UNIVERSITÀ DEGLI STUDI DI MILANO

FACOLTÀ DI SCIENZE AGRARIE E ALIMENTARI

PhD School in Agriculture, Environment and Bioenergy
XXXV cycle

Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy

Photoperiodic regulation of rice stem elongation by the gene *PINE1*

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A.A. 2022/2023

Abstract

In several plant species, inflorescence formation is accompanied by stem elongation. While these two processes need to progress harmoniously, until recently no genetic link had been found that could integrate and coordinate both pathways. Only a recent study has shed light on a new and unexplored pathway that, via the *PREMATURE INTERNODE ELONGATION 1 (PINE1)* gene, links flowering with stem elongation [1]. PINE1 is a zinc finger transcription factor able to repress stem elongation during vegetative growth. During floral induction, the florigenic signal can silence *PINE1* expression, triggering internode elongation. This thesis aims to explore the mechanism of action of PINE1 and the potential exploitation of this pathway for rice breeding for agriculture. The hypothesis that PINE1 could be regulated by genes involved in the flowering machinery has been tested by generating overexpressors of the known flowering related genes *OsMADS14, OsMADS15* and *FTL1* to assess their phenotype regarding stem elongation. Indeed, *FTL1*, the expression of which is promoted by florigens, could be involved in *PINE1* inhibition during flowering. Previous literature on genes similar to *PINE1* reported interaction with co-repressors proteins. Thus, some of the recurring interactors were cloned to test whether they can effectively interact with PINE1. The data suggests that *PINE1* is able to form a complex with TOPLESS (TPL) proteins, which may function in transcriptional repression through chromatin remodeling. The recognition of PINE1 by TPL depends on the presence of EAR-motifs, which are also important for PINE1 function overall.
As PINE1 is a regulator of stem elongation and plant height is an important breeding trait, we attempted to modify plant height by modulating PINE1 expression by introducing mutations in its promoter region. Some of the generated plants did show strong dwarfism, indicating that some mutation in the promoter region lead do a PINE1 overexpression. For the same purpose of finding ways to control plant height, we also identified some candidate genes controlled by PINE1 using RNA sequencing and created knock-out mutations using the CRISPR/Cas9 system to further explore their role in stem elongation.
Overall, this work establishes the framework for a new pathway that controls plant architecture and has implications for crop improvement. Further studies will be needed in the coming years to elucidate the entirety of this cascade of events from flowering to stem elongation and to explore the potential applications of this research for agriculture.

Contents

1. Introduction

It is well-known that rice (*Oryza sativa*) is a crucial species and the third most important crop in terms of production scale, surpassed only by maize and wheat. With over half of the world's population relying on it as a staple food, rice provides approximately 50% of the total caloric intake for 520 million people [2].

The earliest archaeological evidence suggests that *Oryza sativa* was domesticated from its wild ancestor *Oryza rufipogon* in South-East Asia about 10,000 years ago. However, there is an ongoing debate about whether modern rice originated from a single domestication event or multiple independent domestications in various Asian regions. During this period, *Oryza glaberrima* was also domesticated in Africa, but its cultivation remained of minor significance and will not be further discussed in this thesis [3], [4].

Several traits were selected during the domestication process, and many of the causal genes are now known. The best-known domestication gene is probably *Shattering 4 (Sh4)*, where a partial loss-of-function mutation results in a significant reduction in grain shattering [5]. Another widely recognized domestication gene is *SEMI-DWARF 1* (*Sd1*), which was targeted during domestication through the spread of an allele that causes a reduction in tiller length in cultivated rice [6].

Figure 1. Rice lodging [8]

Exactly *Sd1* would much later play a crucial role in the 20th century green revolution, a period of significant agricultural improvement. The use of high-yielding varieties, coupled with mechanization, use of fertilization and pesticides brought many positive changes for agriculture. Nonetheless, plants that produced more seeds also faced new problems that needed to be addressed. Indeed, the total weight of grains produced by a panicle became a limiting factor for the underlying stem. The increased weight of the panicle causes the stem to bend more, and sometimes even lay completely on the ground. This phenomenon is called lodging (Fig. 1). Traditional low yield varieties rarely exhibit this problem since their panicles do not weigh as much [7].

Lodging is a significant issue: empty grain, abortive grain and viviparous germination are all greatly increased following lodging [9]. To prevent lodging, researchers turned to the introduction of a dwarf trait present in the Taiwanese variety Deegeo-woo-gen. By crossing, the dwarf trait was introgressed into many high yielding varieties, leading to a consistent increase in yield due to the newly acquired resistance to lodging. Many years later Ashikari et al. [10] and Monna Lisa et al. [11] were able to identify the causal gene for the dwarf phenotype, which was a loss-of-function mutation of *Sd1*. They discovered that *SD1* encodes a GA20-oxidase, a key enzyme for Gibberellin biosynthesis.

The lack of that GA20-oxidase is responsible for rice dwarfism, meaning that the stem of these plants is much shorter than normal, or more precisely, the length of internodes is affected. By preventing yield losses linked to lodging, the Green Revolution was able to double the yield of both rice and wheat using the same amount of agricultural land [7]. While plant height is probably the most relevant trait related to lodging, other factors can also influence it. The silica content in the stem, cross-sectional area of the stem, and number of vascular bundles, by influencing mechanical support, are all factors that affect lodging resistance [12].

1.1 Rice stem architecture

The rice stem, like stems of other species belonging to the Poaceae family, consists of nodes and internodes that develop from the shoot apical meristem (SAM) as it grows. Every time a leaf is produced, a corresponding node is also produced. The node serves as a junction between that leaf sheath and the stem. Each node also supports an axillary meristem (or bud meristem) and two rows of adventitious roots (or crown roots) (Fig. 2). Even nodes located in the aerial part possess coronary root primordia that can develop into true roots under the right conditions. Each node is connected to upper and lower nodes through a complex and organized vascular system, extensively described by Yamaji and Ma [13]. Additionally, the vasculature of each leaf is connected to

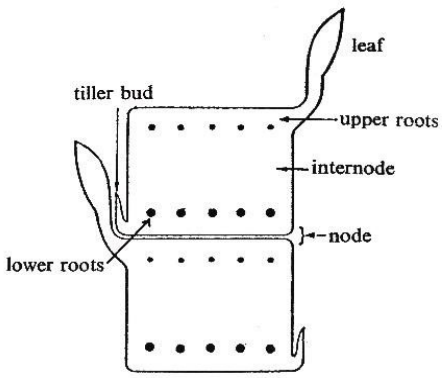

Figure 2. Representation of rice stem architecture, which is composed of nodes and internodes, from which adventitious roots emerge [14]

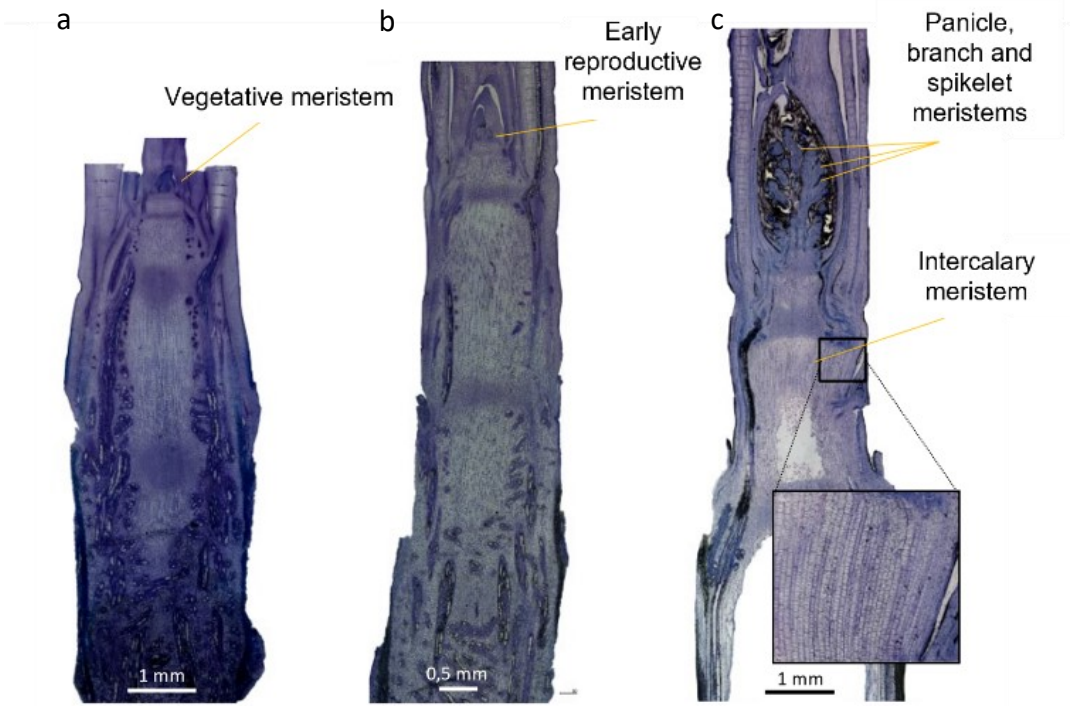

*Figure 3. Rice stem and SAM grown under long days (**a**). 14 days after shift from long day to short day (**b**), and 21 days after the shift (**c**). Source: Giulio Vicentini, unpublished data.*

the stem vasculature at each node, enabling the transport of photosynthates to the sink organs of the plant and the arrival of water and mineral resources from the roots. Internodes, on the other hand, are the sections of tissue that exist between nodes and are mainly composed of vertical vascular bundles that extend from the base of the plant to the SAM/panicle. During the vegetative phase of the life cycle, the internodes do not elongate, resulting in a short stem that is only around 1-2 cm long.

Being originally from southeast Asia, which has a tropical climate, rice has developed as a short-day species. During the summer, when heavy rains occur, vegetative growth takes place, while during the fall, when temperature in those areas are still high, the shorter day length induces flowering. With exposure to short days, the transition from a vegetative meristem to an inflorescence meristem starts. The SAM is still located at the base of the plant, in an enclosed space. The panicle cannot fully develop there, both due to a physical lack of space and since exposure to the environment is necessary for seed dispersion. During the formation of the panicle, internode elongation occurs, which raises the inflorescence during its development, permitting heading at maturity (Fig. 3 and Fig. 4).

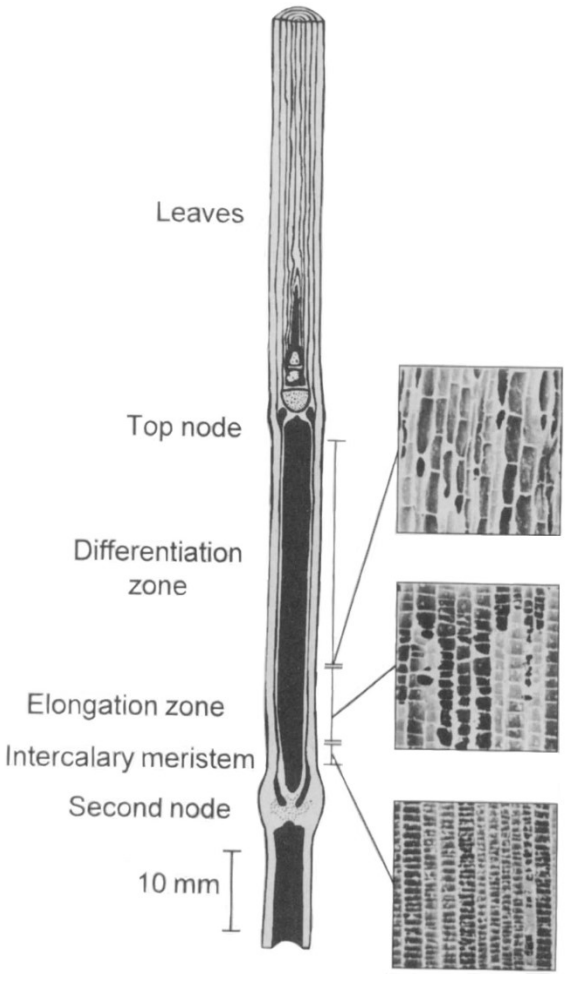

Figure 4. Differences of an internode into its 3 zones: intercalary meristem, elongation zone and differentiation zone [15]

Internode elongation occurs in a very precise way. First of all, not all internodes elongate. The "oldest" internodes, i.e. the lowest ones, actually remain unelongated. Once the flowering stimulus occurs, the SAM stops producing nodes and leaves. From that point forward, only the upper 4 or 5 internodes (the most recently formed ones) retain the ability to elongate. Elongation occurs thanks to the "activation" of intercalary meristems, which are also called rib meristem due to the same parallel cell division plane in the whole meristem.

Intercalary meristems are ring-shaped meristems located just above each node. During vegetative growth they are inactive, but during flowering they are essential for internode elongation. Their mitotic activity is responsible for the increase in cell number during stem elongation. On top of that, the newly formed cells gradually distend over time, contributing to the elongation overall. In each internode 3 zones can be distinguished: 1) the intercalary meristem, with high mitotic activity; 2) the elongation zone, where we find young cells which are not dividing but are expanding and 3) the differentiation zone, with fully

differentiated cells that reached their final dimension (Fig. 4).

Being ring-shaped, the activity of the intercalary meristem causes the elongation only of the outer layer of cells, while the central part of each internode becomes stretched. For this reason, during internode elongation cells are quickly torn apart, leaving an empty cavity inside each elongated internode [14].

1.2 Rice flowering pathway

The pathway of rice flowering has been well studied, as heading date is a trait of great agronomical interest.

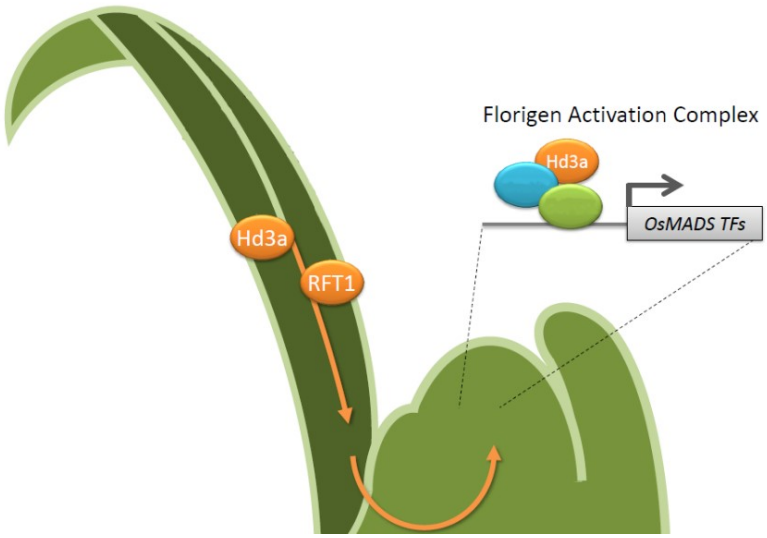

Figure 5. Hd3a and RFT1 are rice florigens: proteins synthesized in the leaves and transported via the phloem to the SAM, where they form a protein complex that promotes flowering initiation by activating the expression of genes necessary for flowering, such as OsMADS transcription factor [18].

As mentioned previously, under long days rice undergoes vegetative growth, which happens as phytochromes perceive the long daylength and promote the expression of a wide variety of flowering inhibitors, which repress the expression of florigenic proteins in the leaves. As the days become shorter, the balance shifts to an abundance of flowering promoters over inhibitors, leading to the expression in the leaves of the two rice florigens *Hd3a* and *RFT1*. Those two proteins enter the phloem and are transported to the SAM. In the SAM, the two rice florigens associate in the Florigen Activation Complex (FAC), a protein complex that modifies gene expression and promotes the transition from a vegetative to an inflorescence meristem, ultimately leading to the development of a panicle (Fig. 5) [16], [17]. So the leaves are the organ able to perceive the photoperiod due to phytochromes, while the florigens Hd3a and RFT1 are proteins which act as mobile signals, transporting the information from the leaves to the SAM, which then undergoes a transition to an inflorescence.

This behavior of rice occurs in the areas where it was first domesticated. But rice has been able to adapt to many different environments due to a wide variety of mutations in flowering activators and repressors, thus permitting flowering at different latitudes [19]–[21]. The Italian climate is also not suitable for the short day flowering of rice, so even Italian varieties present mutations in such flowering regulators to permit flowering in a photoperiod-independent way. In contrast, the Japanese variety Nipponbare has a strong dependance on photoperiod. This variety is frequently used in genomic studies, being the first rice variety with

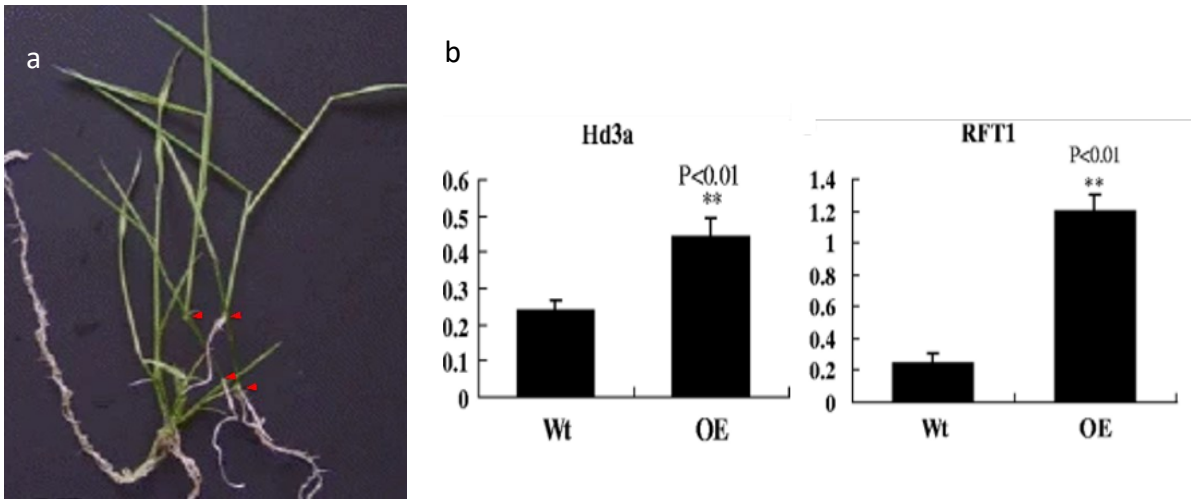

Figure 6. ***a****: OsMADS15 overexpressing plants. Red triangles points to nodes.* ***b****: florigens expression in in WT and OsMADS15 overexpressing plants (OE) [28].*

completely sequenced genome [22]. When florigens enter the SAM and form the previously mentioned FAC, a cascade of events takes place. Among the first genes targeted by the FAC are the MADS-box genes (or OsMADS), that belong to a family of transcription factors known since a long time to be involved in flowering. In fact, research on flowering from the 1990s to the early 2000s largely focused on OsMADS. A review by Yamaguchi and Hirano (2006) summarizes what was known at the time regarding the role of OsMADS in flowering and panicle architecture [23].

The MADS-box TFs OsMADS14, OsMADS15, OsMADS18 and OsMADS34 are required for flower development as the quadruple knockout mutant *osmads34/osmads14/osmads15/osmads18* shows stem elongation (although very much delayed) with new leaves developing instead of flowers [24], [25]. The FAC is able to bind to C-box motifs (GACGTC) [26], which are present in the promoter regions of all the above mentioned *OsMADS* genes. Among these OsMADS, the two which seem to have the biggest impact on flowering are OsMADS14 and OsMADS15. Overexpression of *OsMADS14* under the *UBIQUITIN* promoter leads to very premature flowering in rice calli, so no actual plant can develop [27] while overexpression of *OsMADS15* leads to extremely early flowering of plants with early internode elongation [28]. However, these overexpressors show also a higher level of florigens expression, meaning that by overexpressing the OsMADS gene, a positive feedback mechanism leads to the expression of the florigens and then of the whole flowering machinery (Fig. 6) [28].

Other genes which are activated upon arrival of the florigens into the SAM are FT-like genes (FTL). In particular FTL1 is one of the most upregulated genes during flowering [29]. Those genes are closely related to the florigens themselves, being extremely similar in terms of aminoacidic sequence. FTL1 is not expressed in the leaves but it is expressed in the SAM during floral transition, and very recently it was reported that it is an important factor during flowering, both in regulating the meristematic transition and in controlling panicle architecture [29]. These experiments could conclude that both *OsMADS* and FTL genes are

main actors during the first phases of the floral induction and overall during the whole flowering process.

1.3 Role of hormones in stem elongation

Stem elongation is a process strongly driven by hormones: gibberellins (GA) and brassinosteroids being the major ones. GAs were firstly discovered in rice, as the pathogenic fungus *Gibberella Fujikuroi* caused rice aerial organs to over-elongate, causing stunted growth (Fig. 7). The study of this mechanism led to the discovery of gibberellins [30].

Figure 7. Gibberella fujikuroi infected plant on the left. Healthy plant on the right [31].

GAs are synthesized from geranylgeranyl diphosphate through multiple oxidative steps. GA1 and GA4 are typically the bioactive GAs in plants, while GA3 and GA7 are minor products mainly produced by fungi like the previously mentioned *Gibberella Fujikuroi* [30]. The degradation of GAs occurs due to the activity of GA2oxidase (GA2ox). Interestingly, some GA2ox are present as a ring layer below the meristem during vegetative growth, but this layer vanishes after floral induction. Speculation suggests that this happens to protect the SAM from GAs, thereby avoiding differentiation of indeterminate cells [32]. GA signaling is also well studied. GAs are tetracyclic diterpenoid hormones, therefore hydrophobic. As such, they can pass through membranes as protonated acids. Indeed, the GAs receptor is a nuclear protein called GID1. When GAs bind to GID1, conformational changes to GID1 itself permit the binding of the GID1-GA complex to rice DELLA protein SLENDER RICE 1 (SLR1) (Fig. 8). SLR1 is a growth repressor, that binds and

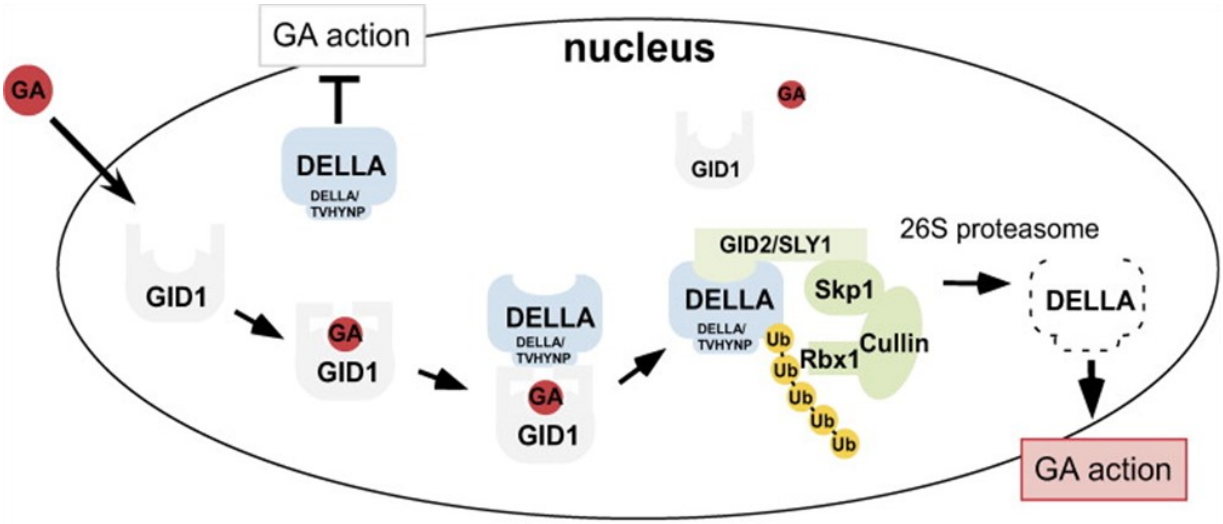

Figure 8. Pathway for GA signaling. GA can bind to its nuclear receptor GID1. Once that happens, rice DELLA protein SLR1 can bind the complex and is then subjected to ubiquitination [34].

activates growth repressing transcription factors/regulators, or inhibits growth promoting transcription factors/regulators. When SLR1 binds to GID1-GA complex, it can be recognized by an E3 ubiquitin ligase (GID2), leading to its degradation through the proteasome 26s pathway. Thus GAs promote growth indirectly by degradation of a growth repressor [33]–[35].

Typically GAs are synthesized where they exert their actions, in elongating tissues. The work of Kaneko et al. [36] showed that enzymes involved in GA metabolism were highly expressed in growing tissues such as germinating seeds, in the basal part of elongating internodes, corresponding to the location of the intercalary meristem, and in the developing panicle (Fig. 9). As mentioned previously, the importance of GAs for stem elongation is also evident from the semidwarf phenotype of *semi-dwarf 1 (sd1)* plants selected during the Green Revolution.

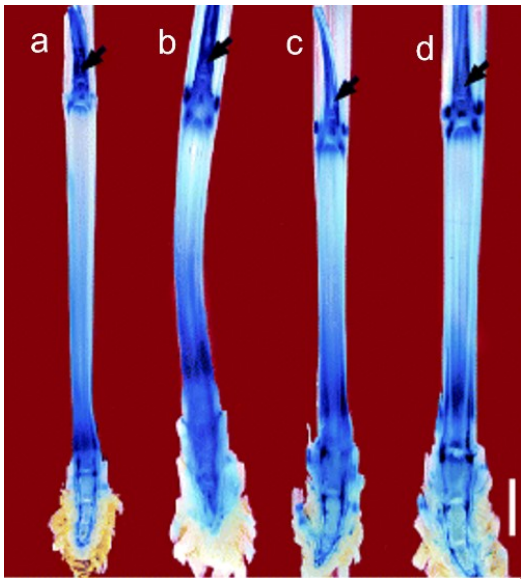

Figure 9. Localization of GUS activity under control of the promoter of different genes involved in GA biosynthesis or signaling. ***a:*** *OsGA3ox2.* ***b****: OsGA20ox2.* ***c****:Ga.* ***d****: SLR1 [36].*

While GA is likely the major hormone responsible for stem elongation, the process involves the interplay among many hormones. It is indeed known that auxin coming from the developing panicle is necessary to promote GA biosynthesis in the stem [37]. Brassinosteroids were discovered due to their effects in promoting stem elongation. Deficient and insensitive brassinosteroid mutants usually display dwarfism with a specific pattern of internode elongation: the second internode usually is unelongated or strongly shortened while the others are almost unaffected. Presumably, different internodes have different levels of brassinosteroid sensitivity [38].

1.4 Role of PINE1 in stem elongation

Ultimately, it is clear that flowering and stem elongation are tightly linked process, and their coordination is essential to achieve reproductive success. Such a relationship implies some kind of molecular connection between the two processes. Such a link was identified in 2019 as PREMATURE INTERNODE ELONGATION 1 (PINE1) [1]. The focus of this thesis is the molecular characterization of *PINE1* gene function.

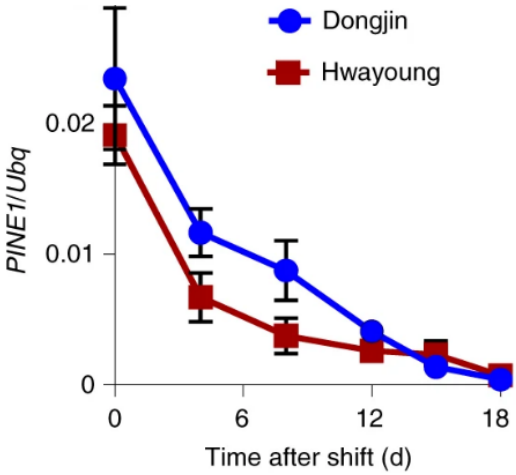

Figure 10. PINE1 expression during floral induction in two different rice varieties [1].

PINE1 was isolated by studying the changes in gene expression at the shoot apical meristem (SAM) during floral transition. By performing an RNA-sequencing of SAM during vegetative growth

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PINE1       MEAPPSLSIVDEDGFVIDLSLTLGLTSPPPSPGGASPSIPPGRGGGGGTSGGDNNRGSRG 60
PROG1       -MDPSSASWPAPASPPVELSLSLPAAAARNRDEAAPTAIVDGK---------------- 42
               * * *     .  ::***:*  ::      .*  :*  *:

PINE1       GGNGGGGGGVRLFPCLFCNKKFLKSQALGGHQNAHKKERSVG--WNTHLYLPAGVAAATT 118
PROG1       --------QVRLFPCLFCAKTFRKSQALGGHQNAHRKERVAGGSWNPNVYGDGGGSA--- 91
                    ********* *.* ***********:*** .*  ** ::*  .* :*

PINE1       TTTTTTAMAVPDMVGMPTHQMSSMALHSCRPHQGSHVTA--ADIATLAAPPHYTVDHGVA 176
PROG1       --------------SMPIASHGVTAAGSSTAADGRWCGGAASDDDTTAAPM-PSLGSGSA 136
                         .**  . .  *  *.   :*    .  :*  * ***   ::. * *

PINE1       GIASGGGDSSVGWRQRQREAGGEKQRQVDLNLKL   210
PROG1       ALGAGAGFAST---ERGSSGGGVAGEELVLELGL   167
            .:.:*.* :*.   :*  ..**   .:: *:* *
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Figure 11. Alignment of PINE1 and PROG1 protein sequences. Highlighted in the yellow rectangle are the EAR-motif, easily recognizable by the sequence LXLXL, and the C2H2 zinc finger domain. Alignment done with CLUSTAL OMEGA web tool.

under long days (LDs) and after exposure to short days (SDs), changes in gene expression during floral induction were identified. Among the genes that were differentially expressed during flowering induction, *PINE1* was the most downregulated gene encoding for a transcription factor. Meaning that *PINE1* is expressed during vegetative growth while its expression decreases with flowering (Fig. 10) [1]. *PINE1* encodes for a C2H2 zinc finger transcription factor, meaning it has 2 Cysteines and 2 Histidines that bind to a zinc atom to form a structure (called Zinc Finger) able to interact with the DNA [39]. This family of transcription factors is quite large, with 189 members in rice, many of which play critical roles in plant development [40]. One of the best-known examples in rice is *PROSTRATE GROWTH 1*

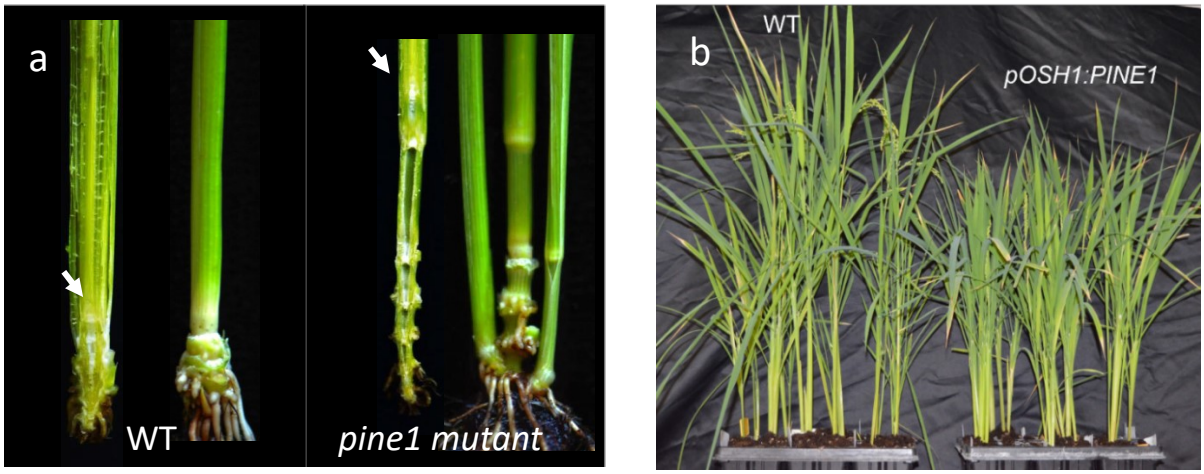


*Figure 12. **a**: Comparison between WT and pine1 knockout mutant, the white arrow point indicates the SAM. **b**: Comparison of WT and PINE1 overexpressing plants [1].*

(PROG1), which is a gene strongly associated with the domestication history of rice. Wild rice ancestor *Oryza rufipogon* displays a prostrate growth habit due to PROG1 activity in promoting a wide tiller angle. During domestication, a mutation that changed one amino acid inhibited PROG1 activity, resulting in a shift from a prostrate to an erect culm architecture that was favored by artificial selection [41]. PROG1 is an apt comparison to PINE1, not just

because they are both C2H2 zinc finger proteins, but also because they both contain two Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR)-motifs in their structure. Furthermore, these EAR-motifs share similar locations within PINE1 or PROG1 proteins: one is towards the N-terminal while the other is at the very C-terminal (Fig. 11).

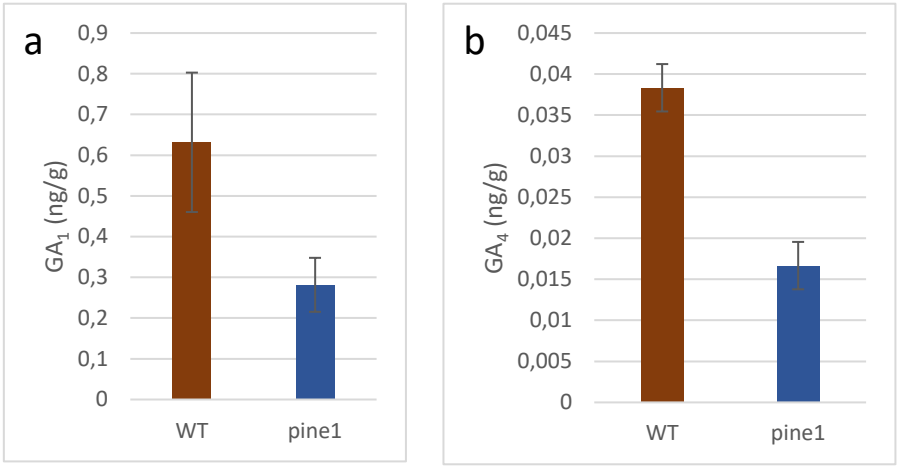


*Figure 13. Bioactive GA (GA_1 in **a** and GA_4 in **b**) quantification in WT and pine1 knockout mutant [1].*

PINE1 plays a critical role in inhibiting internode elongation during vegetative growth. Indeed, *pine1* knock-out mutants display internodes elongation (Fig. 12) starting around 15 days after germination, in a process independent from flowering (thereafter the name of the gene). This implies that PINE1 acts as a repressor of stem elongation. This is further corroborated by RNA-sequencing data, which reported PINE1 as more expressed under LDs (when internodes need to be kept unelongated) and less expressed during flowering, permitting internode elongation. Further experiments confirmed that some components of the flowering machinery are able to inhibit *PINE1* expression during flowering, leading to the elongation of the last formed internodes.

When *PINE1* was expressed under the *OsACTIN2 (ACT2)* promoter (a strong and ubiquitous promoter) plants were sterile, potentially due to ectopic expression of *PINE1* in floral organs. On the other hand, when PINE1 was overexpressed under the *OSH1* promoter (specifically expressed in the SAM area), plants showed repressed growth (Fig. 12), with a panicle that did not fully emerge due to a deficit in internode elongation, suggesting PINE1 as an inhibitor of internode elongation [1].

As GA is the primary hormone responsible for stem elongation, it has been investigated whether PINE1 is involved in GA metabolism. Surprisingly, bioactive GA quantification showed that WT plants contained more bioactive GA than *pine1* mutants (Fig. 13), indicating that the *pine1* internode elongation is not caused by excess GA. The expression of genes involved in GA biosynthesis also supported this finding. On the other hand, treatment with exogenous GA revealed that *pine1* plants have increased GA responsiveness, as they elongate more with higher GA concentrations, whereas WT plants are insensitive to exogenous GA application (Fig. 14). These results suggest that PINE1 plays a role in GA sensitivity [1].

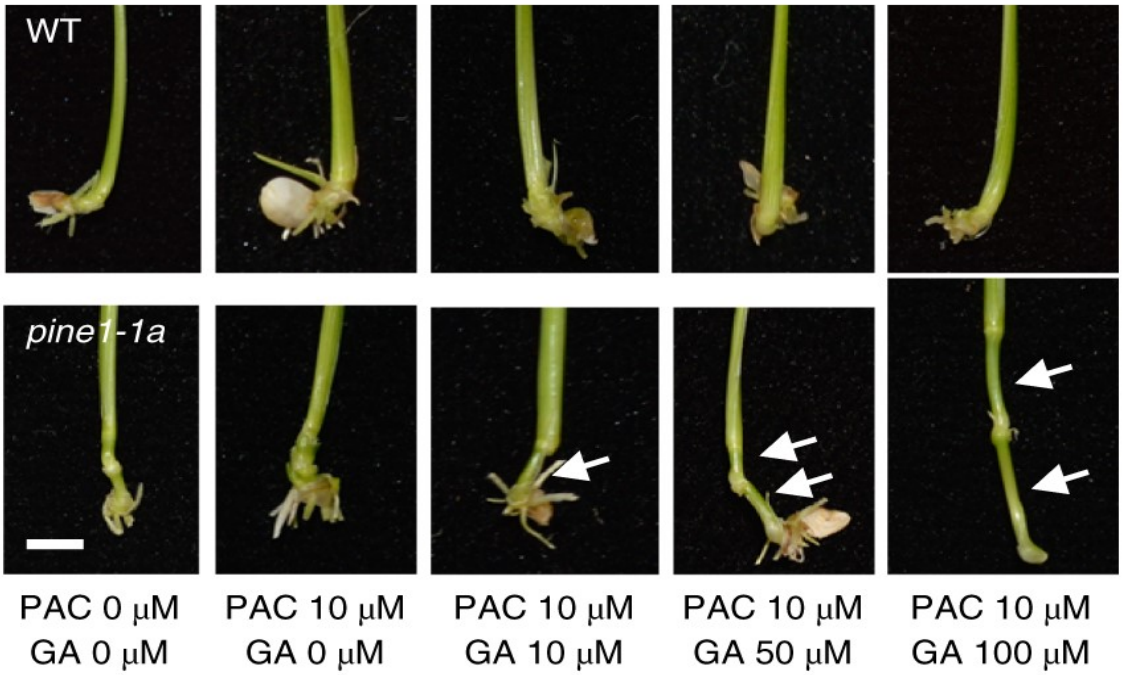


Figure 14. Exogenous GA_3 treatment on WT (TOP) and pine1 knockout mutant (BOTTOM). PAC=Paclobutrazol, a strong inhibitor of GA biosynthesis used to even the level of endogenous GAs in the different samples. White arrows indicate internodes [1].

To detect the exact location of *PINE1* expression *in vivo*, a construct was used that expresses the green fluorescent protein (GFP) under the control of the *PINE1* promoter region. Confocal analysis revealed that *PINE1* is expressed throughout the stem, with a strong signal in nodes, especially lower nodes of unelongated stems. Weaker expression is also present at the insertion site of leaves in the stem and around the SAM in plants grown under LD conditions, and, consistently with RNA-seq data, it is no longer expressed after SDs exposure. *PINE1* expression is higher in nodes, but is weaker in unelongated internodes, with expression present on the internal side of the leaf sheath and near the vascular bundles. Localization of *PINE1* expression across the stem supports its role in regulating stem elongation during vegetative growth and flowering (Fig. 15) [1].

The same gene was also discovered by the lab of Prof. Motoyuki Ashikari at Nagoya University (Japan) while cloning QTLs responsible for stem elongation in deepwater rice. Ashikari et al., named the gene *DECELERATOR OF INTERNODE ELONGATION 1 (DEC1)* as an allelic variant present in deepwater rice that allows internode elongation under flooding [42].

Deepwater rice is a type of rice that exhibits a distinct growth habit when submerged (Fig. 16). Those varieties grow in flood prone areas and are adapted to survive flooding [15]. Submergence leads to anoxia, which causes an increase in ethylene content since ethylene is not soluble in water and remains entrapped within tissues.

Ethylene is a gaseous phytohormone which triggers a unique pathway of deepwater rice. Deepwater rice has genes called *SNORKEL1 (SK1)* and *SNORKEL2 (SK2)* which are absent in the other rice varieties. Those genes mediate the response to the increased ethylene by promoting GA biosynthesis and reducing SLR1 levels. That causes internodes to elongate, and that elongation maintain the apical portion of the plant above the water level [43]. Besides this mechanism, Ashikari's group discovered that in deepwater varieties is also present an allelic variant of *PINE1/DEC1* with differences in its own promoter region. In deepwater rice, *PINE1/DEC1* expression decreases drastically during submergence, contributing to internode elongation. Moreover, exogenous GA treatment inhibits *PINE1/DEC1* expression in deepwater rice but not in "normal" rice, further indicating different regulations in the two variety. These findings suggest that *PINE1* is not only controlled by the flowering process, but its expression can change also in response to anoxia in deepwater rice [42].

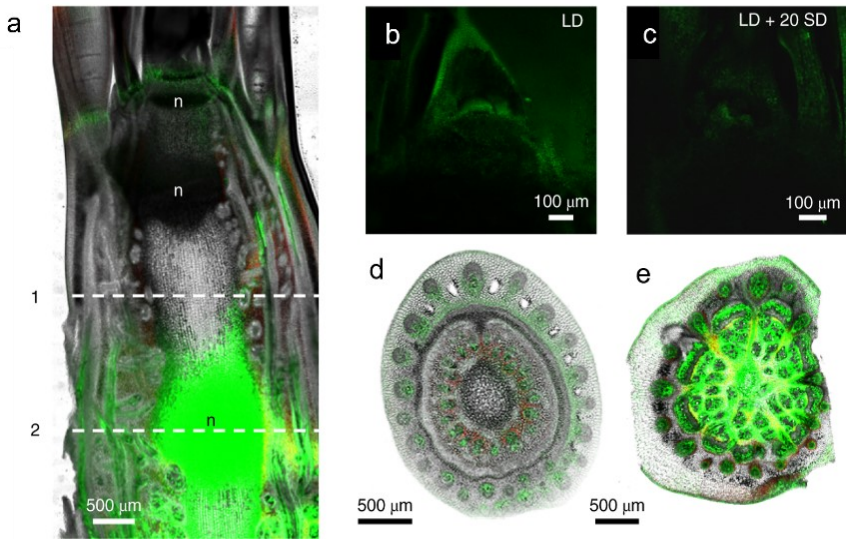


Figure 15. Analysis of transgenic plants carrying the construct pPINE1:GFP. ***a****: Longitudinal section of a rice stem. 1 and 2 indicate respectively an internode and a node.* ***b****: GFP signal in a vegetative shoot apical meristem.* ***c*** *inflorescence meristem.* ***d****: Cross section of the stem at an interrnode level.* ***e****: cross section of a rice stem at a node level [1].*

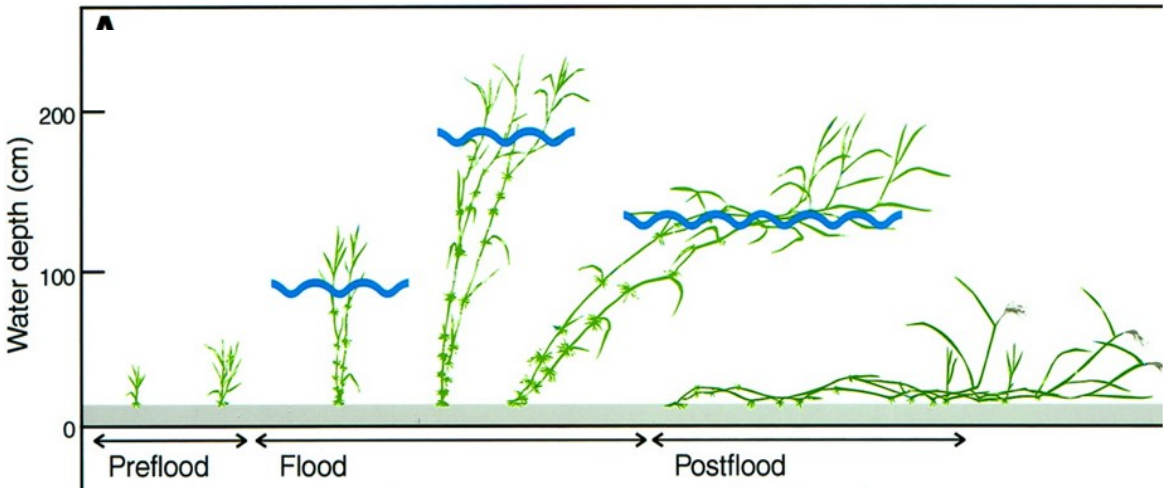


Figure 16. Deepwater rice elongate internodes if exposed to submergence [15].

As mentioned earlier, SLR1 is the sole DELLA protein in rice and its degradation is the only identified mechanism through which GAs exert their role of growth promoters. Notably, SLR1 decreases equally in deepwater rice and normal rice upon exogenous GA treatment, implying

that SLR1 degradation alone is not enough to induce stem elongation and hinting at the possibility that PINE1 operates through a separate pathway from SLR1 [42].

1.5 Use of CRISPR/CAS9

PINE1 mutation was obtained through CRISPR/Cas9 technology. CRISPR is a genome editing system that allows for targeted mutations. It derives from a molecular defensive system of bacteria to protect themselves from viruses. In particular Cas9 derives from *Streptococcus pyogenes*, but many other similar proteins are present in other Bacteria. Cas9 is an endonuclease, that can cut DNA. The cut is not dependent on protein-DNA recognition as in the case with restriction enzymes. Instead, the high precision of CRISPR/Cas9 is achieved by the binding of a guideRNA (gRNA) to the Cas9. gRNA contains a sequence called spacer, which is user-defined and binds to a complementary DNA sequence directing the Cas9 to the genomic target site. The gRNA also contains a scaffold, which is a sequence recognized and bound by Cas9. To trigger a double-strand break, the CRISPR/Cas9 system requires two conditions: first, the guide RNA must bind and anneal to a complementary sequence in the genome, and second, a specific adjacent sequence called protospacer adjacent motif (PAM) must be present. For the commonly used Cas9 enzyme, the PAM sequence is NGG, which must flank the complementary sequence. Once these two conditions are met, the Cas9 enzyme can bind to the DNA and cause a double strand break (Fig. 17). The DNA repair process, usually non homologous end joining, then attempts to repair the cut. But while doing so, random mutations are frequently introduced. If these mutations occur in coding sequences, they frequently lead to frame-shift mutations and gene knock-out [44].

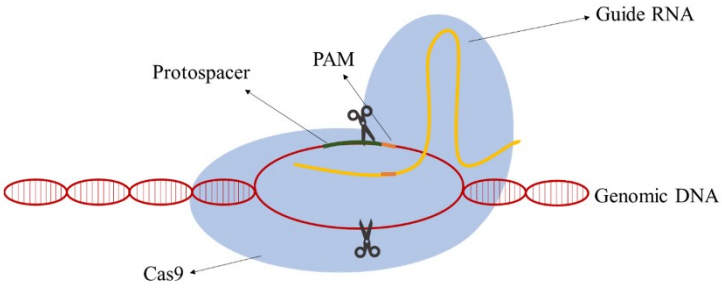


Figure 17. Representation of the Cas9 binding to a complementary region of the guideRNA and causing a double strand break [45].

The *pine1* knock-out mutants mentioned before was obtained by integrating a Cas9 and a proper gRNA into the plant genome using *Agrobacterium tumefaciens*. Multiple guideRNAs can be used simultaneously to obtain big deletions spanning among them [47]. This approach will be further explained in the results section since we used it to investigate the activity of different regions in PINE1 promoter.

1.6 The EAR-motif functions and interactome

As PINE1 function is still unknown, to characterize it we started by analyzing its conserved domains. Indeed, as mentioned before, PINE1 contains two EAR-motifs.

EAR-motifs are small protein motifs defined by the sequences LXLXL or DLNXXP. EAR-motifs were the first repression motif reported in plants and one of the principal mechanisms of plant

gene regulation. Among known EAR-motif containing proteins we find the AUX/IAA proteins. Those are repressors of the Auxin response, they bind to Auxin Responsive Factors preventing the activation of Auxin responsive genes. Similarly to the dynamic with GAs and SLR1, AUX/IAA are ubiquitinated and degraded in presence of high levels of Auxin [48], [49].

The way EAR-motifs influence gene expression is through epigenetic regulation, which involves chemical modifications of DNA and the proteins around which it is wrapped, called histones. DNA and histones form the chromatin. Histones can be packed loosely or tightly around DNA, depending on a variety of chemical modifications. A loose group of histones forms Euchromatin, which is transcriptionally active since it renders DNA accessible to the transcriptional machinery. Tightly packed histones form Heterochromatin, which is a compact structure not easily accessible, so genes in an heterochromatic zone cannot be promptly expressed.

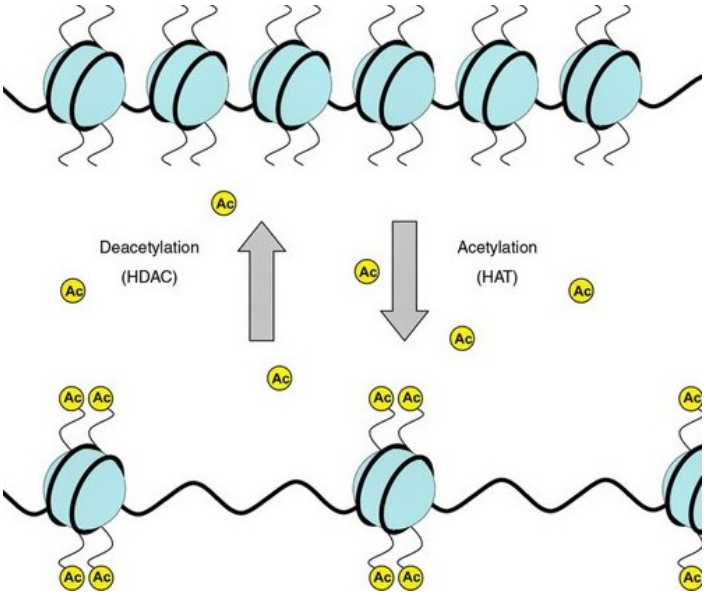


Figure 18. Histone acetylation/deacetylation affect the chromatin by compacting and relaxing its structure, influencing its accessibility to other proteins [46].

Many chemical modifications can change the chromatin state, including histone acetylation/deacetylation. Different Lysine residues in histones can be acetylated or deacetylated, which leads to either loose or compact chromatin and thus to gene expression or silencing, respectively. Acetylation removes positive charges on the histones, thereby decreasing the interaction of the N termini of histones with the negatively charged phosphate groups of DNA. As a consequence, the condensed chromatin is transformed into a more relaxed structure (Fig. 18), associated with greater levels of gene transcription [50].

It has been observed that EAR-motif containing proteins are able to interact with corepressor proteins such as TOPLESS/TOPLESS RELATED (TPL/TPR) or SAP18. These are able to bind histone deacetylase and lead to histone deacetylation

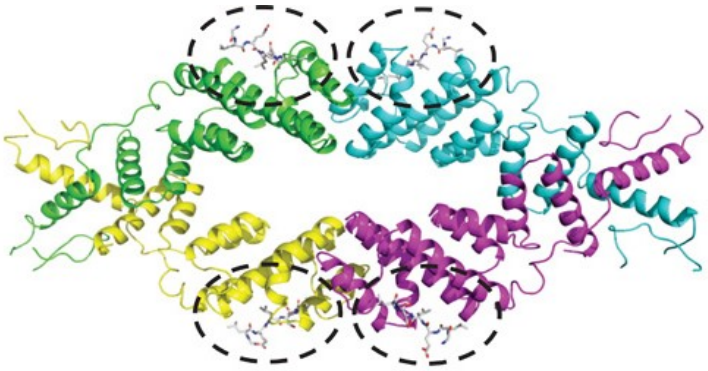


Figure 19. Structure of the TOPLESS tetramer, each TOPLESS molecule is reported in a different color. The black dotted circles indicate the binding sites for the EAR-MOTIFS [51].

and ultimately to gene silencing [48].

TPL proteins can form large complexes, composed by many proteins. Ke et al. [51], and Martin-Arevalillo et al. [52] reported that four TOPLESS proteins arrange themselves into a tetrameric complex (Fig. 19). The hydrophobic EAR-motif can interact with a hydrophobic groove present in the TOPLESS proteins. Each of the four TPL can bind to an EAR-motif transcription factor. Additionally, it seems that in *Arabidopsis thaliana* TPL work in conjunction with HISTONE DEACETYLASE 19 (AtHDA19) to get a proper shoot pole formation. Furthermore, AtHDA19 was shown to co-immunoprecipitate with Arabidopsis TPR1, indicating an *in vivo*

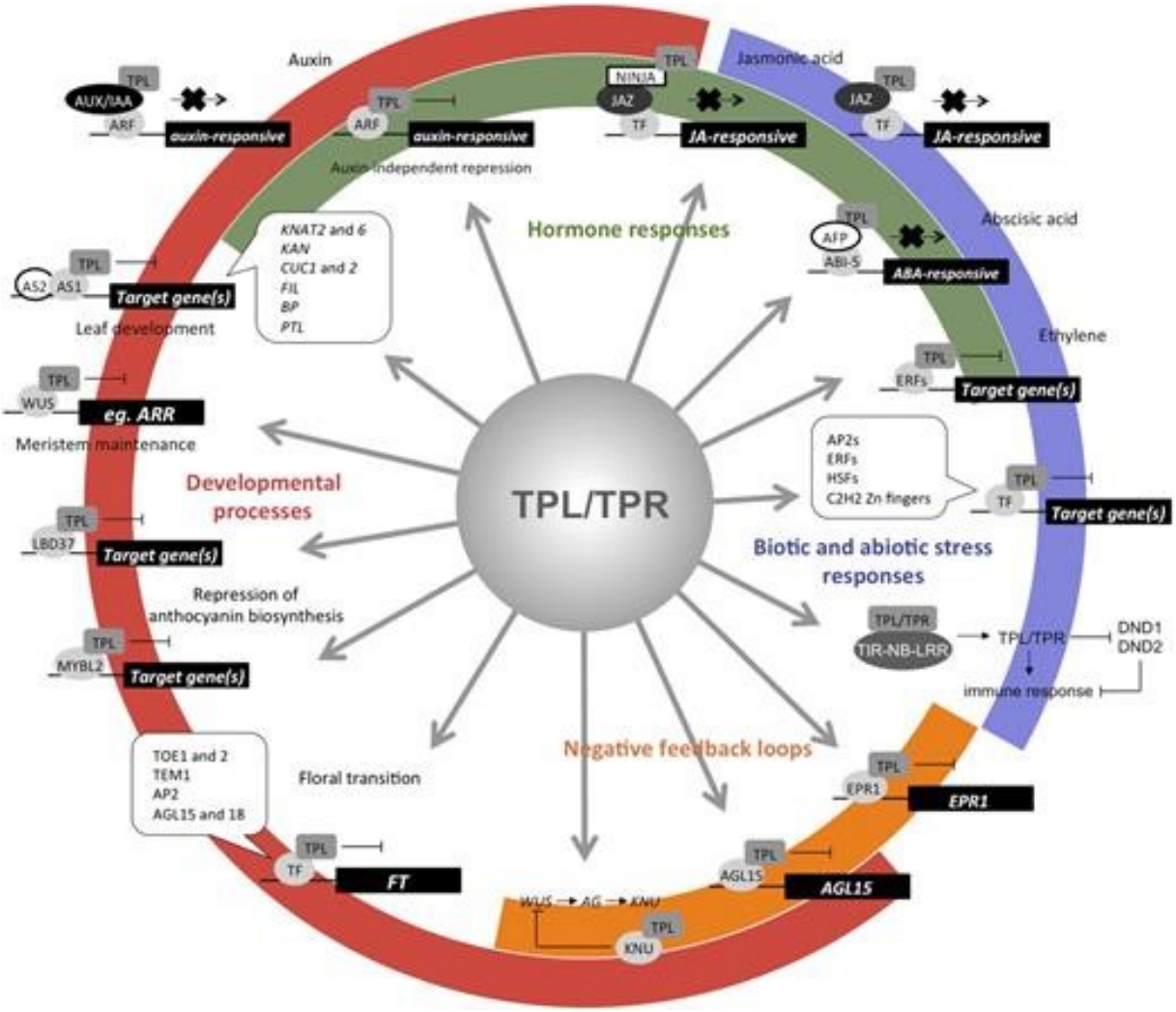


Figure 20. Representation of the many roles where TOPLESS and TOPLESS-RELATED proteins are involved [54].

interaction between the two proteins. The genetic and *in vivo* association of TPL and TPR proteins with histone deacetylase and the ability to interact with several EAR-motif containing proteins suggest an epigenetic link between the EAR motif, TPL and chromatin modification via histone deacetylation [53]–[56]. Actually, the interaction EAR motif-TPL is solely a part of TOPLESS interactome. TPL/TPR family modulate gene expression in a wide variety of processes, including hormone signaling, stress response and the control of flowering time (Fig. 20) [54]. This diversification in TPL/TPR involvement can be explained by the fact that the DNA binding specificity is given from the transcription factor that binds to TPL, which is only a "tool" to mediate the process of transcriptional repression.

2. Aim of the thesis

The purpose of this thesis is to investigate a novel pathway for internode elongation in rice. The pathway is initiated by the perception of a short day photoperiod and culminates in internodes elongation during the development of rice inflorescence. However, the mechanisms that transduce photoperiodic changes into the activation of this pathway are not well understood, and only recently have some signals been explored. As a result, most of the mechanisms involved in this pathway are still largely unknown.

To address this, the thesis organically develops three key aspects of PINE1 function.

Firstly, the upstream pathway, which is essentially what controls PINE1 expression. PINE1 is expressed in long day, during the vegetative growth, and is repressed in short day, requiring regulations in both instances.

Secondly, the mechanism of action of PINE1, specifically how it is able to regulate gene expression, its interaction partners, and the important regions and motifs within the PINE1 protein to proper function.

Finally, the thesis investigates the genes controlled by PINE1. Its downstream genes will be explored by RNA-sequencing, and some of the genes may have a direct role in the rice internode elongation pathway.

3. Materials and methods

3.1 Plant conditions and measurements

The rice ((*Oryza sativa*) cultivar 'Nipponbare' was used in this study. Nipponbare is a temperate japonica cultivar the genome of which was the first whole sequenced genome among monocot plants. Rice plants were grown in long day (LD; 16-h light/8-h dark) or short day (SD; 10-h light/14-h dark) conditions with day and night temperatures set at 28 °C and 24 °C, respectively. *hd3a rft1* double mutant plants were grown for 1.5 months in LD contitions before being separated and left for an additional 3 months in either SD or LD regimes. Subsequently, leaves were removed to expose the stem, and internode length was measured using a caliper. For the internode measurements on the knockout genes obtained from the RNA-seq, internodes were measured when the panicle was ready to be harvested. To evaluate statistical significance an unpaired t-test was performed.

3.2 Rice transformation

The protocol for rice transformation commences with seed sterilization. Rice seeds, which had previously been cleaned of their glumes, were placed in a 50 ml tube for the sterilization process. The tube was filled with a 70% ethanol solution, and the seeds were agitated for one minute. Afterward, the ethanol was drained, and the tube filled with commercial bleach and a drop of detergent (Tween20). The tube was once again agitated for 30 minutes. Subsequently, the bleach was replaced fresh bleach, and the tube was agitated again for another 30 minutes. To remove any remaining bleach residue, 6-8 subsequent cleaning steps with sterile H_2O were performed. After the final cleaning step, the water was drained, and the seeds were placed in Petri dishes containing callus induction medium. The seeds remained in the medium for 3-4 weeks, kept in the dark at 28°C.

Before transformation, calli were thoroughly cleaned to remove the shoots and seeds. They were then placed in a 50ml tube with 35ml co-cultivation medium and *A. tumefaciens* containing the desired construct to an optical density (O.D. 600) of 1. Calli were agitated in the *A. tumefaciens* solution for 5-10 minutes, and excess liquid was removed by blotting on sterile paper. The calli were then transferred to solid co-cultivation medium and kept in the dark at 28°C for 3 days.

Calli were washed 5 times in sterile H_2O and 3 times in sterile H_2O containing 400mg/l Cefotaxime to eliminate *A. tumefaciens.* They were then cultured on selection plates at 28°C in darkness for a month. This step was performed to kill untransformed cells and allow transformed cells, which are resistant to the selective antibiotic, to grow. The regenerated tissue was then transferred to regeneration media and kept under LED lights at 28°C for 2 months. The plantlets were finally transferred to sterile plastic boxes with rooting media, and after 2 weeks, they were planted in soil under LD conditions.

Below is the recipe for preparing 1 liter of the aforementioned media.

Callus Induction:

N6 MACRO I	100ml
N6 MACRO II	100ml
B5 MICRO	1ml
Proline	0.5g
Glutamine	0.5g
Caseine amino acids	0.3g
Sucrose	30g
Gamborg B5 vitamins	112mg/ml is a 1000x solution to add post-autoclave
FeNaEDTA	36mg/ml is a 1000x solution to add post-autoclave
2,4D	3mg/L to add post-autoclave
pH	5.8
Agar	7g

Co-cultivation:

R2 MACRO I	100ml
R2 MACRO II	100ml
R2 MICRO	1ml
Glucose	10g/L
Gamborg B5 vitamins	112mg/ml is a 1000x solution to add post-autoclave
FeNaEDTA	36mg/ml is a 1000x solution to add post-autoclave
2,4D	2mg/L to add post-autoclave
Acetosyringone	150 µM
Agar	7g (only for the solid media)
pH	5.2

Selection:

N6 MACRO I	100ml
N6 MACRO II	100ml
B5 MICRO	1ml
Proline	0.5g
Glutamine	0.5g
Caseine amino acids	0.3g
Sucrose	30g
Gamborg B5 vitamins	112mg/ml is a 1000x solution to add post-autoclave
2,4D	2mg/L to be added post-autoclave
FeNaEDTA	36mg/ml is a 1000x solution to be add post-autoclave
Cefotaxime	200mg/L is a 500x solution to be add post-autoclave
Vancomycin	100mg/ml is a 1000x solution to add post-autoclave
Hygromycin/Geneticin	50mg/L to add post-autoclave
pH	6.0
Agar	7g

Regeneration:

N6 MACRO I	100ml
N6 MACRO II	100ml
B5 MICRO	1ml
Proline	0.5g

Glutamine	0.5g
Caseine amino acids	0.3g
Sucrose	30g
Gamborg B5 vitamins	112mg/ml is a 1000x solution to add post-autoclave
FeNaEDTA	36mg/ml is a 1000x solution to add post-autoclave
BAP	1mg/ml is a 333x solution to add post-autoclave
NAA	10mg/ml is a 1000x solution to add post-autoclave
pH	5.8
Agar	5g

Rooting:	
Murashige & Skoog media	4.4g
Sucrose	50g
pH	5.8
Agar	5g

N6 MACRO I: KNO_3 (28.3g/L), $(NH_4)_2SO_4$ (4.63g/L), KH_2PO_4 (4g/L), $MgSO_4*7H_2O$ (1.85g/L)
N6 MACRO II: $CaCl_2*_2H_2O$ (1.66g/L)
B5 MICRO: $MnSO_4*H_2O$ (10g/L), KI (0.75g/L), H_3BO_3 (3g/L), $ZnSO_4*7H_2O$ (2g/L), $CuSO_4$ (0.025g/L), $Na_2MoO_4*2H_20$ (0.25g/L), $CoCl_2*6H_2O$ (0.025g/L)

3.3 Cloning methods

The genes for yeast two-hybrid and FRET/FLIM assays were amplified from cDNA derived from rice vegetative SAM using primers containing ATTB gateway recombination sites. The amplified genes were then recombined with pDONR207 using BP clonase II (Invitrogen) and transformed into *E. coli* via electroporation. The presence of the correct clone was verified in obtained colonies, and purified minipreps were prepared using the Macherey-Nagel plasmid purification kit. The plasmid was then recombined with the final vector using LR clonase II (Invitrogen). The final vectors for yeast two-hybrid were pGADT7 and pGBKT7, with each tested gene cloned into both plasmids. For FRET/FLIM experiments, the final vectors were pABIND-GW-GFP (in which TPL3 was cloned) and pABIND-GW-mCherry (in which PINE1 and HDAC15). Clones of PINE1 with a mutation of the N-terminal EAR-motif were obtained via overlapping PCR. Constructs of *pPINE1:PINE1* with a mutated N-terminal EAR-motif were obtained by separately amplifying the promoter, and two portions of the gene to introduce the mutation via overlapping PCR, and then digesting both the promoter and gene with SacI (the site present at the beginning of the 5'UTR of PINE1). The resulting digested products were purified using the Macherey-Nagel Gel and PCR purification kit. The purified products were ligated using T4 ligase from PROMEGA and amplified in its entirety before cloning into pDONR207. The resulting clones were then recombined with pMPGWB410 and transformed into *E. coli* and then *A. tumefaciens*. The construct for the editing of the *PINE1* promoter consists of alternating gRNA and tRNA sequences and was made by synthesis. The synthesized part was then digested with FokI (present at both extremities) and ligated into pRGEB32 that had been previously digested with BsaI. The resulting construct was then transformed into *A. tumefaciens* which was subsequently used to transform rice. For CRISPR/Cas9 or CRISPR/VQR cloning, the gRNA is first selected. The CRISPR-P online tool was used to design the gRNA as

two complementing primers. These primers were annealed to each other in a ramp-down program in a thermal cycler, after which they were ligated into a predigested (BsaI) pRGEB32 (containing a *Cas9*) or pC1300-UBI:VQR (containing a *VQR*), and then subsequently transformed in rice.

3.4 FRET/FLIM analysis

A. tumefaciens transformed with one of the plasmids used for FRET/FLIM was cultured overnight in selective liquid YEB media. 50mL of the culture was pelleted at 4500 RCF for 10 minutes at room temperature. The supernatant was thereafter discarded and the pellet was resuspended in resuspension solution (10 mM $MgCl_2$, 10 mM MES-KOH pH 5.6, and 100 µM acetosyringone) to reach an O.D. 600 of 0.1. Hereafter the various plasmids were mixed: an equal volume of *A.tumefaciens* suspension carrying pABIND-GW-GFP, pABIND-GW-mCherry and p19 were added to a falcon. This suspension was then used for agroinfiltration of 1 month old leaves of *Nicotiana benthamiana* by the use of a needleless syringe.

Two days after the infiltration the plants were sprayed with a solution containing 17-β-estradiol which induces synthesis of the transgenic proteins. 6 hours after this treatment, leaf tissue was sampled and 20 nuclei per sample were analyzed using SP8 DIVE FALCON spectral multi-photon FLIM microscope (Leica microsystems, Germany). GFP and mCherry fluorophores were excited using the laser PM930 and PM light 1045 respectively. The former was then detected in the spectra range of 493-547nm while the latter of 586-650nm. Images were then processed using the software LAS-X FLIM/FCS module. An unpaired t-test was used to evaluate statistical significance.

3.5 cDNA synthesis and gene expression analysis

Only SAM or stem tissue was used for gene expression analysis. 4-8 samples were collected in triplicate, immediately placed in liquid nitrogen after collection, and ground using a tissue homogenizer or pestle and mortar, depending on the sample. Next, 500 µL of TRIzol reagent was added to the sample, and after 5 minutes at room temperature, 200 µL of chloroform was added and mixed by inverting the tube. After centrifugation for 15 minutes at 21000 RCF at 4°C, the supernatant was transferred to a new RNase-free tube and 175 µL of pre-chilled propan-2-ol was added. The tube was shaken by hand and placed on ice for 10 minutes before centrifugation for 20 minutes at 21000 RCF at 4°C. The supernatant was discarded, and the pellet was washed with 500 µL of pre-chilled 70% ethanol and centrifuged for 10 minutes at 21000 RCF at 4°C. The pellet was dried at room temperature, resuspended in 50 µL of H_2O, and treated with 5,7 µL of turboDNAse buffer (Ambion) and 1 µL of TurboDNAse (Ambion). After 30 minutes at 37°C, 5 µL of NaAc 3M was added to each sample, followed by 2.5 volumes of ethanol. The samples were mixed by hand, left overnight at -20°C, and then centrifuged for 20 minutes at 21000 RCF at 4°C. The supernatant was discarded, and the pellet was washed with 500 µL of pre-chilled 70% ethanol, centrifuged for 10 minutes at 21000 RCF at 4°C, and dried at room temperature before being resuspended in 10 µL of H_2O. RNA was then quantified using the IMPLEN P300 Nanophotometer.

For cDNA synthesis, 1000ng of RNA was mixed with 1 µL of 70 µM oligo dT and H_2O up to 5 µL. The mix was heated at 70°C for 5 minutes and then retrotranscribed using RT II (PROMEGA) at 42°C for 60 minutes, followed by 15 minutes at 70°C. Before real-time PCR, cDNA was diluted with 80 microliters of H_2O. The real-time PCR was performed using SYBR green 2x Master mix (thermoscientific) in an eppendorf Mastercycler realplex^2.

3.6 Yeast transformation and yeast two-hybrid

Both AH109 and Y187 yeast strains were cultured overnight in 10ml YPAD medium at 30°C and 250 rpm. The following day, 300 ml of YPAD media was inoculated with the yeast culture to achieve an O.D. 600 of 0.1, followed by incubation at 30 °C and 230rpm until the culture reached an O.D. 600 between 0.4-0.6. Next, the cultures were divided into 50 ml tubes, centrifuged at 850 RCF for 5 minutes at room temperature, the water drained, and the pellet resuspended in 10 ml sterile H_2O, and all tubes combined into a single tube for each strain. Then, the two tubes were centrifuged, the water drained and the pellet resuspended in 1.5 ml TE/LiAc. A volume of 100 µl of cells was added to 1.5 ml tubes containing 1000 ng of the plasmid of interest and 5 µl of preheated DNA carrier at 95 °C for 5 minutes. Tubes were vortexed and add 600 µl of PEG/LiAc added. After vortexing again, the tubes stayed at 30°C and 200 RPM for 30 minutes in a shaker. Finally, 70 µl of DMSO was added, the tubes inverted for mixing, incubated at 42 °C for 15 minutes, and then placed on ice for 90 seconds.
The tubes were then centrifuged for 10 seconds at 6000 RCF and the supernatant was removed. The pellet was resuspended in 500 µl of TE buffer and 200 µl were spreaded onto selective media: SD-W for the plasmid with the binding domain, and SD-L for the plasmid with the activation domain. After 3 days at 30 °C, colonies were visible. A colony was inoculated from each plate in 3 ml of SD media with the corresponding drop-out selection and grew overnight at 30 °C with shaking at 250 rpm. Next, two liquid colonies containing the proteins whose interactions need to be tested were mixed in a well of a PCR plate. In particular, were 10 µL from each colony and 80 µL of YPAD+10% PEG were mixed in each well.

The PCR plate was left overnight at 30 °C for mating to occur. The next morning, 2 µL were picked from each well and placed in each selective medium. The selective media used included SD-W-L (to confirm mating), SD-W-L-H-A, SD-W-L-H+5mM 3AT, SD-W-L-H+10mM 3AT, SD-W-L-H+20mM 3AT. 3AT stands for 3-amino-1,2,4-triazole, and is an inhibitor of histidine biosynthesis. 3AT is used to determine the selective strength of a media. Increasing it results in a more selective media, and viceversa.
After 5-7 days at 30 °C, the plates were checked for any yeast growth. To perform this experiment with controlled O.D. 600 as in Fig. 25d, colonies were inoculated from the SD-W-L plate in the same liquid media and grew overnight in a shaker at 30 ° C at 230 rpm. O.D. 600 was measured the following day, then colonies were diluted at the desired O.D. 600 and 2 µL were plated in the same selective plate.

Each yeast two-hybrid experiment was repeated three times, each time AD and BD were tested in both sides of the interaction.

3.7 RNA-sequencing

WT and *pine1* stem were sampled from plants 20 days after germination. eight stem per sample were taken, in triplicate. RNA was extracted with TRIzol method as described before. Sequencing was performed by Novogene (UK) using 150bp paired ends reads, non-stranded illumina sequencing. RNA-seq yielded 46 milion reads from each sample. Raw reads were trimmed and filtered to eliminate low quality/shorter reads by using fastq-mcf [57]. Quality checking of trimmed reads was done by using fastQC [58]. STAR [59] was then employed to generate a rice genome index using the Nipponbare genome (Oryza_sativa.IRGSP-1.0.47.gtf, https://rapdb.dna.affrc.go.jp/download/irgsp1.html) and to map the reads. On average, 92% of the reads were uniquely mapped in rice genome. HTSEQ-COUNT [60] was used to count the number of reads for each gene and finally DESEQ2 [61] was used to normalize the reads in each sample and to isolate differentially expressed genes. Differentially expressed genes were filtered by Log_2FoldChange ≤ -1.5 or Log_2FoldChange ≥ 1.5.

3.8 Construct for the mutation of PINE1 promoter

As previously mentioned, mutations in PINE1 promoter were achieved through a construct consisting of an alternation of guide RNA and tRNA which are cleaved at both ends, freeing the gRNA oligonucleotides. Here in Fig. 21 I report the sequence of such construct. The portions marked in green corresponds to the tRNA, while in red are the eight gRNA which targets different region in PINE1 promoter, while in blue there is the guideRNA scaffold, which is necessary for the gRNA to bind to the Cas9.

Such construct was synthesized by GENEWIZ (Germany) and was subsequently cloned into rice expression vector pRGEB32.

Figure 21. Construct used to generate multiple mutations in PINE1 promoter. The red regions are the actual guideRNA which targets PINE1 promoter, while the blue regions correspond to a scaffold necessary to bind the guide to the Cas9. The blue sequences are tRNA that will be cleaved at both ends.

3.9 Primer table

In table 1 is reported a list of primer (5' –> 3' sequence) used throughout this thesis:

Cloning of OsMADS overexpressors	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTCGAGGTCATTCATATGCTTGA	primer fw su pactin with cloning site attb1
	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTCCTTTAGCCCTTGATGG	primer fw su posh1 with cloning site attb1
	ACGTGGATCCAGGGTTGGGGGGATGGGA	primer rv su pactin with cloning site bamHi
	ACGTGGATCCGAGAGAAGCTCAAGACACGCAG	primer rv su posh1 with cloning site attb1
	ACGTGGATCCATGGGGCGGGGGAAGGTG	primer fw osmads15 with cloning site bamhi
	ACGTGGATCCATGGGGCGGGGCAAGGTG	primer fw osmads14 with cloning site bamHi
TOPLESS and SAP18 cloning	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGTCGCTCAGCCGG	FW topless LOC_Os01g15020 with attb1 site
	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTATCTTTGGATTTGGTCTGCAG	RV topless LOC_Os01g15020 with attb2 site
	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGTCGCTGAGCCGG	FW topless LOC_Os03g14980 with attb1 site
	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTATCTTTCTGGTTGATCAGAACT	RV topless LOC_Os03g14980 with attb2 site
	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGTCGCTTAGCAGGG	FW topless LOC_Os08g06480 with attb1 site
	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAGACTTCTGGTTTGTTAGCT	RV topless LOC_Os08g06480 with attb2 site
	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGGGAAGGGGCGAG	FW sap18 (Os02g0122000) with attb1 site
	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAATAGATTGCAACACTCAAGT	RV sap18 (Os02g0122000) with attb2 site
	CTAATGAAGCGATTGAGGCCA	FW topless Os01g0254100 to check pDONR207 clones
	GCTCTACTGAAGCTGACGGA	RV topless Os01g0254100 to check pDONR207 clones
	TTGGGATGTCTGAGGAGGTG	FW topless Os03g0254700 to check pDONR207 clones
	TGGGACATGGACAACACAAG	FW topless Os03g0254700 to check pDONR207 clones
	CTGCGGATTCTGAACATCTGA	FW topless Os08g0162100 to check pDONR207 clones
	TCTTGGCTGCTGGAGATGAA	FW topless Os08g0162100 to check pDONR207 clones
gRNA for C-terminal EAR motif of PINE1	GGCACTAGCTAGAGCTTCAGGTTG	Forward guide for PINE1 C.term EAR motif for VQR
	AAACCAACCTGAAGCTCTAGCTAG	Reverse guide for PINE1 C.term EAR motif for VQR
gRNAs to CRISPR downstream genes of PINE1	GGCAAGGCGAATTACAGGAAGCAG	guide fw per crispare Os06g0271500
	AAACCTGCTTCCTGTAATTCGCCT	guide rv per crispare Os06g0271500
	GGCATTAGATGGACAAAGAGGTCG	Forward Os03g0115800 gRNA CRISPR
	AAACCGACCTCTTTGTCCATCTAA	Reverse Os03g0115800 gRNA CRISPR
	GGCATCGCCGCCCCAACATCTGGA	Forward Os07g0442800 gRNA CRISPR
	AAACTCCAGATGTTGGGGCGGCGA	Reverse Os07g0442800 gRNA CRISPR
	GGCAGGAGGTGGCAGTAATAACTT	guide fw to CRISPR Os07g0153150
	AAACAAGTTATTACTGCCACCTCC	guide rv to CRISPR Os07g0153150
primer to obtain clones of PINE1 with mutation on N- and C-terminal EAR motif	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGAGGCTCCCCCTTCTCTTTC	fw on PINE1 with attb1 cloning site
	CTCTCGGTGACGCTGGGCCTGACA	fw on PINE1 to mutate the N-terminal EAR-motif
	TCAGGCCCAGCGTCACCGAGAGGTC	rv on PINE1 to mutate the N-terminal EAR-motif
	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAGAGGAGCATATGCAAGGGG	primer fw on PINE1 promoter with attb1 cloning sites
	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGTTGAGATCAACCTGCCTCTG	rv on PINE1 to truncate the C-terminal EAR, with attb2 site
	CCATTTCTCATCACTGAGCTG	fw on 5'UTR of PINE1
	GGGAGGTAGAGGTGGGTGTT	rv on PINE1
primer to clone Histone deacetylase 15 into pDONR207	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGTCTGATATGAGGTC	LOC_Os07g06980 fw Histone deacetylase 15 with attb1 site
	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGGGCTCTGCTTCATCAT	LOC_Os07g06980 rv Histone deacetylase 15 with attb2 site
Primer for real time PCR	GCTCTTCCCTTGCCTCTTCT	fw on PINE1 for qPCR
	GGGAGGTAGAGGTGGGTGTT	rv on PINE1 for qPCR
	AATGGGACCAGACACAACCT	fw on OsMADS14 (Os03g0752800)
	AATGGGACCAGACACAACCT	rv on OsMADS14 (Os03g0752800)
	CACGAGATCTCCGTCCTCTG	fw on OsMADS15 (Os07g0108900)
	AGCGCTCATAACGTTCAAGG	rv on OsMADS15 (Os07g0108900)
	CGGATCTCCGACGATTGTAT	Os03g0115800 FW for qPCR
	AAGGACCATCTTCCTGAAAGG	Os03g0115800 FW for qPCR
	GCAACCTTGTTTCGCAGGAT	Os07g0442800 primer fw for qPCR
	TGAGCTTCACCGGACTTTAACA	Os07g0442800 primer rv for qPCR
	TGGGACAAGTCCTGAATTTTG	fw on FTL1 (Os01g0218500) for qPCR
	TAGAATCGGTGGGAGCATTT	rv on FTL1 (Os01g0218500) for qPCR
For the pOSH1:FTL1 overexpressing vector, the construct used is published in Giaume et al. 2023 [27]		

Table 1. List of primer sequences (5'->3') used in the current study.

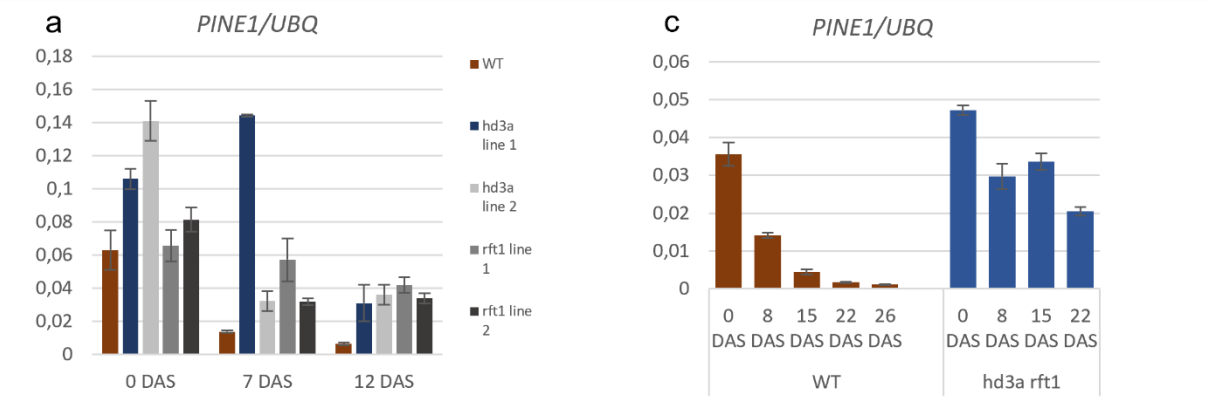
4. Results

4.1 *PINE1* expression depends on florigens and their downstream genes

We have previously provided data that support the notion that *PINE1* functions downstream of the florigens and that both Hd3a and RFT1 are sufficient to downregulate it [1]. To further support this idea, we hypothesized that Hd3a and RFT1 are also necessary to reduce *PINE1* transcription. To this end, we evaluated the expression of *PINE1* in single mutants of the florigens *hd3a* and *rft1*, as well as in the double mutant of both.

As mentioned previously, *PINE1* expression is suppressed during short day exposure, when floral induction happens. We found that *PINE1* expression was not as strongly repressed in the single mutants of either *hd3a* or *rft1*, suggesting that both florigens are responsible for its repression and have overlapping functions (Fig. 22a). To conduct this experiment, two independent lines were used for each florigen: *hd3a* line 1 and line 2 carry different one base pair deletion, causing in both cases a frameshift in the protein sequence. Similarly, *rft1* line 1 and line 2 carry different one base pair insertion, again causing the frame shift of the protein sequence (Fig. 22b).

When measuring *PINE1* expression in the double mutant *hd3a rft1*, we observed that *PINE1* transcript decreased very little after exposure to short days (Fig. 22c). This indicates that changes in photoperiod alone have milder and/or slower effect in controlling *PINE1* expression, but rather, the florigens (or their downstream signals) largely determine the repression of *PINE1* during flowering. The double mutant of the florigen used for this experiment contains the *hd3a* alleles -4 and -15 deletions, both causing loss of function mutation, while *rft1* alleles are both +1 insertions, causing frameshift and loss of function of both alleles (Fig. 22d). Interestingly, if given enough time (3 to 5 months), even the double mutant of the florigens elongates its internodes (Fig. 23a). This elongation is not related to flowering as these plants cannot flower at all. These internodes have a uniform length, unlike the typical pattern of internode elongation associated with flowering. Furthermore, this elongation appears to be influenced by photoperiod, with double mutants exposed to SD exhibiting more and longer internodes (Fig. 23e). It is currently unknown whether this is due to a slow *PINE1* inhibition under SD or some other mechanism that is not dependent on the *PINE1* pathway but still influenced by the photoperiod. As previously mentioned, florigens play a significant role in regulating *PINE1* expression. However, there is no evidence to suggest that they directly bind to the *PINE1* promoter to suppress its expression during flowering. The database of binding sites for OsFD1 and OsFD4 (components of the Florigen Activation Complex, together with the florigens) published by Cerise et al. [62] indicates that neither of these proteins bind to the *PINE1* genomic region. Other proteins downstream to the florigen might be responsible for *PINE1* suppression during flowering. OsMADS14, OsMADS15 and FTL1 are well-known proteins involved in flowering that are activated by the florigens. All of them are strong flowering promoters involved also in panicle formation.



b

hd3a	WT		GAGCACCAACCTCAAGGTCACCTATGGCT
	line 1	-1	GAGCACCAA-CTCAAGGTCACCTATGGCT
	line 1	-1	GAGCACCAACC-CAAGGTCACCTATGGCT
rft1	WT		CCATTCGTCCGGATCATAACCTCAG
	line 1	+1	CCATTCGATCCGGATCATAACCTCAG
	line 2	+1	CCATTCGTTCCGGATCATAACCTCAG

d

hd3a	WT		GAGCACCAACCTCAAGGTCACCTATGGCT
	line 1	-15	GAGCACCAAC----------GGCT
	line 1	-4	GAGCACC----TCAAGGTCACCTATGGCT
rft1	WT		CCATTCGTCCGGATCATAACCTCAG
	line 1	+1	CCATTCGATCCGGATCATAACCTCAG
	line 2	+1	CCATTCGTTCCGGATCATAACCTCAG

*Figure 22. **a**: PINE1 expression in the SAM of WT and two independent knockout lines for both hd3a and rft1 during floral transition. DAS=days after shift. **b**: Knockout alleles of the single florigens mutants used in a. Plants were homozygous for the reported mutation. **c**: PINE1 expression in WT and double mutant of both florigens during floral transition. **d**: Alleles present in the double mutant of the florigens used in b.*

As previously noted, overexpression of both OsMADS proteins results in a strong early flowering phenotype. Recent research has also shown that overexpression of FTL1 results in early flowering and a modified panicle architecture [29]. Additionally, overexpression of *OsMADS15* seems to cause also early internode elongation which appears similar to *pine1* phenotype [28]. To identify a protein that may be involved in regulating PINE1 expression between the florigens and *PINE1*, I analyzed the expression of these genes in elongating internodes during flowering and found that all three genes were expressed in the stem (Fig. 23b,c,d). To study the effect of these genes on *PINE1*, I cloned overexpression vectors for OsMADS14, OsMADS15, and FTL1, using *pOSH1:OsMADS14*, *pOSH1:FTL1* and *pACT:OsMADS15*. *pOSH1* is a promoter active only in the SAM region, so the genes under its control will be expressed only there, while *pACT* is a stronger, constitutive and ubiquitous promoter [63]. As previously mentioned, OsMADS overexpressors had already been studied. However, overexpressing OsMADS15 results in a positive feedback on the florigens expression [28] and their overall response cascade, making it difficult to determine whether the observed phenotype is due to the overexpressed gene or from the activation of the flowering pathway altogether. To avoid this issue, we overexpressed these three genes in the *hd3a rft1* double mutant background.

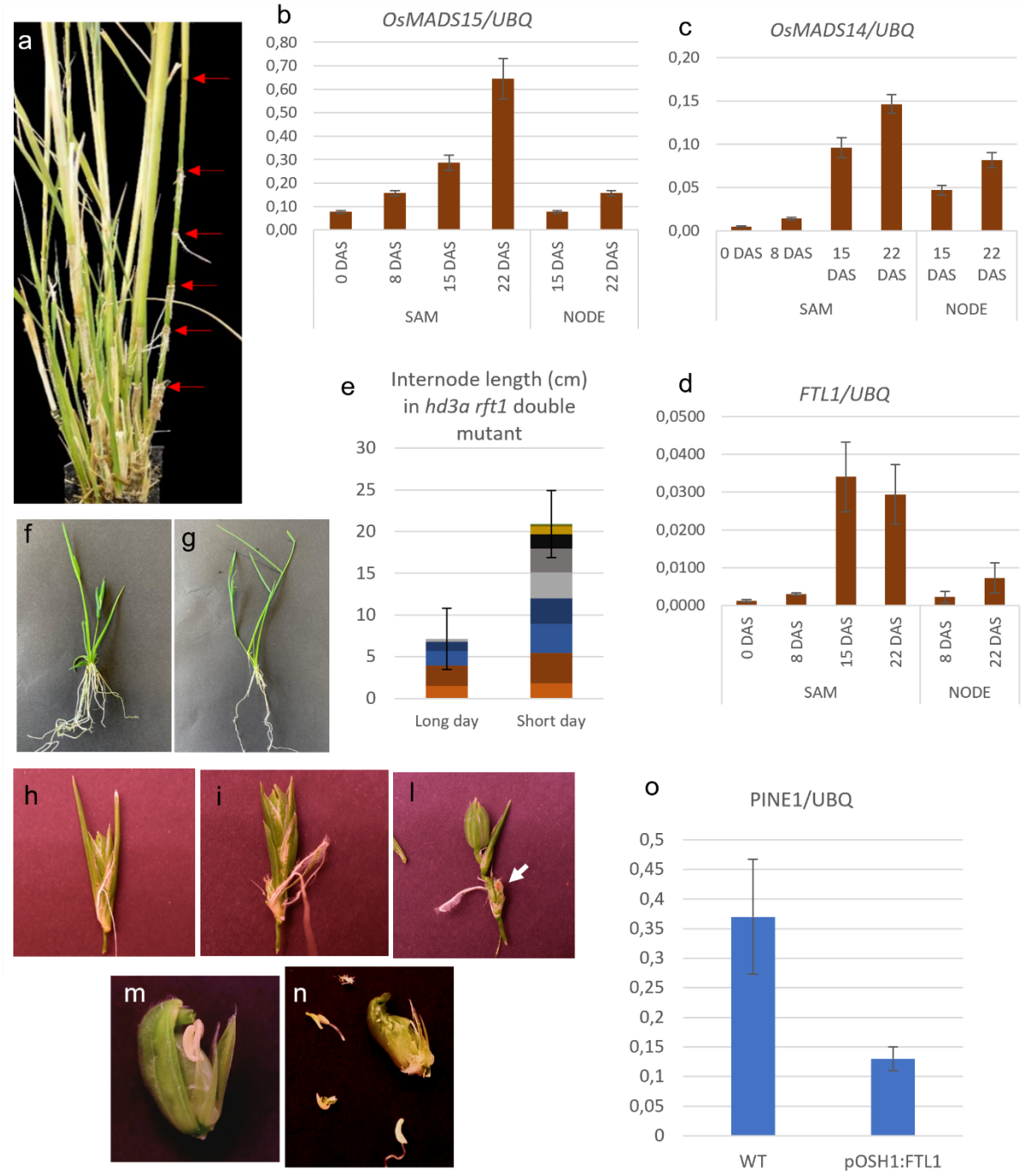


Figure 23. ***a****: Internode elongation in the double mutant of the florigens. Red arrows points to the nodes of a culm.* ***b, c, d****: Respectively OsMADS15, OsMADS14 and FTL1 expression in WT during floral transition both in the SAM and in a node.* ***e****: Representation of internode elongation in the double mutant hd3a rft1 in LD and SD. Standard deviation is on the total height. Plants represented are averaged from population of 15 plants from both photoperiods.* ***f, g****: plants overexpressing FTL1 in a hd3a rft1 background. Both were taken from the rooting phase of the in vitro culture.* ***h,i,l,m,n****: details of the panicle and flowers of plants overexpressing FTL1 in hd3a rft1 background. from left to right is the same inflorescence disassembled. In l the white arrow point to a malformed ovary and no male organs were present in that flower. In m and n the apical flower is being divided in its parts, a deformed ovary and 3 stamens, two of which were deformed.* ***o****: PINE1 expression in a WT tiller and in a FTL1*

OsMADS14 and *OsMADS15* overexpressing plants did not exhibit any different phenotype from the WT, indicating either that the transgene expression was too low or that OsMADS14

or 15 are not sufficient to trigger flowering/stem elongation in the absence of Hd3a and RFT1. Further study will be needed to assess that. On the other hand, overexpression of *FTL1* led to internode elongation in the early stages of plant development, from the rooting phase of the in vitro culture (Fig. 23f,g). The phenotype was comparable to that of the *pine1* phenotype, suggesting that FTL1 is a major *PINE1* inhibitor during flowering. The effect of FTL1 on *PINE1* expression was verified by comparing the *PINE1* expression level in a FTL1 overexpressing tiller and a WT tiller of similar dimension, both kept in long day. *PINE1* expression in the WT resulted around three times more expressed than in the *pOSH1:FTL1* plants. However, this does not necessarily prove that FTL1 is directly responsible for *PINE1* inhibition, as there may be other proteins involved in between the two. Additionally to the internode elongation phenotype, *pOSH1:FTL1* plants showed also extreme early flowering. These plants were able to flower during the rooting phase of the in vitro culture. The inflorescence is altered, presenting a fully formed flower (althought with a deformed ovary and stamens, Fig. 23l,m,n) and an ovary on the side (Fig. 23l). Additionally, in the inflorescence, a number of leaves and a root were present (Fig. 23h,i,l), suggesting that the shift from vegetative to reproductive meristem didn't suppress the production of vegetative tissues. Those phenotypes are probably not related to *PINE1* but probably results from the partial activation of the flowering pathway given by FTL1. Taken together our data suggest that FTL1 alone, a gene whose expression is induced by the florigens, may be able to suppress PINE1 expression during flowering, leading to internode elongation.

4.2 Study of PINE1 promoter regions

Studying the effect of known flowering proteins in stem elongation is one possible method for investigating *PINE1* regulation. Alternatively, the unbiased study of *PINE1* promoter could offer insight on its regulation, as different regions may be responsible for *PINE1* expression during vegetative growth and inhibition during flowering. To identify the 'active' regions of *PINE1* promoter, we employed a CRISPR/Cas9 multiplex approach by utilizing a construct that express different guides simultaneously. To achieve this, we utilized the method previously published by Xie et al. [47].

In eukaryotic cells, RNA polymerase III transcribes tRNAs as one long pre-tRNA that is spliced by tRNA-splicing endonuclease. Transcription terminates after four or more sequential thymidines. The long pre-tRNA contain multiple tRNAs which are divided by tRNAse Z and tRNAse P through cleavage at both ends of each tRNA. A construct expressing alternating sequences of gRNA and tRNA will be cleaved at both ends of each tRNA, causing the release of each gRNA which can then be bound by the Cas9 (Fig. 24a). We employed a private sequencing service to synthesize this fragment, that was then cloned into the rice expression vector pRGEB32. We selected 8 guides to target PINE1 promoter, spanning approximately from -2000bp upstream of the 5'UTR to just before it (Fig. 24d). To confirm editing, we performed PCR analysis on *PINE1* promoter of the resulting plants. As shown in Fig. 24b, some bands are similar in size to the WT promoter, while others are shorter, some significantly so, indicating that major deletions occurred. Indeed, in some plants (Fig. 24e and white arrow in Fig. 23b) the whole 2000bp upstream PINE1 was deleted.

Some resulting plants eventually exhibited a strong dwarf phenotype (Fig. 24c and Fig 24f), affecting the length of all internodes. This suggests that in these plants, a determinant site for *PINE1* inhibition during flowering was deleted from *PINE1* promoter. Consequently, *PINE1* remained highly expressed during flowering, inhibiting internode elongation. However, as these plants had biallelic mutations in the T0 generation, each edited plant had two different mutated alleles of *PINE1* promoter. Therefore, it was impossible to associate a particular mutation with a consequential modification of *PINE1* expression and the resulting phenotype. However, the T1 generation is currently growing, and a complete analysis of the effect of each mutation and its consequences will be carried out shortly. Few plants showed instead a phenotype similar to the one of *pine1* knockout, suggesting that modifications in *PINE1* promoter may have disrupted sites necessary for PINE1 expression, leading to a lack of *PINE1* expression (Fig. 24g).

Taken together, these preliminary results suggests that different regions in *PINE1* promoter are responsible for positively and negatively regulate PINE1 expression, and the approach to edit the promoter is a suitable method to identify these regions. Further study of these regions could aid in identifying the regulatory proteins that bind the promoter and regulate PINE1 expression, as well as precisely locate their binding site. In addition, there is potential agronomic interest, as dwarf plants are more resistant to lodging. As will be explained in the discussion chapter, dwarf plants obtained through gibberellin deficiency have some drawbacks, which may be mitigated using *PINE1* as a dwarfing gene.

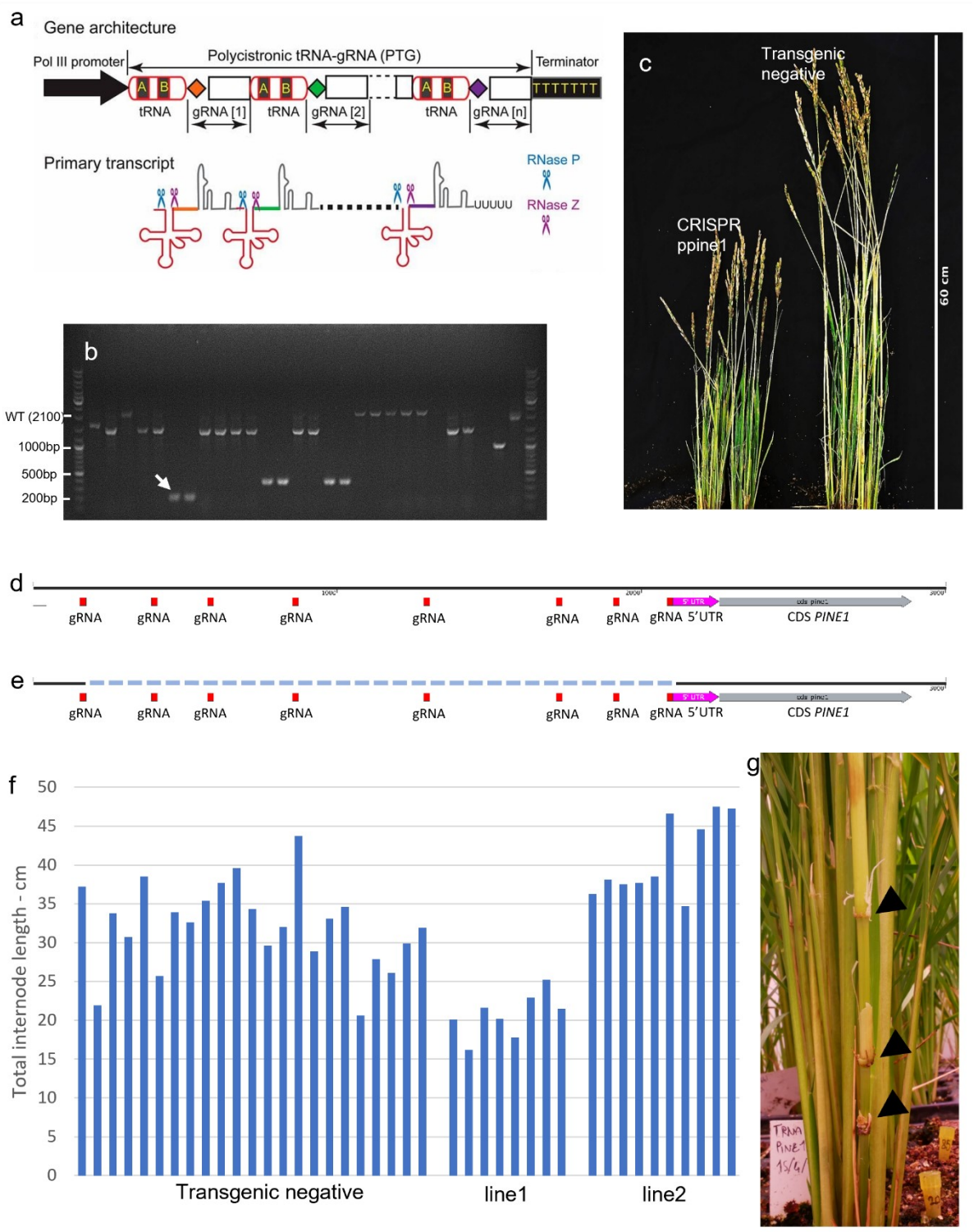


Figure 24. ***a****: Architecture of the transgene necessary to express multiple gRNAs targeting PINE1 promoter* [47]. ***b****: PCR on PINE1 promoter in CRISPR ppine1 plants. The white arrow points to a band with the deletion of the whole promoter, represented in part e of the figure.* ***c****: comparison of CRISPR ppine1 plants on the left with non-transformant plants. Leaves were taken out to expose and measure the stem.* ***d****: representation of the WT PINE1 promoter and the eight gRNA used for its mutation.* ***e****: Representation of the longer deletion obtained in pine1 promoter, where the whole 2000bp upstream of PINE1 is deleted.* ***f****: -Comparison of some non-transformant plants with two of the lines with mutation in PINE1 promoter. Each bar represents the total length of a culm.* ***g****: Plant with mutation on the pine1 promoter which elongates the internodes prior to floral induction. Black triangles points to the nodes.*

4.3 PINE1 interaction with TOPLESS co-repressors

PINE1 serves as a regulatory protein, capable of controlling stem elongation. As said before, its zinc finger motif allows for specific binding on target sites in the genome. In addition, PINE1 also contains two EAR-motifs, known for being strong repressional motif. Previous research has identified various proteins that interact with EAR-motifs, including the corepressor TOPLESS (TPL) and TOPLESS-RELATED (TPR) proteins, SAP18 and ETHYLENE INSENSITIVE 3 (EIN3) proteins. Those are all corepressor, meaning that they are unable to bind DNA themselves but they bind and assist transcription factors such as PINE1.

To investigate the potential interaction of these proteins with PINE1, cDNA was generated from vegetative shoot apical meristems. Following that, TPL1 (Os01g0254100), TPL2 (Os03g0254700) and TPL3 (Os08g0162100), SAP18 (Os02g0122000) (Fig. 25a), EIN3-1 (Os03g0324200), EIN3-2 (Os07g0685700), EIN3-3 (Os09g0490200) coding sequences were amplified. Rice has only three TPL genes and one SAP18, but six EIN3 proteins. We cloned the three most highly expressed in the SAM, since it is the same region where PINE1 is also present. The amplified products were inserted into pDONR207 via gateway system and then transformed into *E.coli* using electroporation. The obtained plasmids were then further recombined into a yeast expression vector for the yeast two-hybrid assay, using plasmids pGADT7 (carrying the GAL4 activation domain) and PGBKT7 (carrying the GAL4 binding domain). These plasmids were subsequently transformed into yeast strains AH109 and Y187, respectively. The transformed yeast cells were then mated to generate a diploid yeast containing both plasmids and expressing the two proteins of interest fused with a domain of the GAL4 transcription factor (Fig. 25b). After mating, the cells were plated on increasingly selective media, where the expression of reporter genes is required for yeast viability.

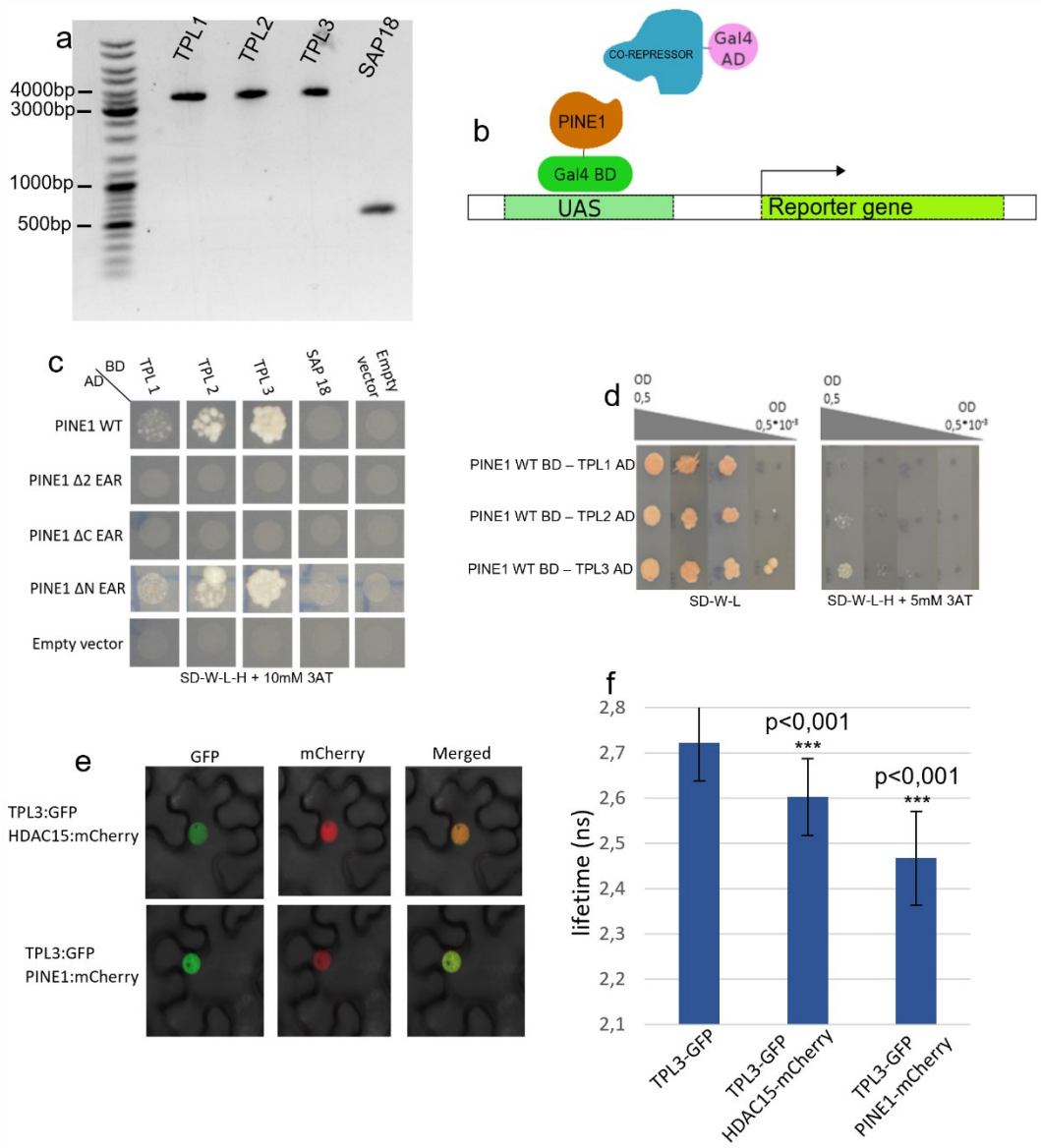


Figure 25. ***a****: PCR on cDNA of SAM to amplify the three TOPLESS genes and SAP18.* ***b****: Working diagram of a yeast two-hybrid. The transcription factor GAL4 is split in binding domain and activation domain. Each are fused with the protein of interest. If the two proteins interact, GAL4 reconstitutes himself and promotes the transcription of reporter genes essentials for yeast growth. Without interaction and without expression of reporter genes, yeast is not viable.* ***c****: Yeast two hybrid shows interaction between PINE1 with TPL2 and TPL3. The interaction disappears without the C-terminal EAR motif.* ***d****: Yeast two hybrid comparing PINE1 interaction with TOPLESS at different cell concentration.* ***e****: Confocal image of TPL3:GFP, HDAC15:mCherry and PINE1:mCherry showing colocalization of both couples tested in Nicotiana benthamiana nuclei.* ***f****: FLIM results for the interaction between TPL3 and PINE1 and TPL3 and HDAC15. The lower the lifetime, the stronger the interaction.*

The interaction between the two proteins of interest results in the reconstitution of a functional GAL4 transcription factor, which causes the expression of these reporter genes, enabling yeast to grow on selective media. Conversely, in the absence of interaction, no yeast growth is observed.

The yeast two-hybrid assay revealed an interaction between PINE1 and TPL2, as well as between PINE1 and TPL3. No interactions were observed with TPL1, SAP18 (Fig. 25c) and the three EIN proteins (data not shown). The interaction between PINE1 and TPL2 appeared weaker than the interaction with TPL3. To confirm this finding, we evaluated these interactions in different selective media and different cell concentration (Fig. 25d). The growth was consistently more pronounced in the PINE1-TPL3 interaction than in the PINE1-TPL2 interaction. We next tested the interaction of modified PINE1 clones, to investigate the requirement of the two EAR motifs for the interaction. The variants included:

- A modified version of PINE1 with the mutation L21V that disrupt the N-terminal EAR motif and a truncation that cuts the last 3 amino acids, eliminating also the C-terminal EAR-motif (PINE1 Δ 2 EAR).
- A truncated clone of PINE1, missing the last 3 amino acids (PINE1 ΔC EAR).
- A clone of PINE1 with the mutation L21V (PINE1 ΔN EAR).

The absence of both EAR-motifs in PINE1 prevented any protein interactions, confirming the essential role of EAR-motifs in TPL recognition. Truncation of only the C-terminal EAR motif also resulted in the inability to interact, further indicating its importance. However, when only the N-terminal EAR-motif was mutated, the interaction was not affected (Fig. 25c).

Collectively, these results suggest that PINE1 is capable of interacting with TPL2 and TPL3, and that the interaction requires the presence of the C-terminal EAR-motif.

Yeast two hybrid is useful for detecting the existence of an interaction, but is a qualitative assay. It is unable to accurately measure the strength of the interaction. For this reason we decided to perform a FRET-FLIM assay to determine the strength of the interaction between TPL3 and PINE1. Initially, we cloned both proteins into a tobacco expression vector, which allowed for the expression of TPL3-GFP and PINE1-mCherry (pABIND-GW-GFP and pABIND-GW-mCherry respectively). GFP and mCherry are both fluorescent proteins with different excitation and emission spectra, such that they are suitable for this type of assay. Transient expression of these two chimeric proteins was obtained by agroinfiltration, i.e. *Agrobacterium tumefaciens* has been introduced into *Nicotiana benthamiana* leaves using a needleless syringe.

At first it has been assessed that the two proteins colocalize in the nucleus. As it is visible in Fig. 25e, TPL3 is in green while PINE1 is in red. In the merged panel (technically called maximum projection) green and red are not distinguishable anymore and the orange color results from the superposition of the two. Then to quantify the strength of the interaction, FLIM was utilized. When a fluorophore is excited by a photon of specific wavelength, it will emit a photon of lower energy and return to its ground state. However, if a second fluorophore in a ground state is close, the energy is transferred to the second fluorophore instead of being lost through photon emission. This energy transfer process is quicker than photon emission. The time taken for a chromophore to return to its ground state from the excited state is referred to as lifetime.

The GFP's lifetime can indicate whether it's emitting energy through photon emission (longer lifetime; no interaction) or is passing its energy to the mCherry (shorter lifetime; interaction

is occurring). A shorter lifetime corresponds to a stronger interaction. To determine the GFP's natural decay rate, we measured the lifetime of TPL3:GFP alone. Next, we measured the lifetime of TPL3:GFP in presence of PINE1:mCherry. Consistent with our earlier findings, the GFP lifetime was significantly decreased, suggesting a robust interaction between the two (Fig. 25c).

4.4 TOPLESS interaction with HISTONE DEACETYLASE

As previously discussed, evidences in literature suggests that TOPLESS proteins interact with histone deacetylase to compact the chromatin. Actually Cheng et al. [64] reported an interaction between TPL2 and HDAC1, suggesting in our case the existence of a complex formed by PINE1-TPL2-HDAC1. To determine whether TPL3, the stronger PINE1 interactor we identified, could interact with histone deacetylases, we employed FRET-FLIM as previously described. Indeed we had found that Histone deacetylase 15 (HDAC15) could co-localize with TPL3 in tobacco nuclei (Fig. 25e), and the half-life of the GFP in presence of HDAC15-mCherry was significantly reduced (Fig. 25f), indicating an interaction between the two protein.

Therefore we propose that PINE1 can form complexes with TPL2 and TPL3, the latter in turn interact with histone deacetylases in order to modulate the expression of downstream genes. Future studies are necessary to further elucidate the role of HDACs in this complex. Furthermore it is likely that PINE1 has other interactors, which identification will require additional research.

4.5 Requirement of the EAR motifs for the function of PINE1 *in vivo*

After investigating the role of PINE1 EAR-motif in interaction with other proteins, I was also interested in understanding the importance of those EAR-motif in rice. A clone was created containing *PINE1* expressed under its own promoter (*pPINE1:PINE1*). However, we also created other clones of PINE1. One where it was missing the N-terminal EAR-motif (ΔN-EAR), and a second one missing the C-terminal EAR-motif (ΔC-EAR). In particular, we employed the same type of mutation used for the previously presented yeast two-hybrid. We performed a complementation test transforming these three constructs into plants with *pine1* knockout background. As a result, around 40% of the plants transformed with the *pPINE1:PINE1* (i.e. the WT version of *PINE1*) recovered the phenotype and didn't show signs of internode elongation (Fig. 26a). The fact that the penetrance was not 100% could be due to variability in the expression of the transgene. Meanwhile all resulting plants containing the other *PINE1* variants modified in the EAR-motif were showing various degree of internode elongation. This indicates that those modified clones of PINE1 were not completely functional, not enough to rescue the *pine1* phenotype. These results imply that both EAR-motif are actually important for a proper PINE1 function.

4.6 CRISPR of PINE1 C-terminal EAR motif

To corroborate the previously shown complementation experiment which suggested that EAR-motifs are essentials for PINE1 function, we introduced a frame-shift mutation in the C-

terminal part of PINE1, mutating essentially only the C-terminal EAR-motif. To obtain such mutation we employed a variant of the Cas9, called VQR, which recognizes a NGA PAM, that is different than the one of the conventional Cas9. Interestingly, both homozygous and

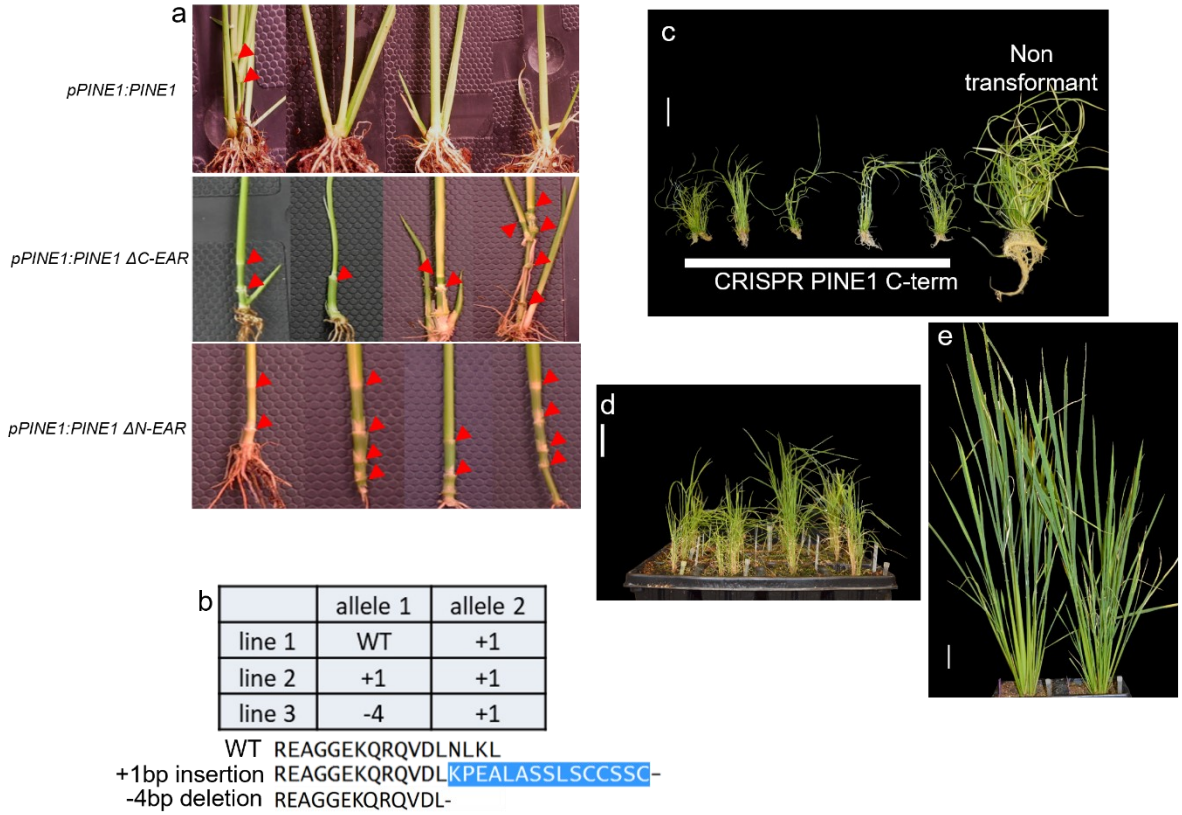


*Figure 26. **a**: Complementation test to verify if modified clones of PINE1 in the EAR-motifs could rescue the phenotype of pine1 knockout. **b**: Independent lines obtained by CRISPR of PINE1 C terminal. Each line contains at least a +1bp insertion allele. The amino acids resulting from the frameshift mutation are reported under the chart. Highlighted amino acids are not present in PINE1 WT. **c**: Effects of frameshift mutation in PINE1 C-term in regenerating plants. Edited plants are the five on the left while the plant on the right is a non-transformant plant. The white bar is 5cm. **d:** plants carrying mutation on the C-terminal of PINE1 after two months in a greenhouse. The white bar is 5cm. **e**: Non-transformant plant after two month in a greenhouse. The white bar is 5cm.*

heterozygous plants with truncated PINE1 C-term obtained were showing an extremely dwarf phenotype (Fig. 26c,d). These dwarf plants remained dwarf for months, while non transformant plants did develop normally (Fig. 26e). Unfortunately, the plants were not healthy enough to make any seeds so we couldn't propagate them. Further checking of the obtained mutations showed that only 3 different sets of mutation were obtained, reported in the Fig. 26b. As shown, each plants carried an allele of PINE1 which had a one base insertion. The resulting frameshift mutation deleted PINE1 C-terminal EAR-motif but at the same time resulted in the shift of the stop codon too, adding extra amino acids to the protein. Since a dwarf phenotype seemed in contradiction with a loss-of-function mutation of PINE1, we hypothesize that the phenotype could be due to these extra amino acids, producing an overfunctional PINE1 allele. Indeed, the DNA binding domain of PINE1 was of course still intact in these plants so PINE1 could have retained a modified regulatory activity. This interpretation

is corroborated by the finding that a heterozygous plant harbouring a WT *PINE1* allele and an overfunctional allele, was dwarf and identical to plants homozygous for the *PINE1* overfunctional allele, indicating dominance of the new allele. Consistently with the role of PINE1 in GA signalling, treatment of the dwarf primary edited plants with GA did not rescue the mutant phenotype.

4.7 Study of PINE1 downstream genes through RNA-seq

To identify genes regulated by PINE1, we conducted an RNA-seq analysis comparing gene expression in *pine1* and WT stems. So RNA was extracted from stem tissues in triplicate and sent to a company facility for Illumina sequencing. Raw reads were analyzed to determine gene expression levels in each sample. Around 22000 genes (rice contains $\approx$50000 genes) were expressed in our samples. Through DESEQ2, 365 differentially expressed genes (filtered by Log_2FoldChange>1.5 or Log_2FoldChange<-1.5 and p-value<0.001) were isolated. Of the 365 differentially expressed genes, 277 are less expressed in the WT, consistently with the putative role of PINE1 as a transcriptional repressor. A gene ontology analysis was performed, however no known genes related to hormone biosynthesis or signaling were identified, and we did not observe any pathway enrichment that could be related to internode elongation (data not shown). In fact, many of the most differentially expressed genes are of unknown function and lack recognizable motifs (Fig. 27a). To validate the expression data obtained through the RNA-seq, we measured the expression of two of the differentially expressed genes. Os07g0442800 has a positive fold change, so it is more expressed in the WT by the RNA-seq (Fig. 27b). Real-time PCR was done in 2 independent WT sample and three independent *pine1* knockout mutant samples. Expression data supported that the gene is more expressed in the WT even in different independent samples (Fig. 27c). The same approach was taken with the gene Os03g0115800, which confirmed yet again the data provided by the RNA sequencing (Fig. 27d and 27e).

To further investigate the potential functions of the differentially expressed genes, we decided to knock-out some of them. Specifically, we chose genes that were highly differentially regulated and expressed at fairly high levels. As many of these genes lack a name or known motif, we will refer to them by their locus name. The mutated genes were Os06g0271500 (which is identified as a putative transposon), Os03g0115800, Os07g0442800 and Os07g0153150. A variety of frame-shift mutations were obtained for all these genes. Os07g0153150 knockout mutant plants are only at the T0 generation (i.e. the plants obtained from the transformed calli) so we do not have phenotypic data yet. However, we were able to perform a preliminary phenotyping analysis on the knockout plants for the other three genes. Specifically, we measured internode length at panicle maturity to determine if any phenotypes were present (Fig. 28a,b,c,d).

The knock-out of two of these genes did not show any clear phenotype regarding internode elongation. However, for the knock-out of Os03g0115800 we observed a significant decrease in the first internode (the uppermost one) length (Fig. 28e,f). As stated before, phenotyping of these genes is only at a preliminary stage, more generations and a larger sample number will be needed in order to better assess any effect of the mutations introduced in those genes.

a

Locus	Description	Log_{10} Fold Change	p-value
Os07g0442800*	Conserved hypothetical protein	13.035	2.89e-28
Os07g0153150*	Conserved hypothetical protein	10.057	8.10e-144
Os04g0668200	latency associated nuclear antigen, putative, expressed	8.856	3.58e-13
Os11g0491600	NB-ARC domain containing protein, expressed	8.216	3.46e-15
Os05g0438201	Conserved hypothetical protein	8.199	7.29e-06
Os11g0233201	receptor-like protein kinase HAIKU2 precursor, putative	7.729	1.23e-09
Os02g0538550	Conserved hypothetical protein	7.159	3.85-08
Os11g0118000	Alpha/beta hydrolase fold-1 domain containing protein	7.155	4.17e-08
Os09g0273600	Hypothetical gene	6.770	7.10e-07
Os08g0340601	Hypothetical protein	6.386	4.10e-06
Os11g0229100	proteophosphoglycan, putative, expressed	-10.182	1.71e-17
Os06g0637800	Hypothetical gene	-10.257	1.30e-17
Os04g0355401	Hypothetical protein	-10.455	2.03e-18
Os08g0340700	Non-protein coding transcript	-10.867	6.73e-20
Os04g0172560	Hypothetical gene	-11.403	7.52e-22
Os12g0632401	Hypothetical conserved gene	-11.434	5.48e-22
Os11g0229000	Hypothetical conserved gene	-11.719	4.81e-23
Os02g0496300	Conserved hypothetical protein	-12.494	4.54e-26
Os03g0115800*	Conserved hypothetical protein	-14.710	1.27e-35
Os06g0271500*	transposon protein, putative, CACTA, En/Spm sub-class	-15.206	5.14e-38

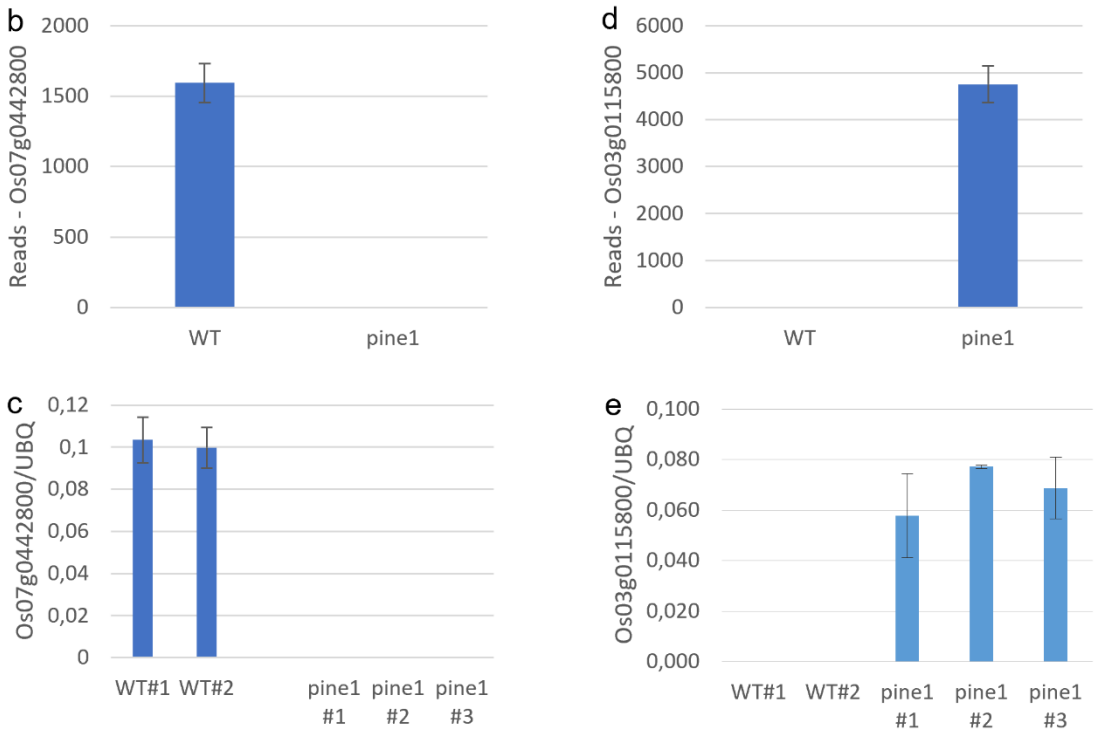


Figure 27. ***a****: 10 most upregulated and downregulated genes resulting from the RNA-sequencing of pine1 knockout mutant. Positive fold change indicates genes that are more expressed in the WT, and viceversa for the negatives fold change. Genes which we mutated with CRISPR are indicated with an asterisk.* ***b:*** *Number of Normalized reads obtained by DESEQ2 by the RNA sequencing for the gene Os07g0442800.* ***c****: Gene expression through real time PCR of the gene Os07g0442800 to validate the RNA-seq data.* ***d****: Number of Normalized reads obtained by DESEQ2 by the RNA sequencing for the gene Os03g0115800.* ***e****: Os03g0115800 expression in WT and pine1 knockout measured with real-time PCR to validate the RNA-seq data.*

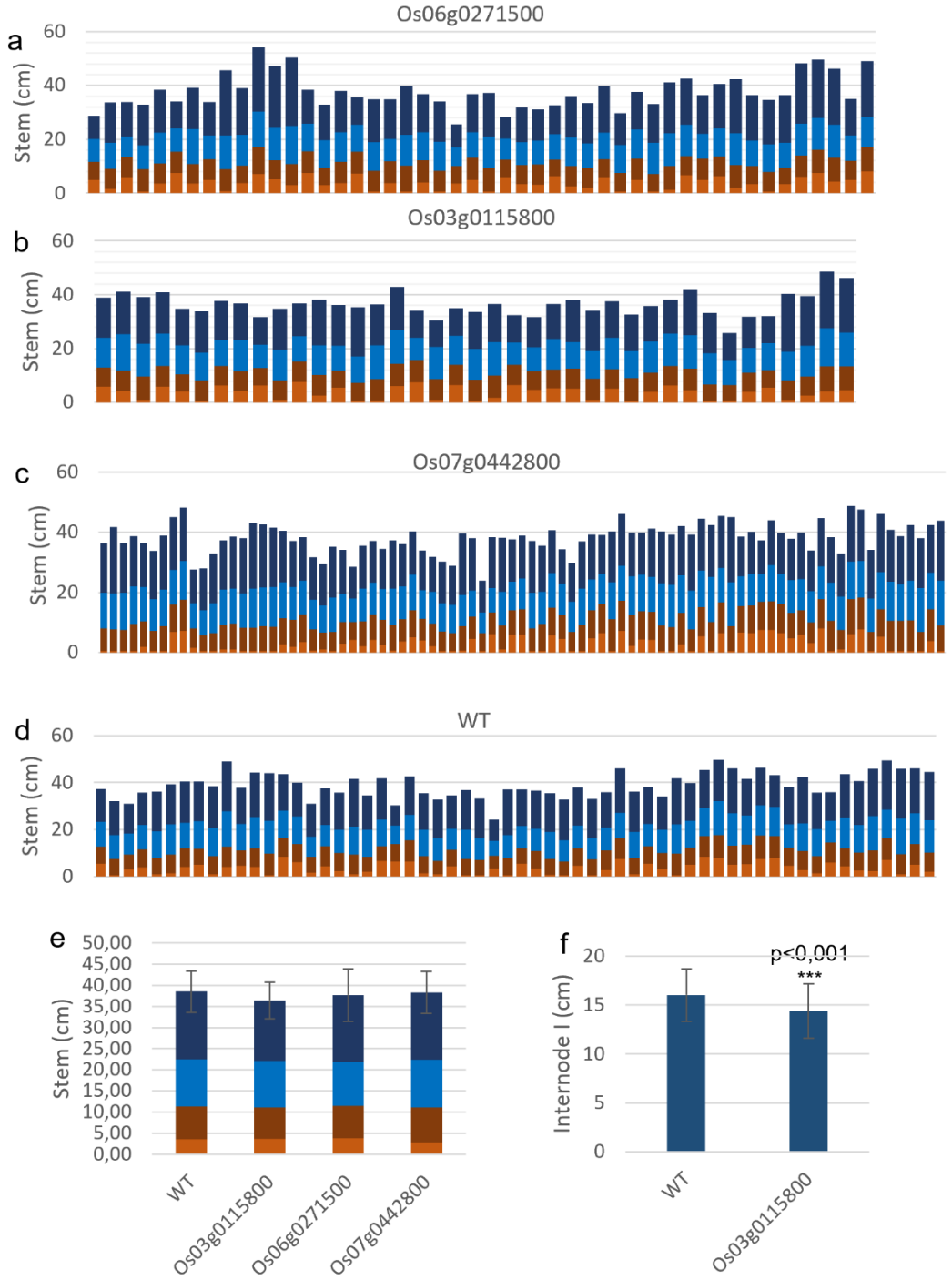


Figure 28. ***a****: Representation of stem in knockout plants of the gene Os06g0271500.* ***b****: Representation of stem in knockout plants of the gene Os03g0115800.* ***c****: Representation of stem in knockout plants of the gene Os07g0442800.* ***d****: Representation of stem in WT plants.* ***e****: Average stem of the WT and the three knockout genes represented in a,b,c,d. Error bars represents the standard deviation on the total height of the plants.* ***f****: Internode I (uppermost internode) average length in WT and Os03g0115800 knockout plants. Error bars represent the standard deviation of the length of that internode alone.*

5. Discussion

5.1 Regulation of PINE1 expression and study of its promoter

Firstly, we observed how the expression of *PINE1* is affected by flowering. Florigens can initiate the process of internode elongation by inhibiting *PINE1* expression. However, even in their absence, overexpression of the florigen-like protein *FTL1* in the SAM can trigger both internode elongation and flowering, although the resulting inflorescence is altered. Internode elongation displayed in such overexpressor closely resembles the phenotype of the *pine1* knockout mutant, and may even be stronger. This suggests that by inducing flowering, the molecular mechanisms responsible for stem elongation can be activated similarly to the knockout of *pine1* alone. We identify FTL1 as a possible repressor of *PINE1* expression, further proof will be needed to corroborate that. It still however remains elusive whether FTL1 could have a direct role in controlling *PINE1* expression by binding to its promoter. Moreover, we noted how internodes elongate even in a double mutant of the florigens, so internode can elongate without a flowering-driven pathway. Elongation is more noticeable if the plants are exposed to short days. We therefore hypothesize that different and independent pathway could lead to the inhibition of PINE1 expression. One which is flowering dependent, which could be mediated by FTL1, and other pathways with milder effects, that are dependent on the photoperiod but not on flowering.

Then we investigated *PINE1* promoter and successfully generated mutations that we hypothesize resulted in sustained high expression of *PINE1* even after flowering, leading to the development of dwarf plants. A more thorough internode measurement and *PINE1* expression analysis in different time points of homozygous T1 plants will be needed to definitely prove the effect of a certain mutation with a modified level of *PINE1* expression, and its link with a phenotype. The plants utilized in this study were still T0 plants (the one regenerated from calli) so most of them carried two different alleles of *PINE1* promoter, making it difficult to discern the effect of each allele. Once we find the mutations responsible for the shortening of the stem we could try to implement those into varieties which still show sensitivity to lodging, to increase their resistance.

Although identifying different mutations in the PINE1 promoter can provide insight into the important sequences for regulating PINE1 transcription, it is challenging to determine which specific protein directly interacts with the promoter to regulate its expression. There are various methods for studying protein-DNA interactions, such as ChIP, Yeast one-hybrid, and pull-down. However, these methods all have a common issue in that they only examine the interaction of one protein at a time and cannot evaluate DNA binding complexes. Thus, instances where the formation of a complex is necessary to bind DNA are very difficult to detect. Additionally, some of these methods are low-throughput so only a small number of proteins can realistically be tested.

Only recently an unbiased approach to find DNA-interacting protein has been used by Wen et al [65] by using a technique called Reverse Chromatin Immunoprecipitation. The technique is similar to a conventional ChIP, but instead of using a tagged protein to precipitate chromatin,

a DNA probe is used to isolate a specific chromatin site and the associated protein, which can then be further analyzed using GC-MS. This method could provide valuable insights into the proteins directly involved in regulating *PINE1*, and when combined with information obtained from mutations in the PINE1 promoter, could yield a comprehensive understanding of how *PINE1* regulation functions.

Understanding the control of *PINE1* expression could also be important as an agronomical perspective. Although GA-deficient rice was the centerpiece of the Green Revolution due to its dwarf phenotype, it has been observed that having less GA has some downsides. It may be useful to first describe the different type of lodging that cereals exhibit. There are three types of lodging: culm bending, culm breaking, and root lodging. Culm bending occurs when plants fail to resist bending pressure from wind and rain, and it generally occurs in the upper internodes. Culm breaking, on the other hand, occurs in the lower internodes when there is too much bending pressure in the higher internodes. Root lodging occurs when the roots are shallow and do not develop well below ground (Fig. 29). A crucial factor for culm breaking type lodging resistance is the physical strength of the culm, which is determined by the morphology and quality of its components. It has been reported that dwarf varieties have reduced culm breaking resistance compared to the original cultivars. This is because higher GA levels increase culm diameter and lignin content, and as a result, GA-deficient dwarf varieties are more prone to culm breaking [7]. Therefore, understanding how to exploit PINE1 in that sense could be beneficial to obtain dwarf varieties which are not GA deficient, and without such problems of GA deficient plants.

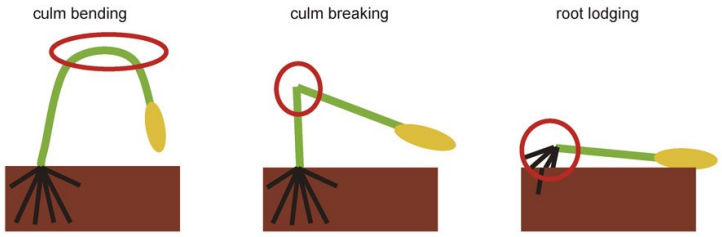


Figure 29. The three diverse types of lodging in cereal: culm bending, culm breaking and root lodging [7].

5.2 Molecular mechanism of action of PINE1

We have observed that PINE1 interacts with TPL corepressors through its C-terminal EAR-motif and we observed the interaction of TPL3 with HDAC15, while other instances in literature indicate interactions between TPL2 and HDAC1. We therefore propose a complex composed by PINE1-TPL-HDAC, which can repress gene expression by compacting the chromatin. In particular, we suggest the possible complexes that PINE1 may form as PINE1-TPL2-HDAC1 and PINE1-TPL3-HDAC15. That however is derived by the study of the interaction of two proteins at a time. A proper experiment should be done in order to check if the three protein do interact together. That could be done through a co-immunoprecipitation assay or testing through confocal microscopy the interaction of three protein, fusing 2 proteins with a split half of the YFP and the third protein with a CFP, which is able to perform FRET with the whole YFP.

While the N-terminal EAR-motif is not directly involved in these interactions, we have demonstrated its importance in PINE1 function overall by *pine1* phenotype complementation

experiment. A further and more precise analysis of the plants carrying clones of PINE1 modified in the EAR-motifs will help determine whether the two EAR-motifs have equal importance or different 'weight' in PINE1 function overall. More in details, internodes measurement and measure of PINE1 expression will be performed soon in these plants, in order to quantify the effect of each EAR-motif.

Although the N-terminal EAR-motif did not participate in any of the interactions we tested, it may still have a secondary role. We also shouldn't exclude the possibility that this EAR-motif might participate in interactions with other proteins that we did not test. Indeed both EAR-motif seems to be quite conserved among Monocots. We can find proteins which are very similar to PINE1 in the major cereals wheat, maize and barley (Fig. 30), suggesting that since their architecture is very similar, they could share the same molecular mechanism regarding stem elongation. We can find a similar protein also in other monocot such as banana and even in eudicots like in tomato (Fig. 30). That corroborate our idea that the N-terminal EAR motif has a conserved function which we were not able to detect with our experiments . As for the

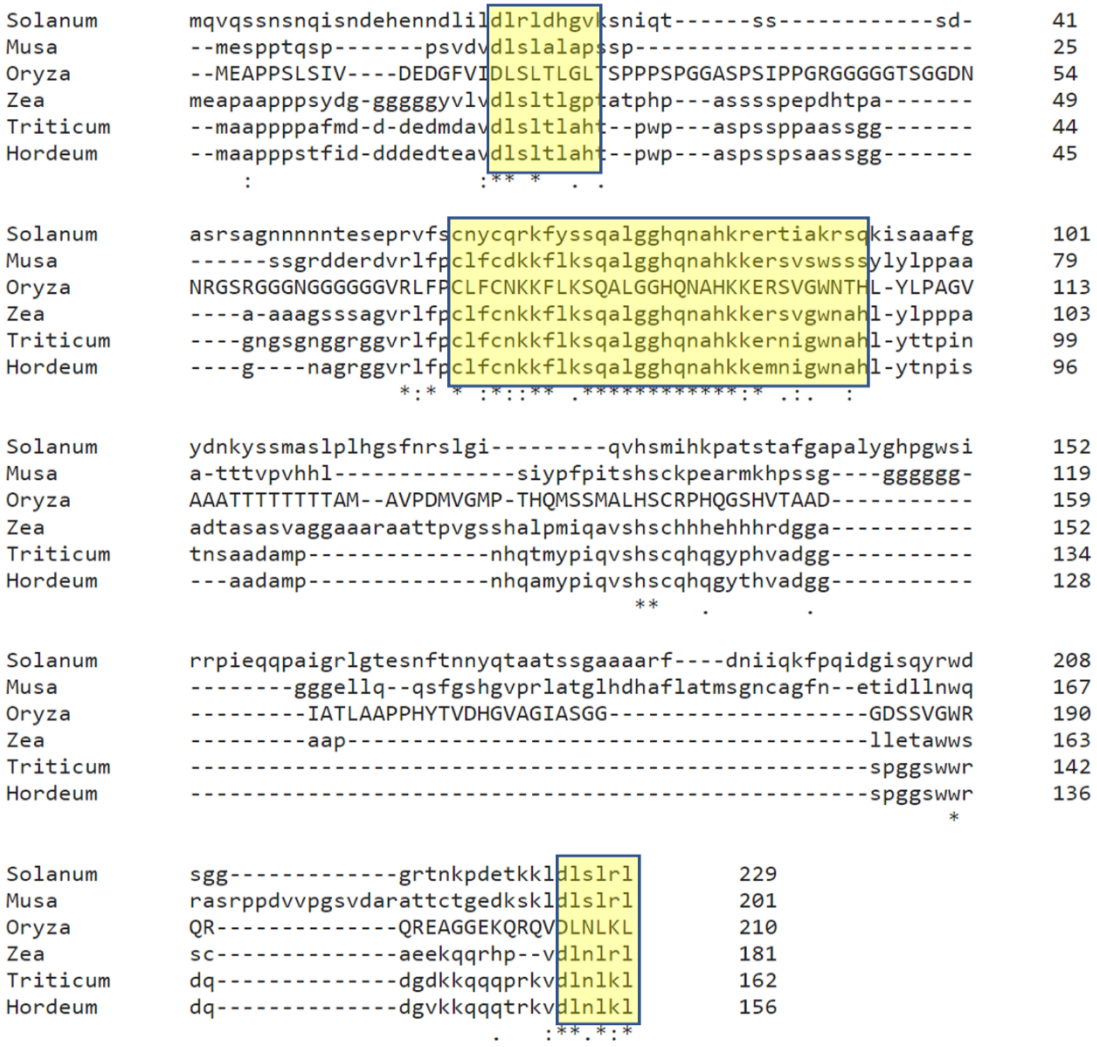


Figure 30. Alignment of PINE1 protein among different species. In the order: Solanum lycopersicum (tomato), Musa acuminata (banana), Oryza sativa (rice), Zea mays (maize), Triticum Aestivum (wheat), Hordeum vulgare (barley). The two smaller yellow rectangle highlights the position of the EAR-motifs. The bigger rectangle highlight the zinc finger motif. Alignment has been done with CLUSTAL-OMEGA web tool.

C-terminal EAR-motif, since we identified at least one of its functions in its interaction with TOPLESS proteins, we may consider testing whether its function is maintained in the "PINE1" of other species by checking if the same interaction is maintained.

We also performed the mutation of the C-terminal part of PINE1 using CRISPR/VQR. We obtained dwarf plants, which was unexpected, and it seemed contradictory with a PINE1 loss of function. However, all the edited plants had one allele with a +1bp insertion which generated a frame-shift of the STOP codon, leading to PINE1 having extra amino acids. Those extra amino acids contain a SMALL UBIQUITIN LIKE MODIFIER (SUMO) interacting motif, which is composed by the amino acids SXS. The addition of this extra motif could be perhaps part of the reason for the unexpected phenotype. *PINE1* with these modifications did probably retain the ability to bind the DNA since the zinc finger motif was unaltered, so the combinations of having extra amino acids while still being able to bind DNA could have led to such unexpected result. In order to confirm whether this +1bp insertion was responsible for such phenotype, the transformation might be repeated in order to obtain different mutations which do not lead to those extra amino acids.

5.3 Downstream pathway of PINE1

We conducted an RNA sequencing to compare the differentially expressed genes between the *pine1* knockout mutant and the WT. However, a gene ontology analysis of the differentially expressed genes did not reveal any major gene that could be responsible for the *pine1* phenotype. It is important to note that RNA-seq cannot identify the direct targets of a transcription factor, and most of the differentially expressed genes probably do not have a role in internode elongation. Nevertheless, we further studied some of the differentially expressed genes, choosing them among the most differentially expressed. We generated knock-out mutants of these genes using the CRISPR/CAS9 system. Although the phenotyping of these plants is still ongoing, we have observed only mild phenotypes so far. In particular, Os03g0115800 had shown a significant difference in the length of the uppermost internode. However, this gene is not conserved even in close species such as wheat, indicating that it might give a small contribution in the overall mechanism of internode elongation.

Indeed, the observed mild phenotypes of our knockout suggest that other genes may be responsible for the *pine1* phenotype, and/or that multiple genes may act in concert. To address this issue, a similar approach to the one used to mutate *PINE1* promoter could be employed. By using multiple guide RNAs, it is possible to mutate many genes simultaneously, thereby making the contribution of different mutant alleles additive. By performing a multiple knockout of genes repressed by PINE1 (so with a negative fold change in our RNA-seq) in a *pine1* knockout background we may complement the phenotype, obtaining a WT-like internode elongation.

As previously mentioned, RNA-seq cannot identify direct targets of a transcription factor. A way to identify them is through the use of Chromatin immunoprecipitation (ChIP), which allows for the identification of binding sites of a tagged protein to the DNA. To tag a protein, a modified version of that protein can be expressed as a transgene with a known small oligopeptide recognized by a proper antibody. In this case, transgenic plants overexpressing

PINE1 tagged with 3xFLAG have already been created. By taking advantage of the tagged protein, it is possible to precipitate with a proper antibody the complex tagged protein-chromatin. The chromatin can then be sequenced to reveal the genomic regions binded by the tagged transcription factor. In addition, the tagged PINE1 allows for *in planta* co-immunoprecipitation followed by gas chromatography-mass spectrometry to identify interacting proteins, including larger complexes such as the one described by Ke et al. [51].

5.4 Regulation of stem elongation beyond PINE1

In addition to the near future research on PINE1, we can also speculate on the broader aspects of rice internode elongation. As previously mentioned, internodes remain unelongated until floral induction, at which point the last-formed internodes (i.e., the younger ones) will be the only ones to elongate. An intriguing question is how newly formed internodes maintain their ability to elongate. Two hypotheses come to mind as to why only specific internodes are capable of elongating. The first is that specific signals from the leaves or the developing panicle, or a combination of both, are capable of activating growth only in those particular internodes. The second hypothesis is that younger internodes retain some sort of dormant meristematic activity that can be activated during flowering. As an internode ages, it may gradually lose that ability to elongate, until it reaches a "maturity" stage when its ability to elongate disappears completely. That last hypothesis could also explain why, even among elongating internodes, the lower ones (i.e. older) are also the shorter ones.

Another interesting and yet unexplored aspect of internode elongation is the temporal pattern with which internodes elongate. Elongation starts from the 4th or 5th internode from the apex. Only after that internode reaches more or less its final length, the next internode (i.e. higher), and only that, commence elongation. This suggests that a signal has to activate each internode individually and/or a signal inhibits elongation of every internode but one. Since elongation begins at lower internodes, it is unlikely that the developing panicle promotes elongation via a signal, as such a signal would activate the upper internodes first, or at least all at once. It is possible that an inhibitory signal from the panicle hinders elongation, and the gradual decrease of that signal over time gradually permits elongation of upper internodes. Perhaps different type of controls pertains to different internodes, as it seems to happen with the BR contribution on internode elongation (BR are often reported to have an effect on the 2nd internode). All in all, the topic of how each internode is different from each and they behave differently is truly fascinating and still almost completely unexplored.

Even though, as stated in the introduction, PINE1 is able to repress gibberellin sensitivity in rice stem, the precise mechanism with which this happen is still unknown, but some data suggest that SLR1 degradation upon GA treatment is not sufficient to kickstart internode elongation, since exogenous GA treatment leads to SLR1 degradation both in deepwater and ‘normal’ rice, but the former responds to excessive GA by elongating the internodes while the latter does not [42]. GA-induced degradation of DELLA proteins acts as a central regulatory switch for GA signal transduction. However some study suggest the existence of a DELLA-independent GA pathway. For example, Tomato (*Solanum lycopersicum*) has only one DELLA gene, *PROCERA* (*PRO*). RNA sequencing using the *pro* mutant suggests that 5% of all GA-

regulated genes in tomato are DELLA independent. Another GA pathway seems to pass through Ca2+, a well known second messenger. One physiological response to GAs is indeed an increase in cytosolic Ca2+, and that increase is independent from the presence of DELLA proteins. In some instance the increase in calcium was found involved with GA feedback regulation in growth. GID1 is localized both in the nucleus and in the cytoplasm, so it might have a role in that GA-dependent cytoplasmatic calcium increase. Furthermore, although GID1 plays essential roles in GA signaling, physiological studies suggest an alternative signaling pathway related to a membrane-localized GA receptor [66]. So the pathway of GA signaling is not yet fully understood and more research will help to place also PINE1 in that pathway.

6. Conclusions

As a final remark, I would like to reassume what we already knew about *PINE1* and the new information acquired during my PhD in a working model of PINE1, highlighting the three aspects previously discussed in the 'aim' chapter: The upstream pathway, the mechanism of action and the downstream genes. This is summarized in Fig.31 and Fig. 32.

Fig. 31 represents rice growing in long day. In such photoperiod rice needs to maintain vegetative features. Among them, internodes need to stay compressed at the base of the plant. A currently unknown transcriptional activator can bind to precise regions in PINE1 promoter (which we are investigating) and allow the expression of PINE1. Our data suggest that PINE1, being present during the long day photoperiod, can bind to TPL2 and TPL3 corepressors. Those can interact with HDAC1 and HDAC15 respectively, suggesting a complex able to repress gene expression by modulating the chromatin state. Since the expression of downstream genes is repressed by such complex, they cannot trigger stem elongation and a vegetative, unelongated stem is maintained.

Fig. 32 represents instead what happens when a rice plant is exposed to a short day photoperiod. The leaves are able to perceive such inductive condition and they produce two florigens: Hd3a and RFT1. Those arrive through the phloem to the SAM where they form a complex called 'FAC' able to regulate gene expression. Among the changes that occur upon the arrival of the florigens, FTL1 expression is strongly promoted. Our data suggest that FTL1 is then able to repress PINE1 expression, either directly or indirectly. PINE1 being absent, its effect on chromatin compaction is lost since the chromatin is not deacetylated anymore, and the genes that were repressed during in long day are now transcriptionally active and they are able to promote the stem elongation necessary for the panicle emergence. Moreover, since internode elongation also happen in the non flowering plants *hd3a rft1,* we hypothesize the existence of an additional pathway, independent from flowering but only from the photoperiod, which is able to inhibit PINE1 expression in a milder way compared to flowering.

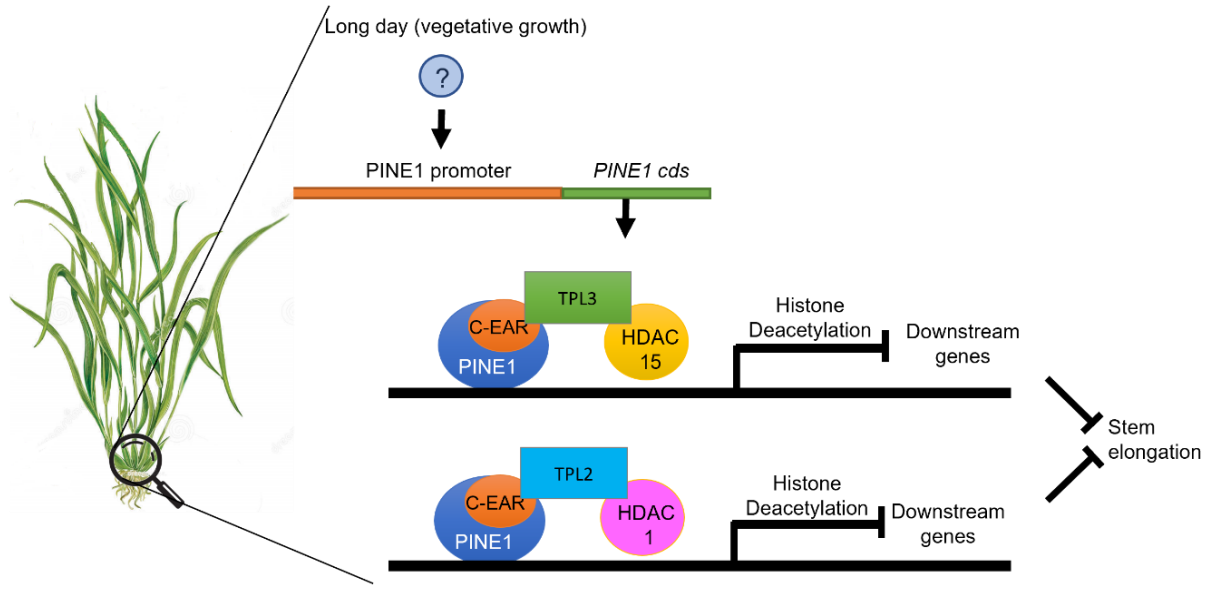


Figure 31. Representation of PINE1 role and its pathway in maintaining the internode unelongated during vegetative growth.

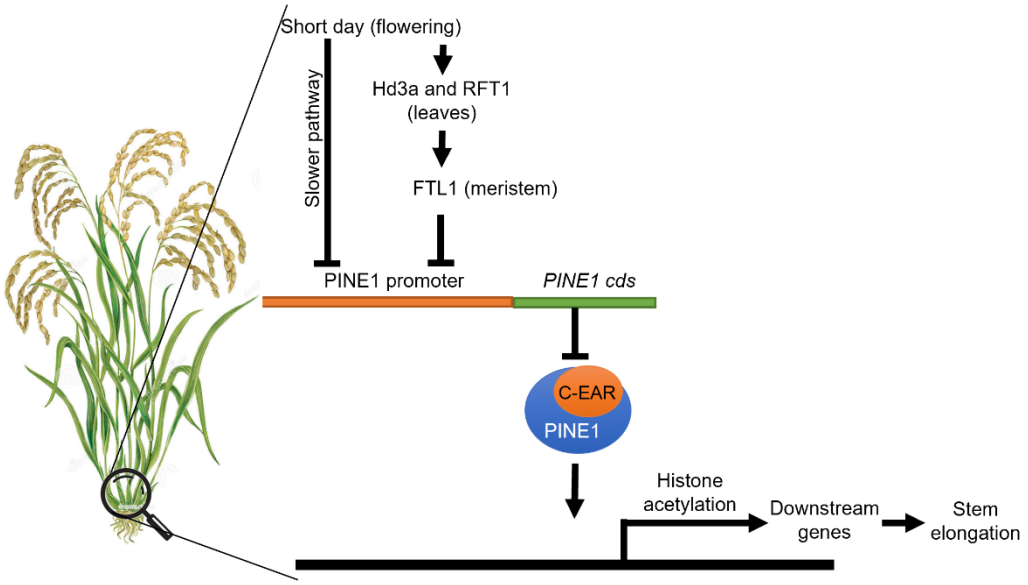


Figure 32. Representation of the pathway leading to PINE1 inhibition and the consequent internode elongation during floral induction.

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