hiei et al., 2008 and Sahoo et al., 2011 [166,167]. The media recipes for rice calli induction and transformation are reported at the end of this section.

All the destination vectors used in our laboratory confer resistance to hygromycin, and progressive calli selection was executed at 50 mg/L and 100 mg/L concentrations of this antibiotic. Positive transformation events were further confirmed through PCR and regenerated plants were transferred to soil for seed propagation (T0 plants).

For FTL1-TbID lines, the plants deriving from the seeds of T0 generation – hence T1 plants - were used for protein detection and immunoprecipitation assays, whereas flowering time experiments and PL proteomic analyses were carried out on T2 lines.

CONFOCAL MICROSCOPY

In *N. benthamiana* microscopy observations, a Nikon Eclipse Ti2 inverted microscope was used, equipped with a Nikon A1R+ laser scanning device and the NIS software (https://www.microscope.healthcare.nikon.com/EU/products). YFP was excited with a 488-nm laser and the emission was recorded in a range of 505-550 nm. For leaf chlorophyll, excitation parameters were the same, but the emission rays were recorded in the 663-738 nm range.

For rice meristem visualization during phenotyping and proteomic sampling of FTL1-TbID lines, we utilized the Normasky microscope Nikon Elicpse E600. Meristem samples were cleared with ethanol prior to observation.

ANALYSIS OF GENE EXPRESSION AND CHROMATIN IMMUNOPRECIPITATION

Rice Hd1-TbID leaf samples were recollected under LD conditions after approximately 1 month from the transfer to soil and RNA was extracted using NucleoZOL (Macherey-Nagel™) and then cleaned from remaining DNA using Turbo DNase (Thermo Scientific™). The cDNA was synthesized with ImProm-II Reverse Transcriptase (Promega™).

RT-PCR was carried out in a RealPlex2 thermocylcer (Eppendorf™), using the Maxima SYBR Green qPCR Master mix (Thermo Scientific™). Analysis of Hd1-TbID was carried out in technical triplicates, using the amplification of the ubiquitin transcripts as a normalizer.

The protocol for chromatin immunoprecipitation is the one reported by Haring et al., 2007 [168].

A Bioruptor® Plus sonication device was employed for chromatin shearing. For immunoprecipitation of FLAG-Hd1-bound chromatin, protein A-coated Dynabeads (Thermo Scientific™) were conjugated to anti-flag M2 mouse monoclonal antibody (Merck™).

RT-PCR was carried out on antibody-treated and non-antibody-treated chromatin samples, as well as on input samples (pre-cleared chromatin before antibody application) for data normalization.

All primers used for RT-PCR and ChIP-PCR are reported in the primers list at the end of this section.

PROTEIN EXTRACTION AND IMMUNOPRECIPITATION

N. benthamiana and rice samples were sampled in liquid nitrogen and cold-grinded with a pH neutral buffer (4M Urea; 50mM Tris-HCl pH 7.5; 150 mM NaCl; 2mM EDTA; 0,2% 2-mercaptoethanol; 0,1 % SDS; Pierce Protease Inhibitor by Thermo Scientific™). Afterwards, proteins were extracted by centrifuging at 14.000 rpm/4°C for 30 minutes, and the supernatant was

recollected. Protein content was quantified by Quick Start Bradford 1x Dye Reagent (Bio-Rad™). For leaf total-proteome analysis, protein concentrations were normalized, and leaf extract were nitrogen-frozen to be sent to MS. For Western blot analyses instead, protein extracts were boiled in 4X Laemmli sample buffer (Bio-Rad™) and run into polyacrylamide gels.

In immunoprecipitation assays, freshly extracted proteins were normalized in concentration and incubated overnight at 4°C with antibody-coated magnetic beads: Dynabeads MyOne Streptavidin C1 (Invitrogen™) or RFP-Trap Magnetic Agarose (Chromotek™). The day after, washing steps were executed with a non-denaturing buffer (50 mM TrisHCl pH 7.5; 150 mM NaCl; 2 mM EDTA; 0,01% SDS; Pierce Protease Inhibitor by Thermo Scientific™) and proteins were eluted from the beads by boiling in 2X Laemmli sample buffer (dilution of 4X Laemmli sample buffer) for 10 minutes. The so-obtained immunoprecipitation pulldowns were either employed for western blot analyses or immediately nitrogen-frozen for MS.

Only in the PL immunoprecipitation experiments, protein samples were filtered through Zeba Spin Desalting Columns, 7K MWCO (Thermo Scientific™) immediately after protein extraction following the instructions of the manufacturer.

The antibodies used for western blot visualizations are reported in the antibodies list at the end of this section.

MASS SPECTROMETRY ANALYSIS

Three biological replicates were processed for rice leaf proteome analyses and PL *N. benthamiana* and rice meristem pulldowns. Proteomic and statistical analyses on raw data were performed by the EMBL Proteomics Core Facility in Heidelberg, Germany (https://www.embl.org/groups/proteomics) . MS was done with an Orbitrap Fusion Lumos ETD machine.

STATISTICAL ANALYSES

For proteomic results, log fold change and false discovery rate (fdr) were calculated, as reference values for protein differential abundance and data meaningfulness. The Limma package was used to analyze the dataset and proteins were assigned the following definitions, based on statistical parameters: hit (fdr<0,05 & log fold change ≥2), candidate (fdr<0,2 & log fold change ≥1.5) or no hit (all the remaining). Proteins were classified as enriched if significantly more abundant in one specific, considered condition. Dot plot analysis was carried out for KEGG and the 20 mostly enriched GO terms in both Biological Process (BP) and Molecular Function (MF) categories. For RT-PCR and flowering time experiments, standard deviation was calculated, and T-test was performed. Results were considered significant with a pvalue ≤ 0,05.

PRIMERS LIST

Cloning

CaMV p35S (Fw) + AttB4	GGGGACAACTTTGTATAGAAAAGTTGCCTGAGACTTTTCAACAAAGGGT
CaMV p35S (Rv) + AttB1r	GGGGACTGCTTTTTTGTACAAACTTGCGTCCTCTCCAAATGAAATGAAC
YFP (Rv) with stop codon + AttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTTGTACAGCTCGTCCATGC
Hd1 (Fw) + AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAATTATAATTTTGGTGGCAACG
Hd1 (Rv) without stop codon + AttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGAACCATGGAACAGTA
FTL1 (Fw) + AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAGCGGGCGGGGGAGG
FTL1 (Rv) without stop codon + AttB2	GGGGACCACTTTGTACAAGAAAGCTGGGTGCATTCTTCTTCCTCCGGTT
pFTL1 (Fw) + AttB4	GGGGACAACTTTGTATAGAAAAGTTGCCGTTTTGTTGCCCGATTCCCT
pFTL1 (Rv) + AttB1r	GGGGACTGCTTTTTTGTACAAACTTGCCACCACCACCCAGCCTTATA
pGOS2 (Fw) + AttB4	GGGGACAACTTTGTATAGAAAAGTTGCCAAGTTTCTGCACCGTTTTCACC
pGOS2 (Rv) + AttB1r	GGGGACTGCTTTTTTGTACAAACTTGCATGGAACTTTGCTGGTGAAA
Maize ubiquitin promoter (Fw) + AttB4	GGGGACAACTTTGTATAGAAAAGTTGCCGTCGTGCCCCTCTC
Maize ubiquitin promoter (Rv) + AttB1r	GGGGACTGCTTTTTTGTACAAACTTGCCTGCAGAAGTAACACC
TurboID (Fw) + AttB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTATGAAGGACAACACCGTGCC
3xHA TAG (Rv) with stopcodon + AttB2 contains STOP codon	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGCGTAGTCCGGCAC

Realtime and ChIP PCR

UBQ (Fw)	GACAACGTGAAGGCGAAGA
UBQ (Rv)	CACCAGGTGGAGTGTGGAC
Hd3a (Fw)	CGATCTGCTGCATGCTCAC
Hd3a (Rv)	CCTTAGCCTTGCTCAGCTATTT
PAL4 (Fw)	GATTAGCTGAATAACAGTGGAG
PAL4 (Rv)	GTTGCATGCTTGCCGCTATG
CAD (Fw)	CCGCCTAGCTTGCTTGTTGT
CAD (Rv)	CTCCATTTCGTAGCTAGTTTGC
PAL4 Promoter (Fw) Pair I	CAACCACCACACAGCCACAG
PAL4 Promoter (Rv) Pair I	CGTCGCGGAGTAGGAGAGAA
PAL4 Promoter (Fw) Pair III	ATGTCGCCGAACAAGTCTCT
PAL4 Promoter (Rv) Pair III	CAGGTCTCGCTGATATGTTCC
PAL4 Promoter (Fw) Pair IV	CTCTCGGTCAAGTCCATGCT
PAL4 Promoter (Rv) Pair IV	TGGCAACTGCTCTTCAGATG
PAL4 Promoter (Fw) Pair V	GGAACATCCCTAAATGGAACG
PAL4 Promoter (Rv) Pair V	CGGTCTTCTCGTGGTTTCTC
CAD promoter (Fw)	CAATAGAGGGAATGGAGAGAAGT
CAD promoter (Rv)	GGCTTGCAGCTGGTAGTGTT
Hd3a promoter (Fw)	ACACGTACAGGAAGACGATGC
Hd3a promoter (Rv)	TAGGAGGGCAAGATGATGGC

ANTIBODIES LIST

Anti-YFP mouse monoclonal, Agrisera™ (AS18 4177)

Anti-RFP mouse monoclonal, Agrisera™ (AS15 3028)

Anti-H3 rabbit polyclonal, Agrisera™ (AS19710)

Anti-HA rabbit polyclonal, Agrisera™ (AS12 2220)

Anti-FLAG M2 mouse monoclonal, Merck™ (F3165)

Streptavidin, HRP conjugate Millipore™ (18-152)

MEDIA RECIPES FOR RICE CALLI TRANSFORMATION

Basal NB-medium	
N6 Macro I (10x)	100 ml/L
N6 Macro II (10x)	100 ml/L
MS FeNaEDTA (1000x)	1 ml/L
B5 Vitamins (1000x)	1 ml/L
B5 Micro (1000x)	1 ml/L
Proline	500 mg/L
Glutamine	500 mg/L
Casamino acids (CEH)	300 mg/L

Basal R2-medium	
R2 Macro I (10x)	100 mL/L
N6 Macro II (10x)	100 mL/L
R2 FeNaEDTA (1000x)	1 mL/L
R2 Micro (1000x)	1 mL/L
LS Vitamins (400x)	2,5 mL/L

Callus Induction Medium pH = 5,8	
Basal NB-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Agarose type I	3 g/L

Co-cultivation Medium Liquid pH = 5,2	
Basal R2-medium	
Glucose	10 g/L
2,4-D	2 mg/L
Acetosyringone	150 µM
Co-cultivation Medium Solid pH = 5,2	
Co-cultivation Medium Liquid	
Agarose type I	7 g/L

Selection Medium I pH = 6	
Basal R2-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Cefotaxime	400 mg/L
Vancomycin	100 mg/L
Hygromycin	50 mg/L
Agarose type I	7 g/L

Selection Medium II pH = 6	
Basal NB-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Cefotaxime	400 mg/L
Vancomycin	100 mg/L
Hygromycin	100 mg/L
Agarose type I	7 g/L

Pre-Regeneration Medium pH = 5,8	
Basal NB-medium	
Sucrose	30 g/L
Cefotaxime	100 mg/L
Vancomycin	100 mg/L
Hygromycin	50 mg/L
ABA	5 mg/L
6-BAP	2 mg/L
NAA	1 mg/L
Agarose type I	7 g/L

Regeneration Medium pH = 5,8	
Basal NB-medium	
Sucrose	30 g/L
BAP	3 mg/L
NAA	0,5 mg/L
Agarose type I	7 g/L

Rooting medium pH = 5,8	
Basal MS medium	
Sucrose	50 g/L
Plant agar	2,5 g/L

Basal NB-medium	
N6 Macro I (10x)	100ml/L
N6 Macro II (10x)	100ml/L
MS FeNaEDTA (1000x)	1ml/L
B5 Vitamins (1000x)	1ml/L
B5 Micro (1000x)	1ml/L
Proline	500mg/L
Glutamine	500mg/L
Casamino acids (CEH)	300mg/L
Basal R2-medium	
R2 Macro I (10x)	100ml/L
N6 Macro II (11,3x)	88,5ml/L
R2 FeNaEDTA (1000x)	1ml/L
R2 Micro (1000x)	1ml/L
LS Vitamins (400x)	2,5ml/L
Modified Callus Induction Medium	
Basal NB-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Agarose type I	3 g/L
pH = 5,8	
Co-cultivation Medium Liquid*	
Basal R2-medium	
Glucose	10 g/L
2,4-D	2 mg/L
Acetosyringone	150 µM
pH = 5,2	
Co-cultivation Medium Solid*	
Co-cultivation Medium Liquid	
Agarose type I	7 g/L
pH = 5,2	
Selection Medium I	

Basal R2-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Cefotaxime	400 mg/L
Vancomycin	100 mg/L
Hygromycin	50 mg/L
Agarose type I	7 g/L
pH = 6	
Selection Medium II	
Basal NB-medium	
Sucrose	30 g/L
2,4-D	2 mg/L
Cefotaxime	400 mg/L
Vancomycin	100 mg/L
Hygromycin	100 mg/L
Agarose type I	7 g/L
pH = 6	
Pre-Regeneration Medium	
Basal NB-medium	
Sucrose	30 g/L
Cefotaxime	100 mg/L
Vancomycin	100 mg/L
Hygromycin	50 mg/L
ABA	5 mg/L
6-BAP	2 mg/L
NAA	1 mg/L
Agarose type I	7 g/L
pH = 5,8	
Regeneration Medium	
Basal NB-medium	
Sucrose	30 g/L
BAP	3 mg/L
NAA	0,5 mg/L
Agarose type I	7 g/L
pH = 5,8	
Rooting medium	

Basal MS medium	
Sucrose	50 g/L
Plant agar	2,5 g/L
pH = 5,8	

Note: all the chemical compounds used in our experiments were produced by Duchefa™.

SUPPLEMENTARY MATERIAL

All supplementary tables are available at the following link:

https://unimi2013-my.sharepoint.com/:f:/g/personal/daniele_chirivi_unimi_it/EjAlHclt-29KkjarzToxnIMB6_-LYZhN9m7zpkEClMdu3g?e=Fn3j6Y

In case of any problem in the file download, please contact me at daniele.chirivi@unimi.it .

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