The rice Phytochrome-Interacting Factor 14 – *a regulator of cold, jasmonic acid and light related genes*

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“Never stop dreaming,
never stop believing,
never give up,
never stop trying, and
never stop learning.”

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List of Abbreviations

3-AT	3-amino-1,2,4-triazole
a.a.	amino acid
ABA	Abscisic Acid
AOS	Allene Oxyde Synthase
AP2	APETALA2
APB	Active Phytochrome B Binding domain
ARF	Auxin response factor
At	*Arabidopsis thaliana*
AUX/IAA	Auxin/Indole 3-Acetic Acid
bHLH	basic Helix-Loop-Helix
BiFC	Bimolecular Fluorescence Complementation
BLAST	Basic Local Alignment Search Tool
bp	base pair
BR	Brassinosteroid
BZR	Brassinazole-Resistant
C2H2	Cysteine2/Histidine2
CAMV35S	Cauliflower Mosaic Virus 35S promoter
cDNA	complementary DNA
CBF	C-repeat Binding Factor
COP	Constitutive Photomorphogenic
Cry	Cryptochrome
ºC	Degrees Celsius
DAI	Days After Imbibition
DEG	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
DRE	Dehydration Responsive Element
DREB	Dehydration Responsive Element Binding Factor

EDTA	Ethylene Diamine Tetraacetic Acid
EMSA	Electrophoretic Mobility Shift Assay
ERF	Ethylene Response Factor
g	gravitational force
g	gram
GA	Gibberellin
GUS	β-Glucuronidase
h	hour
ha	hectare
HA	Hemagglutinin
HD	Homeodomain
HFR	long hypocotyl in far-red
His	Histidin
HOS	High expression of osmotically responsive gene
Hyg	Hygromycin
JA	Jasmonic Acid
JAZ	Jasmonate zim-domain
KD	Kinase Domain
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilogram
L	Litre
LUC	Luciferase
M	Molar
m^{-2}	meter square
m	meter
μg	microgram
μL	microlitre
μM	micromolar
μmol	micromol

min	minute
Mb	Mega base pair
mg	miligram
mL	mililitre
mM	milimolar
ng	nanogram
nm	nanometer
nmol	nanomole
Os	*Oryza sativa*
PBE	PIF Binding E-box
PCR	Polymerase Chain Reaction
Pfr	Phytochrome active form
Phy	Phytochrome
Phot	Phototropin
PIL	Phytochrome-Interacting Factor 3 Like
PIF	Phytochrome-Interacting Factor
Pr	Phytochrome inactive form
Prx	Peroxidase
RNA	Ribonucleic Acid
RNAi	RNA interference
rpm	rotations *per* minute
RR	Response Regulator
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
RT-qPCR	quantitative/real time RT-PCR
s	second
SAUR	Small Auxin Up RNA
SDS	Sodium Dodecyl Sulfate
t	ton
TF	Transcription Factor

Trx	Thioredoxin
T-DNA	Transfer-DNA
UTR	Untranslated region
UV	Ultraviolet
UVR8	UV Resistance locus8
v	volume
WT	Wild Type
Y1H	Yeast One-Hybrid
Y2H	Yeast Two-Hybrid
YFP	Yellow Fluorescent Protein

Summary

Rice (*Oryza sativa* L.) is the staple food for more than half of the world population, and it is very sensitive to adverse environmental conditions. It is also very important for Portugal, which is the biggest rice consumer in Europe with a consumption of 14.8 kg/capita/year. Nowadays, due to climate changes and competition with other crops, the arable land for rice is decreasing. To overcome this and feed the growing world population, keeping the prices affordable, it is estimated that rice yield needs to grow 1.0–1.2% annually beyond 2020. Therefore, it is urgent to develop rice with higher grain yield and more resistant to adverse environmental conditions. To achieve this goal, we need to understand better the molecular mechanisms by which rice plants regulate their growth and development according to the environmental conditions. Light plays a crucial role in plant growth and development, not only due to its function in photosynthesis but also as a signal to regulate gene expression. Light is perceived by plant photoreceptors (e.g. phytochromes) that modulate the activity of Phytochrome-Interacting Factors (PIFs). PIFs are transcription factors and are considered a central hub between light, environmental stimuli, and internal signals. Before our study, the rice PIF14 (OsPIF14) was identified as binding to the promoter of *OsDREB1B*, a key regulator of cold stress, and shown to interact with phytochrome B. The main goal of our study is to contribute for a better understanding of the crosstalk between light and environmental cues, more specifically to characterize the function and mode of action of OsPIF14. In this study, we identified the binding site and studied the importance of the flanking region for the binding of OsPIF14 to *OsDREB1B* promoter. In addition, we characterized OsPIF14 transactivation activity. Also, we generated OsPIF14 silencing (RNAi::*OsPIF14*) transgenic lines and analyzed their phenotype and gene regulation under dark and light conditions.

We have characterized OsPIF14 as a bHLH group B protein based on homology studies with other bHLH TFs, and showed by transactivation activity studies that OsPIF14 acts as a repressor and can decrease *OsDREB1B* gene

expression. We analyzed in detail the importance of each nucleotide for the binding of OsPIF14 to the *OsDREB1B* promoter, and showed that OsPIF14 binds to two N-box type *cis*-element (CACGCG). The strength of the binding to N-box considerably increases when this *cis*-element is extended to CCACGCGG. These results show that the flanking region is important for the binding of OsPIF14 to the *OsDREB1B* promoter. Additionally, OsPIF14 also binds to other similar *cis*-elements, as is the case of G-box (CACGTG) in which the change of one nucleotide (CACGCG to CACGTG) strongly increases the binding of OsPIF14 to DNA.

To characterize OsPIF14 biological function we generated RNAi::*OsPIF14* lines. These lines were analyzed at seedling stage to monitor their growth and development under constant dark or light/dark cycles. RNAi::*OsPIF14* seedlings show higher percentage of root curling as compared to wild type only under dark conditions. Since this phenocopies the effect of jasmonic acid (JA), we hypothesized that RNAi::*OsPIF14* lines have the JA biosynthesis and/or signaling impaired. In fact, we observed that OsPIF14 binds to the G-box present at the promoter of an important gene of JA biosynthesis, the *Allene Oxide Synthase 1* (*AOS1*) and the transactivation assays showed that OsPIF14 represses *AOS1* expression. Nonetheless, no differences were observed in the expression of *AOS1* between RNAi::*OsPIF14* and WT seedlings, suggesting that other factors might contribute for the regulation of *AOS1*. We have also analyzed the expression of JA signaling pathway genes and found that two *jasmonate zim-domain* (*JAZ*) transcripts are down-regulated in the RNAi::*OsPIF14* lines as compared to WT. Since JAZs are the constitutive repressors of JA signaling, these results suggest that RNAi::*OsPIF14* seedlings might be more sensitive to JA or have a constitutive JA-responsive gene regulation.

In order to identify new OsPIF14 direct and indirect targets and gain new insights into OsPIF14 function, we used microarray to analyze the transcript profile of RNAi::*OsPIF14*, WT, and *phyB* in the transition from dark to light. Only a few genes were identified as being differentially expressed in RNAi::*OsPIF14* as compared to the other lines. All the RNAi::*OsPIF14*

upregulated genes showed to have at least one OsPIF14 binding *cis*-element in its promoter, indicating that these genes could be directly repressed by OsPIF14. These genes are mainly associated with lipid metabolism and cell wall structure and organization, suggesting that OsPIF14 could be involved in growth and cell elongation.

This work provides new insights into the function of OsPIF14 in rice, more specifically, in the regulation of cold, jasmonic acid and light related genes. Our results clearly show that OsPIF14 has the potential to interconnect different environmental cues and, in the future, the analysis of transgenic rice lines will be important to further understand the biological function of OsPIF14 in the crosstalk light/JA/cold.

Sumário

O arroz (*Oryza sativa* L.) é a base da alimentação para mais de metade da população mundial, e é bastante sensível a condições ambientais adversas. No caso de Portugal, o arroz tem particular importância, uma vez que somos o maior consumidor Europeu com 14.8Kg/capita/ano. Hoje em dia, devido às alterações climáticas e competição de outros cereais com maior valor para a agricultura, as terras de cultivo de arroz têm vindo a diminuir. De modo a ultrapassar isto e alimentar a população que continua em crescente, mantendo os preços razoáveis, é estimado que o redimento da produção de arroz tenha que crescer entre 1.0-1.2% todos os anos até 2020. Assim, é urgente desenvolver uma planta de arroz com maior redimento de produção e mais resistente a condições ambientais adversas. Para atingir este objectivo, é necessário perceber melhor os mecanismos moleculares pelos quais as plantas de arroz regulam o seu crescimento e desenvolvimento em função das condições ambientais. A luz desempenha um papel fundamental para o crescimento e desenvolvimento das plantas, não só pela sua função na fotosíntese, mas também como regulador da expressão génica. A luz é captada por fotoreceptores das plantas (ex. fitocromos) que modulam a actividade dos fatores que se ligam aos fitocromos (PIFs, do inglês Phytochrome-Interacting Factores). Os PIFs são fatores de transcrição considerados peças centrais na relação entre a luz, estímulos ambientais e sinais internos da própria planta. Antes deste estudo, o PIF14 de arroz (OsPIF14) foi identificado como se ligando ao promotor do *OsDREB1B*, um regulador chave de frio, e provado que interage com o fitocromo B. O principal objectivo deste estudo é contribuir para uma melhor compreensão da relação entre luz e estímulos ambientais, mais especificamente no que respeita a caraterização e modo de acção do OsPIF14. Neste estudo identificámos o local de ligação e estudámos a importância da região flanquante para a ligação do OsPIF14 ao promotor do *OsDREB1B*. Caracterizámos ainda, a actividade transcricional do OsPIF14. Para além disso, produzimos linhas de arroz a silenciar o *OsPIF14* (RNAi::*OsPIF14*) e analizámos o seu fenótipo e regulação génica em diferentes condições de escuro e luz.

Nós caraterizámos o OsPIF14 como uma proteína do grupo B dos bHLH, baseado em estudos de homologia com outros factores de transcrição da família dos bHLH. Por intermédio de estudos de transactivação mostrámos que o OsPIF14 reprime a expressão do *OsDREB1B*. Além disso, analisámos em detalhe a importância de cada nucleótido para a ligação do OsPIF14 ao promotor do OsDREB1B, e mostrámos que o OsPIF14 liga-se a dois elementos do tipo N-box (CACGCG). A força de ligação para a N-box aumenta consideravelmenete quando este elemento é estendido para CCACGCGG. Estes resultados mostram que a região flanqueante é importante para a ligação do OsPIF14 ao promotor do *OsDREB1B*. Para além disso, o OsPIF14 liga-se a outros elementos semelhantes, como é o caso da G-box (CACGTG) no qual a alteração de um nucleótido (CACGCG para CACGTG) aumenta consideravelmente a ligação do OsPIF14 ao DNA.

De modo a caraterizar a função biológica do OsPIF14, produzimos linhas de arroz RNAi::*OsPIF14*. Estas linhas foram analisadas no estadio de plântula, para monitorizar o seu crescimento e desenvolvimento em escuro constante ou ciclos de luz/escuro. As plântulas RNAi::*OsPIF14* mostraram maior percentagem de enrolamento da raiz do que as selvagens, apenas quando crescidas em escuro constante. Uma vez que este fenótipo mimetiza os efeitos do ácido jasmonico (JA), hipotetizámos que as linhas RNAi::*OsPIF14* possam ter uma desregulação nas vias de biosíntese e/ou sinalização do JA. Na verdade, observámos que o OsPIF14 se liga à G-box presente no promotor de um gene importante da via da biosíntese do JA, o *Allene Oxide Synthase 1* (*AOS1*). Através de ensaios de transativação mostrámos que o OsPIF14 reprime a expressão do *AOS1*. No entanto, não são observadas diferenças na expressão do *AOS1* entre as plântulas RNAi::*OsPIF14* e as selvagens, o que sugere que possam haver outros factores a regular o *AOS1*. Analizámos também a expressão de outros genes da via do JA e observámos que dois *jasmonate zim-domain* (*JAZ*) estão reprimidos nas linhas RNAi::*OsPIF14* comparativamente ao selvagem. Uma vez que os JAZ são os repressores constitutivos da sinalização do JA, estes resultados sugerem que as

plântulas RNAi::*OsPIF14* possam ser mais sensíveis ao JA ou apresentar uma regulação constitutiva dos genes regulados pelo JA.

De modo a identificar novos alvos, directos ou indirectos do OsPIF14, e para adquirir novos conhecimentos sobre a função do OsPIF14, usámos microarrays para analisar os transcritos das linhas RNAi::*OsPIF14*, selvagem e *phyB* na transição do escuro para a luz. Foram identificados poucos genes como sendo mais regulados nas linhas RNAi::*OsPIF14* comparativamente com as restantes linhas. Todos os genes identificados como estando mais expressos nas linhas RNAi::*OsPIF14* mostram ter pelo menos um elemento de resposta no seu promotor ao qual o OsPIF14 se liga, indicando que podem ser reprimidos directamente pelo OsPIF14. Esses genes estão maioritariamente relacionados com o metabolismo de lípidos e estrutura e organização da parede celular, sugerindo que o OsPIF14 possa estar envolvido no crescimento e elongamento celular.

Este trabalho fornece novos dados sobre a função do OsPIF14 em arroz, mais especificamente, na regulação de genes envolvidos no frio, ácido jasmonico e luz. Os nossos resultados mostram claramente que o OsPIF14 tem o potencial de interligar diferentes estimulos ambientais e, no futuro, a análise de plantas de arroz transgénicas será importante para perceber melhor a função biológica do OsPIF14 na relação luz/JA/frio.

Table of Content

Chapter 1

General Introduction and Research Objectives

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1. The importance of rice

Rice (*Oryza sativa* L.) belongs to the family Poaceae (Gramineae) and is one of the world's oldest and most consumed cereals worldwide. It is estimated that more than half of the world population relies on rice to survive, especially in Asia, which accounts for 87% of the global rice consumption (Maclean et al., 2013). Domestication of wild rice is believed to have started about 9,000 years ago in the middle Yangtze and upper Huai rivers of China (Molina et al., 2011; Maclean et al., 2013). Nowadays, rice is produced worldwide, in a wide range of locations and under a variety of climatic conditions, from the wettest areas in the world to the driest deserts, except in Antarctica, where no crops are grown (Maclean et al., 2013). The highest rice yields have traditionally been obtained in high-latitude areas that have long day length and where intensive farming techniques are practiced or in low-latitude areas that have high solar radiation (Maclean et al., 2013). This data clearly show the importance of light, namely the intensity and day length, in rice productivity. Rice cultivars can be divided into two major groups, Asian rice, *Oryza sativa*, cultivated worldwide, and the African rice, *Oryza glaberrima*, cultivated in West Africa. In addition, *Oryza sativa* can be subdivided into two subspecies, *indica* and *japonica,* which is believed to be the result of independent domestication in India and China, respectively (Gross and Zhao, 2014). *Indica* varieties are grown throughout the tropics and subtropics. Traditionally, they have a tall stature, weak stem, droopy leaves and long grains. These varieties' grains are drier and flakier when cooked due to their higher amylose content. On the other hand, *Japonica* varieties have a short and erect stalk with round grains. These varieties grow in cooler zones of the subtropics and the temperate zones, the grain has low amylose content, making them moist and sticky when cooked (Maclean et al., 2013).

In Europe, around 80% of rice production takes place in Italy, Spain, and Russia, with a further 10% in Greece and Portugal (Maclean et al., 2013). The production yield (t/ha) in Europe is higher than in the rest of the world (6t/ha compared to 4.37t/ha, respectively) being Greece and Spain the most productive countries (Maclean et al., 2013). In 2010, Europe produced 4,319 million tons of

rice. However, this was not enough to overcome the demand, and 1,400 million tons still had to be imported. In Europe and on average, rice consumption reaches 5.2kg/capita/year, which is approximately 10% of the world average. This value is only overcome by countries such as Greece, Spain, and Portugal with 7.1, 11.5 and 14.8kg/capita/year, respectively. In Europe, *Japonica* is the most cultivated rice, however long *indica*-type grain varieties are developing a new market niche as a gourmet food. According to the report "EU RICE ECONOMIC FACT SHEET" published in 2015 by European Commission, the import of Basmati rice variety already represented 30% of total Europe rice imports. This trend appears to be related to the increased mobility of immigrants from Southeast Asia, who introduced aromatic rice, into markets (Maclean et al., 2013).

Global rice consumption is expected to increase during the next years along with the world population growth. Due to the pressure on rice lands from urbanization in the developing world, climate changes, and competition from other high-value crops, the land for rice cultivation is decreasing. It is therefore urgent to increase the actual yield of rice production. It has been predicted that, to feed the still-growing world population and keep the prices affordable, rice yield needs to grow by 1.0–1.2% annually beyond 2020 (Maclean et al., 2013). Therefore, it is highly important to develop rice varieties with higher yield and more resistant to biotic and abiotic stresses. To achieve this goal, we need to understand better the mechanisms by which rice plants regulate their growth, development and how they cope with adverse environmental conditions.

2. Rice as a model plant

Arabidopsis is well established as the model for plant biology research, however, since dicotyledons differ in many aspects of development from the main cereal crops (rice, maize, and wheat) (Izawa and Shimamoto, 1996), a model plant for cereals is needed. Rice is a diploid species (2n=24), which has a relatively short life cycle (3-6 months), a fully sequenced and relative small genome size (~390Mb), especially when compared to other monocots such as maize or wheat. Thus, rice has emerged as a model plant for monocots due to the increased number

of molecular tools available. The number of genetic tools and T-DNA mutant lines available, as well as the high efficiency of rice transformation, has been crucial to making this cereal a model plant (Izawa and Shimamoto, 1996; Shimamoto and Kyozuka, 2002).

3. The importance of light for plant growth and development

Plants, as sessile organisms, cannot move to avoid adverse conditions, but they regulate their growth and development according to the environmental cues. Light is essential for plants, not only as an energy source for photosynthesis but also as an external signal that modulates gene expression and consequent plant growth and development. For instance, under constant dark, plant growth processes are constantly stimulated in the attempt to rapidly reach the sunlight. This process is called skotomorphogenesis and since it is a heterotrophic process, it can only be sustained for a short period. In Arabidopsis, this process is characterized by plants with long hypocotyl, closed cotyledons, apical hook formation to protect leaves before they reach soil surface, and inhibition of chlorophyll synthesis. After light stimuli, there is a reprogramming of processes, including gene expression, leading the switch from heterotrophic to autotrophic growth, a process called photomorphogenesis. Apical hook opens, cotyledons become green, and the photosynthesis process starts. Autotrophic plants can sense light intensity and wavelength. Blue, red and far-red are the wavelengths most perceived by the leave photoreceptors. For instance, under the shade of a competitor plant, the shorter plant receives the light that is filtered by the leaves of the taller plant. Therefore the shorter plant perceives the filtered light as a signal to induce growth and development. Typically this filtered light has low red/far-red ratio and induce stem elongation, suppress lateral development, such as leaves and branches, and accelerate flowering, a process known as shade avoidance response (Björn, 2015; Zdarska et al., 2015). Moreover, some plants, such as lettuce do not germinate unless they are exposed to light. Red is the essential wavelength, but germination can be prevented if seeds are exposed to far-red after the red (Björn, 2015). In this context, light plays an important role during whole plant life cycle, from seed

germination to flowering. In addition, plant response to biotic and abiotic stress is also dependent on the light. For instance, FR light alters the expression of herbivore-induced genes, increasing the performance of herbivore attack (Izaguirre et al., 2006), and Arabidopsis show increased tolerance to cold at short days as compared to long days (Lee and Thomashow, 2012).

4. Plant photoreceptors

Plants can perceive sunlight wavelength from UV to far-red through specific photoreceptors, such as UVR8, phototropins, cryptochromes, and phytochromes. Each photoreceptor includes a specific chromophore responsible for light absorption except for UVR8 in which the wavelength is absorbed by tryptophan residues (for more details about plant photoreceptor structure consider the reviews (Möglich et al., 2010; Jenkins, 2014)).

4.1. UV Resistance locus8

UV Resistance locus8 (UVR8) is a UV-B light (280 to 315 nm) receptor. The *UVR8* encodes a seven-bladed β-propeller protein that forms homodimer (Fig. 1). The homodimer structure is stabilized by the aromatic and charged amino acids that are present in the interface between the two monomers. After 1h of UV-B light exposure, the dimer structure is completely dissociated in two monomers. This process can be reverted by dark with the same rate. In contrast to the other photoreceptors, UVR8 does not have a prosthetic chromophore group. Tryptophan (trp) residues absorb UV-B light and fourteen trp residues were identified in UVR8 structure, six in the β-propeller and seven in the interface between monomers (Fig. 1). The mechanism of UVR8 photoreception is not completely understood, but it is known that these trp residues are involved in monomer and homodimer stability. Mutation of trp amino acids present at the β-propeller results in unstable or non-functional UVR8 receptor, while mutation of trp amino acids present in the interface between monomers affects the conversion from dimer to monomer imposed by UV-B light.

Given that UV light has the potential to damage molecules, such as DNA, plants developed systems to cope with UV light. The Arabidopsis UVR8 regulates the transcription of genes associated with the prevention and repair of UV damage, including those involved in flavonoid biosynthesis, DNA repair, and the amelioration of oxidative damage. The mechanism of action of UVR8 is poorly understood, however, after UV-B light exposure, UVR8 accumulates in the nucleus. UVR8 is able to interact with constitutively photomorphogenic 1 (COP1) that also accumulates in nucleus after UV light exposure. *cop1* mutants showed impaired expression of the same genes regulated by UVR8 under UV light, suggesting that COP1 and UVR8 act together to mediate the photomorphogenic UV-B response. This function of COP1 contrasts with the well established function of repressor of photomorphogenesis (discussed in section 5), these results show that COP1 can have a dual function or other factors can interact with COP1 to regulate photomorphogenesis. For more details about structure and biological function of UVR8 please consider the review (Jenkins, 2014).

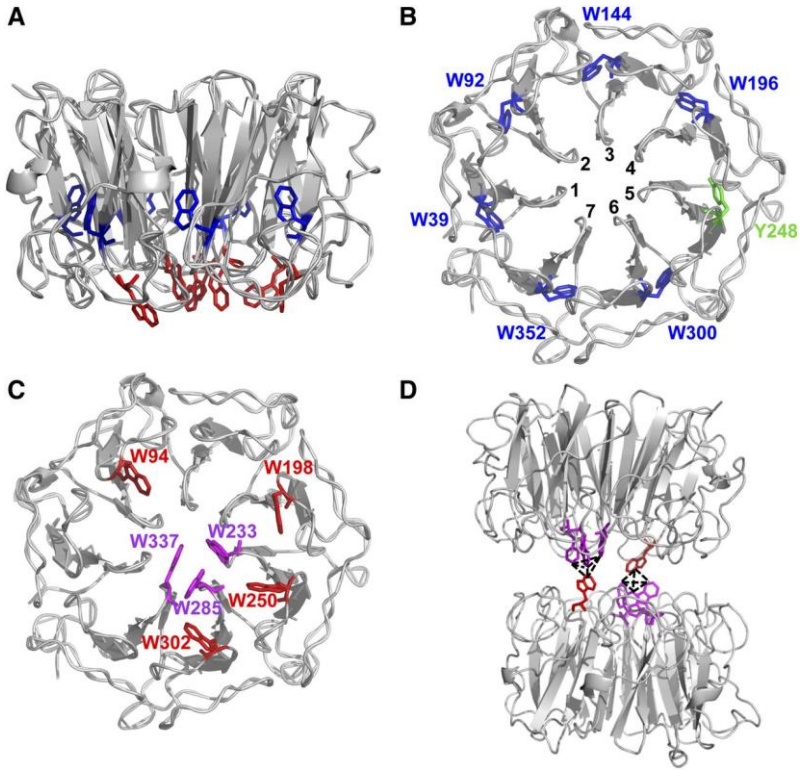

Figure 1 (from (Jenkins, 2014)) Tryptophan (W) residues in UVR8 protein. A. The arrangement of all w residues, except the w400, in monomer viewed from the side. B. The arrangement of the core w residues in dimer viewed from the top. Each w is associated with a different β-propeller and y248 closes the aromatic ring. C. The arrangement of the interface w residues viewed from the top. D. Electrostatic forces between the w residues from the two monomers. Blue w residues belong to the core protein, red and purple to the. Purple w residues are considered the triad and are fundamental for dimerization.

4.2. Phototropins

Phototropins are UV-A and blue light receptors constituted by a photosensitive N-terminal and a serine/threonine kinase domain (KD) (Fig.2) (Briggs et al., 2001). The N-terminal, comprises two LOV (light, oxygen, or voltage) domains, each bound to a flavin mononucleotide (FMN) molecule. Dark-adapted LOV domains absorb maximally near 447nm. After irradiation, there is a conformational change in phototropin structure allowing the formation of a covalent bond between the FMN and a cysteine of the LOV domain. This reaction occurs in microseconds and the bioactive molecule, which absorbs maximally near 390nm, is formed (Briggs et al., 2001; Christie, 2007). Therefore, phototropins are able to perceive UV-A and blue light and play a role in regulating light-dependent processes that are important for photosynthesis and plant growth. In Arabidopsis, two genes encoding phototropins were identified, *PHOT1* and *PHOT2* which have been associated with root and hypocotyl phototropism (Sakai et al., 2001), stomatal opening (Kinoshita et al., 2001), chloroplast leaf movement/accumulation (Sakai et al., 2001) and leaf expansion (Sakamoto and Briggs, 2002).

4.3. Cryptochromes

Cryptochromes are UV-A and blue light photoreceptors, which show a peak of absorption near 450nm. These photoreceptors are constituted by two domains, the N-terminal PHR (Photolyase Homologous Region) and the C-terminal CCT domain (Cryptochrome C-Terminal extension) (Möglich et al., 2010; Liu et al., 2011b). In Arabidopsis, three genes encoding cryptochromes were identified. Cryptochromes 1 and 2 (*AtCRY1* and *AtCRY2*) are flavoproteins whose

photosensory domain is similar to DNA photolyases but lack their DNA repair activity (Li and Yang, 2007). The third cryptochrome identified, AtCRY3, belongs to a different class of cryptochromes, the Drosophila, Arabidopsis, Synechocystis, and Homo cryptochrome (CRY-DASH) (Brudler et al., 2003). CRY-DASH can bind DNA and act in transcriptional regulation, but its function as a photoreceptor is not clear (Brudler et al., 2003; Wang et al., 2015). AtCRY3 lacks the cryptochrome c-terminal domain and has a signal peptide that directs it into the mitochondria and chloroplast (Kleine et al., 2003).

The cryptochromes absorb light through their flavin adenine dinucleotide (FAD) chromophore. Blue light reduces the oxidized ground state of FAD forming the active signaling state, the radical $FADH^{\cdot}$. The green light wavelength can reduce further the radical conformer forming $FADH^{-}$, this reduced form abrogates the light signal. FAD is fully oxidized from $FADH^{-}$ after a fixed dark period (Fig.2) (Banerjee et al., 2007; Bouly et al., 2007). Moreover, it has been shown that blue light, in opposition to red and far-red, induces cryptochromes phosphorylation. The phosphorylation seems to be a signaling mechanism that is important for the function and regulation of cryptochromes activity (Shalitin et al., 2002; Shalitin et al., 2003). Arabidopsis CRY1 is nuclear and cytoplasmatic (Guo et al., 1999; Wu and Spalding, 2007), while CRY2 is exclusively nuclear (Guo et al., 1999). Together with PHOT1 and PHOT2, CRY1 and CRY2 regulate hypocotyl bending (Ohgishi et al., 2004) and stomatal opening (Mao et al., 2005). Moreover, it was shown that cryptochromes act together with phytochromes to regulate hypocotyl growth, chlorophyll accumulation, cotyledon expansion, anthocyanin accumulation (Neff and Chory, 1998), and flowering time (Guo et al., 1998). In rice, three cryptochromes, OsCRY1a, OsCRY1b, and OsCRY2, were identified (Hirose et al., 2006). OsCRY1a and OsCRY1b are homologous to AtCRY1 and when overexpressed in Arabidopsis are localized in the nucleus, inhibit hypocotyl growth, and induce accumulation of anthocyanin under blue light (Matsumoto et al., 2003). Moreover, OsCRY1a/b are responsible for the blue-light de-etiolation response in rice, while OsCRY2 is involved in the promotion of flowering time (Hirose et al., 2006). Furthermore, neither the

expression nor transcript stability of *OsCRYs* is affected by light, however, OsCRY2 protein is degraded by light in a phytochrome B-dependent manner (Hirose et al., 2006).

4.4. Phytochromes

Phytochromes are the plants red/far-red light photoreceptors (Fankhauser, 2001; Takano et al., 2009). Phytochromes (phys) are holoproteins constituted by the chromophore group, a linear tetrapyrrole phytochromobilin, bound to a cysteine in the N-terminal of the phytochrome apoprotein (PHY) (Rockwell et al., 2006). The PHY is constituted by two major domains. The photosensory core domain, and the HKRD (histidine kinase-related domain) localized at the c-terminal. The photosensory core domain can be further divided into three domains, the PAS (Per, ARNT, Sim) domain, the GAF (cGMP phosphodiesterase/adenyl cyclase/FhlA) domain and the PHY (phytochrome-specific) domain (Fig.2). Phys are synthesized in the cytosol in the inactive form (Pr), which has a maximum absorption in red light (near 660nm). After red light absorption, phytochrome conformation changes and the nuclear localization signal is exposed (Chen et al., 2005). The active phytochrome form (Pfr) is translocated to the nucleus (Huq et al., 2003; Fankhauser and Chen, 2008), where it interacts with nuclear proteins, such as Phytochrome-Interacting Factors (PIFs) (Ni et al., 1998), promoting PIF phosphorylation and degradation via the proteasome (Al-Sady et al., 2006; Shen et al., 2007; Ni et al., 2013). Pfr is rapidly converted back to Pr by far-red light (~740nm) or more slowly by dark in a process called dark reversion. In this context, the binding to PIFs is lost, and PIFs can regulate gene expression (Ni et al., 1999).

In Arabidopsis, five phytochromes (phyA – phyE) were identified (Kircher et al., 2002). These are divided into two groups, light labile (phyA) and light stable (phyB – phyE) (Hirschfeld et al., 1998), however even the light stable phyB is degraded after a period of red light in a process dependent of PIFs (Jang et al., 2010). The characterization of single and multiple phytochrome mutants showed that phytochromes have distinct roles (Reed et al., 1994; Neff and Chory, 1998).

For instance, phyA promotes while phyB inhibits germination under far-red light and phyA inhibits hypocotyl growth under far-red while phyB inhibits under red light (Reed et al., 1994). However, phyA and phyB also have overlapping functions in the de-etiolation under red light, such as cotyledon development and hook opening (Reed et al., 1994) or under white light, for instance in the inhibition of hypocotyl elongation or increasing chlorophyll content (Neff and Chory, 1998). These functions are due to the characteristics of each phytochrome to perceive light wavelength and fluence (radiation incident per unit surface area per unit time). The very low fluence responses (VLFR) are initiated after perception of intensities as little as 1nmol m^{-2}. Low fluence responses (LFRs) occur in the range of 10–1000 μmol m^{-2}, while high irradiance responses (HIRs) require continuous light with a total fluence typically in excess to 10 mmol m^{-2}. Interestingly, only phyA perceives very low fluence rate wavelengths from 300nm to 780nm and the FR-HIR light to induce germination (Shinomura et al., 1996; Shinomura et al., 2000), this may be a mechanism of seeds to perceive light when they are under the soil. On the other hand, the classic red/far-red LFR is mediated by all phytochromes (Rockwell et al., 2006). In addition, Devlin and Kay have reported that within red light wavelength phytochromes are able to distinguish fluence rate, being phyA more important for low fluence rate while phyB is more important for high fluence rate (Devlin and Kay, 2000). In this context, it is established that phyA is more important for far-red and very low intensities responses and phyB is more important for red light responses. The other phytochromes seem to play a secondary role in light perception by acting together with phyA and phyB in light responses. In rice, three phytochromes (phyA – phyC) were identified (Takano et al., 2005), being the sole red/far-red photoreceptors (Takano et al., 2009). The use of single and multiple phytochrome mutants have shown that rice and Arabidopsis phytochromes share similar functions. For instance, the inhibition of rice coleoptile growth due to the VLFR is mediated by phyA (Takano et al., 2001) while the LFR is mediated primarily by phyA for far-red and phyB for red light. However, double mutants show that all phytochromes act together to regulate coleoptile growth (Takano et al., 2005). Moreover, it was shown that rice

phytochromes play an important role in the regulation of seminal root elongation (Shimizu et al., 2009), being phyA responsible for far-red inhibition and phyA and phyB for red light inhibition. Furthermore, it was shown that phyB delays flowering time under long (14L/10D) and short days (10L/14D). However, phyC also delays flowering time under long days, while phyA acts synergistically with phyB and phyC to regulate flowering time under long days (Takano et al., 2005).

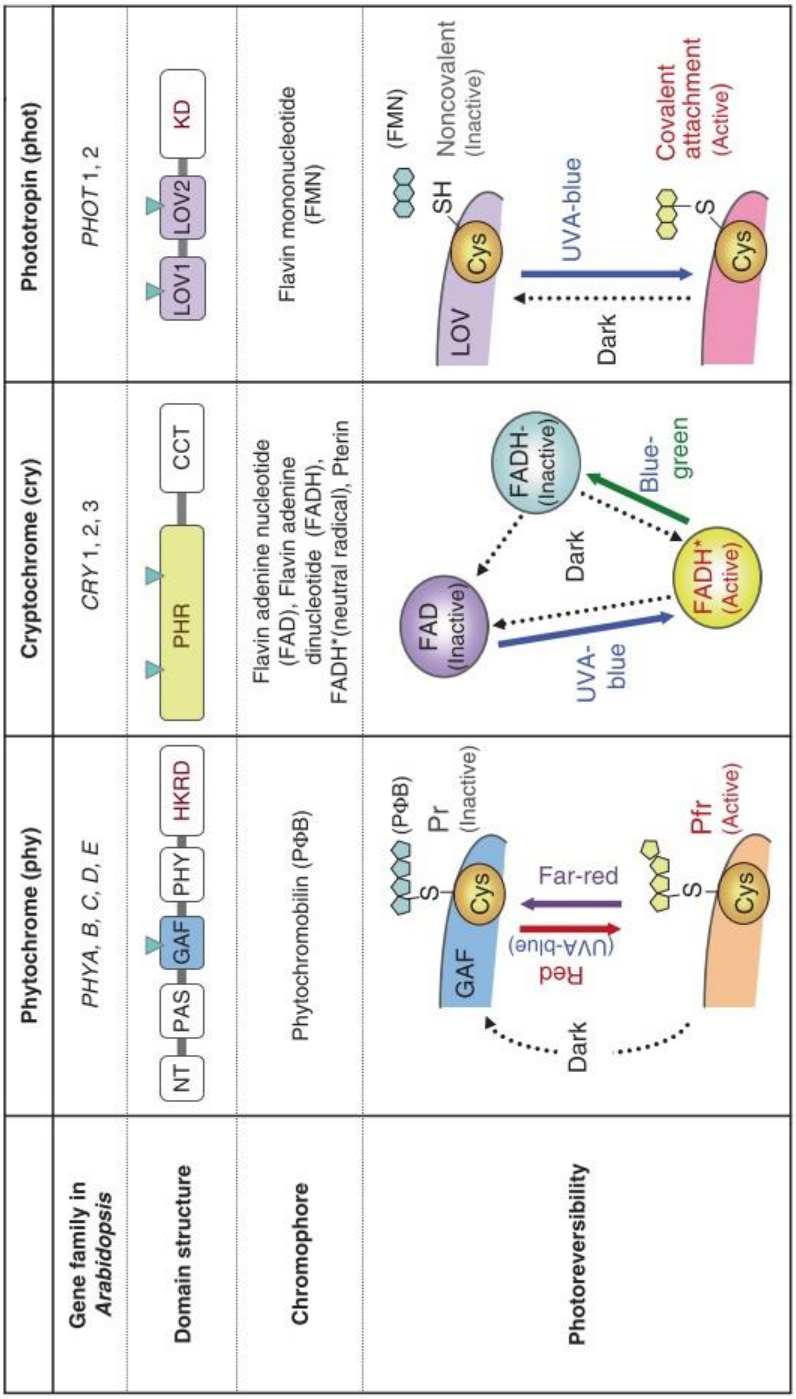
Phytochrome (phy)
Cryptochrome (cry)
Phototropin (phot)
Gene family in Arabidopsis
PHYA, B, C, D, E
CRY 1, 2, 3
PHOT 1, 2
Domain structure
NT
PAS
GAF
PHY
HKRD
PHR
CCT
LOV1
LOV2
KD
Chromophore
Phytochromobilin (PΦB)
Flavin adenine nucleotide (FAD), Flavin adenine dinucleotide (FADH), FADH*(neutral radical), Pterin
Flavin mononucleotide (FMN)
Photoreversibility
(PΦB)
Pr
(Inactive)
S
Cys
GAF
Far-red
Red
(UVA-blue)
Dark
Pfr
(Active)
FAD
(Inactive)
FADH-
(Inactive)
Blue-
green
UVA-
blue
FADH*
(Active)
(FMN)
SH
Noncovalent
(Inactive)
LOV
UVA-blue
Dark
Covalent
attachment
(Active)

Figure 2 (adapted from (Kami et al., 2010; Möglich et al., 2010)**).** Schematic representation of the main plant photoreceptors. Arabidopsis has five genes encoding phytochromes (*phyA-E*), three encoding cryptochromes (*cry1-3*) and two encoding phototropins (*phot1-2*). The domains of each photoreceptors group are schematically represented by the position of the chromophore attachment marked with an arrowhead. Phytochromes have an NT (N-terminal) extension followed by the three domains that yield the photosensory core; the PAS (Per, ARNT, Sim) domain, the GAF (cGMP phosphodiesterase/adenyl cyclase/FhlA) domain and the PHY (phytochrome-specific) domain and the C-terminal that comprises the HKRD (histidine kinase-related domain). Cryptochromes are composed of a PHR (photolyase homology region) and a CCT (cryptochrome c-terminal extension) domain. Phototropins have two LOV (light, oxygen, voltage) domains in their N-terminal and a Ser/Thr protein KD (kinase domain). Phytochromes have the linear tetrapyrrole phytochromobilin (PΦB) as a chromophore which is covalently bound to a Cys residue in the GAF domain. Red and far-red light switch the PΦB between the Pfr and the Pr conformers upon isomerization of a double bond between the third and the fourth ring of the tetrapyrrole. Cryptochromes have two chromophores; the FAD (Flavin Adenine nucleotide) and the pterin acting as an antenna pigment. After UV-A/blue light absorption FAD is reduced to the active form, the radical FADH, which is converted back to the full oxidized form (FAD), via blue/green light through the formation of $FADH^-$ or directly via dark reversion. Phototropins use FMN (flavin monocleotide) as a chromophore. Under dark, FMN is bounded noncovalently to each of the LOV domains. After absorption of UV-A/blue light FMN binds covalently to a Cys of the LOV domain.

5. Light signal transduction: the Phytochrome-Interacting Factors perspective

Given that light is essential for plant growth and development, the photoreceptors are key elements for plants. A large number of elements acting downstream of the photoreceptors have been identified. However, we will focus on phytochrome interacting factors (PIFs). PIFs are getting more attention from researchers due to their ability to integrate environmental and internal signaling. Here, we will present and discuss the several layers of light signal transduction, focusing on the importance of PIFs for plant growth taking into consideration that they do not act alone and can interconnect light, hormones, biotic, and abiotic stress. Most of the work performed to characterize the biological function of PIFs has been carried out in Arabidopsis, and we will present it here. In section 6, we will describe what is known about the rice PIFs.

5.1. PIFs subfamily

In Arabidopsis, 162 basic/helix-loop-helix (bHLH) transcription factors (TFs) were identified (Toledo-Ortiz et al., 2003) and 15 constitute the PIF subfamily (Leivar and Quail, 2011). PIF3 was the first PIF identified in Arabidopsis and received its name due to the interaction with phytochrome A and B C-terminal domain (Ni et al., 1998). Among the 15 bHLH proteins that compose the PIF subfamily, seven were proven to bind to phytochromes in Arabidopsis (AtPIF1, 3, 4, 5, 6, 7, and PIL1) (Table1) (Leivar and Quail, 2011; Luo et al., 2014). Therefore, PIFs were associated with red/far-red light perception and signal transduction, playing a central role in phytochrome signaling. PIFs are constituted by the active phytochrome B-binding (APB) and a conserved basic/helix-loop-helix (bHLH) domain. Interestingly, only PIF3 and PIF5 contains the active phytochrome A-binding (APA) site between APB and bHLH domains (Leivar and Quail, 2011). The bHLH domain contains approximately 60 amino acids divided into two domains, the basic domain, and the helix-loop-helix (HLH) domain. The basic domain is constituted by approximately 15 amino acids with the overall basic charge being fundamental for DNA binding. The HLH is constituted by two amphipathic α-helices separated by a loop allowing the formation of homo- or hetero-dimers between bHLH TFs (Toledo-Ortiz et al., 2003). Other bHLH members of the PIF subfamily either do not bind to phytochromes or this interaction was not proven. For instance, the long hypocotyl in far-red 1 (HFR1) interacts with PIFs and inhibit their capacity to bind to DNA showing that the regulation of PIF subfamily proteins is complex and not only dependent on phytochromes (Hornitschek et al., 2009).

The study of *pif* single and multiple mutants has been fundamental to unveil PIFs biological role. These studies have shown that PIFs have distinct functions (Table 1). For instance, PIF1 acts as a principal regulator of seed germination under dark, PIF3 is crucial for ethylene-induced hypocotyl elongation, PIF4 is the major regulator of high-temperature responses, and PIF7 is the major regulator of auxin biosynthesis in shade conditions. However, PIFs also show overlapping functions, for instance, PIF4 and PIF7 showed to regulate cold acclimation, PIF4

and PIF5 act together to regulate blue light-induced hypocotyl elongation and PIF1, PIF3, PIF4, and PIF5 act synergistically to regulate skotomorphogenesis (long hypocotyls, agravitropic growth of hypocotyl, apical hook formation, and the inhibition of cotyledon opening under dark) (Table 1).

5.2. PIFs transcriptional regulation

PIF genes are expressed differently during seed and plant development (Jeong and Choi, 2013). *PIF1, PIF4, PIF5* and *PIF7* show similar expression being more expressed in seedling and leaf as compared to root, flower, and fruit. *PIF3* and *PIF8* are less expressed in roots showing similar expressed in seedling, leaf, flower, and fruit. *PIF6* is more expressed in flower and fruit as compared to the other developmental stages (Jeong and Choi, 2013). Moreover, during seed maturation, *PIF6* is the most expressed *PIF* (Jeong and Choi, 2013), being consistent with its observed role in seed dormancy (Table 1). Taken together, these results are consistent with the observed synergistic role of PIFs on the regulation of skotomorphogenesis, but also the distinct function of PIF6 in the regulation of seed dormancy (Table 1). In rice, all the *PIFs/PILs* show higher expression in leaf (mature and young) suggesting that rice and Arabidopsis PIFs/PILs might have similar functions (Jeong and Choi, 2013)

The expression of *PIFs* is also regulated by light. Etiolated Arabidopsis exposed to white light shows induction of both *PIF4* and *PIF5* and rapid repression of *PIL1* (Yamashino et al., 2003). PHOT1 and PHOT2 are negative regulators of the blue light-induced expression of *PIF4* and *PIF5* (Sun et al., 2013). These results are consistent with the role of PIF4 and PIF5 on the regulation of blue light-induced hypocotyl growth (Table 1). Moreover, it was shown that the expression of both *PIF4* and *PIF5* is regulated by the circadian rhythm. Both *PIFs* show a peak of expression after dawn (Yamashino et al., 2003) that is maintained independently of the day length (short (8h/16h) or long (16h/8h) days) (Nomoto et al., 2012).

It was demonstrated that the evening complex (EC), formed by three proteins (early flowering 3 (ELF3) and 4 (ELF4) and the transcription factor lux arrhythmo

(LUX)) represses the expression of *PIF4* and *PIF5*. LUX binds to the LUX binding site (LBS; GATWCG) in the promoter of both PIFs and ELF3, and ELF4 are recruited to reconstitute the evening complex (Nusinow et al., 2011). The EC is diurnally regulated and peaks at dusk, therefore, *PIF4* and *PIF5* expression is repressed in the early evening (Nusinow et al., 2011).

In addition, it has been reported that *PIFs* are also regulated by alternative splicing. *PIF6* expression increases during seed maturation, having a peak at the stage of dry seed. During this process, an alternative splicing form is formed (*PIF6-β*). *PIF6-β* encodes a truncated protein with intact N-terminal but without the bHLH domain. Interestingly, Arabidopsis plants overexpressing *PIF6-β* showed increased germination frequency by an unclear mechanism (Penfield et al., 2010).

PIF expression was also shown to be regulated by internal and external stimuli (hormone, nitric oxide and abiotic stress) (Jeong and Choi, 2013). ACC (ethylene precursor) induces *PIF3* expression and repress *PIF4*. It was shown that the Ethylene-Insensitive 3 (EIN3) TF directly binds to the specific EIN3 Binding Site (EBS; CTCTGC)) elements in the *PIF3* promoter to activate its transcription. Since the hypocotyl elongation in the *pif3* mutant was insensitive to ACC, PIF3 seems to be an essential component required for ethylene-induced hypocotyl elongation in light (Zhong et al., 2012). Other hormones, such as brassinosteroids, auxins and jasmonate were shown to repress the expression of *PIF5*, *PIF4*, and *PIF8*, respectively (Jeong and Choi, 2013). The crosstalk between light/PIFs and hormones will be addressed later on.

In addition to light and phytohormones, PIF genes are also modulated by Nitric oxide (NO), which was reported to repress PIF gene expression. NO-deficient mutants show longer hypocotyls under red light, but not under blue or far-red and showed enhanced expression of *PIF1*, *PIF3*, and *PIF4*, suggesting that NO might regulate hypocotyl by down-regulating *PIF* expression. Consistent with this hypothesis, the quadruple *pif* mutant (*pifq*; *pif1*, *pif3*, *pif4*, and *pif5*) showed to be insensitive to NO-triggered hypocotyl shortening (Lozano-Juste and León, 2011). Moreover, PIF3 was shown to induce the expression of PIL1 and PIF6 showing a

complex regulatory feedback mechanism (Leivar and Monte, 2014; Soy et al., 2016).

Abiotic stresses, such as high salinity (200mM NaCl), cold (8ºC), and heat (30ºC), were also shown to regulate *PIF* gene expression. Briefly, NaCl induces the expression of *PIF6*, while cold and high temperature induce the expression of *PIF1* and *PIF4* (Jeong and Choi, 2013). In rice, 3h of abiotic stress alter *PIFs* gene expression (Jeong and Choi, 2013). Drought and 200mM NaCl induce *OsPIL11* and *OsPIL15* and repress *OsPIL13*, while cold (4ºC) and heat (42ºC) repress *OsPIL13* and *OsPIL14*, respectively. The crosstalk between PIFs and low temperature will be discussed later on.

The regulation of *PIFs* transcript level seems to be important for the regulation of some particular processes in which PIFs are involved. However, the study of PIF protein regulation, especially its stability, interaction with other proteins, and competition to bind DNA, has been shown to play a critical role in the mechanisms by which PIFs regulate plant development and growth.

Table 1. Phytochrome interaction, degradation and biological role of Arabidopsis and rice PIFs/PILs

Species	PIF/PIL	Accession number[a]	Interaction		PIF/PIL degradation[b]	Biological role[c]		
			phyA	phyB		Hypocotyl elongation	Shade avoidance	Other
Arabidopsis	PIF1/PIL5	AT2G20180	Yes [1]	Yes [1]	FR and R [2]	Promote [1]		Inhibit seed germination [1]; neg. regulate chlorophyll biosynthesis [3]; skotomorphogenesis [4]
	PIF3	AT1G09530	Yes [5]	Yes [5]	FR and R [6,7]	Promote [8]		Ethylene-induced hypocotyl elongation [9]; neg. regulator chlorophyll biosynthesis [4]; skotomorphogenesis [4]
	PIF4	AT2G43010	No [10]	Yes [10]	R [11]	Promote [12]	Promote [13]	High temperature response [e,14,15,16]; light and hormone cross-talk [17]; blue light-induced hypocotyl growth [18]; cold acclimation [19]; skotomorphogenesis [4]
	PIF5/PIL6	AT3G59060	No [20]	Yes [20,21]	R [11,20]	Promote [22,23]	Promote [13]	Chlorophyll degradation and senescence [24]; blue light-induced hypocotyl growth [18]; flowering time [21] skotomorphogenesis [4]
	PIF6/PIL2	AT3G62090		Yes [21]		Inhibit [25]		Seed dormancy [25]
	PIF7	AT5G61270	No [26]	No [26]	No [26]	Promote [26]	Promote [27]	Affects positively shade-induced auxin biosynthesis [27]; cold acclimation [19,28]
	PIL1	AT2G46970		Yes [29]	Accumulation [29]	Inhibit [29]		Circadian gating of the shade-avoidance hypocotyl elongation [30]

Table 1 (continued)

Species	PIF/PIL	Accession number[a]	Interaction		PIF/PIL degradation[b]	Biological role[c]		
			phyA	phyB		Hypocotyl elongation	Shade avoidance	Other
Rice	OsPIL11	LOC_Os12g41650				Promote (Arabidopsis) [31]		Inhibit hypocotyl elongation and promote cotyledons and leaves growth under red (tobacco) [32]
	OsPIL12	LOC_Os03g43810						
	OsPIL13	LOC_Os03g56950		No [33]				Promote internode elongation under drought [33]
	OsPIL14	LOC_Os07g05010						
	OsPIL15	LOC_Os01g18290						Inhibit seedling growth under dark, regulate auxin pathway and suppress cell wall organization [34]
	OsPIL16	LOC_Os05g04740		Yes [35]				Increase cold tolerance [35]

Definitions:

[a] Accession number (Nakamura et al., 2007; Leivar and Quail, 2011)

[b] Degradation under far-red (FR) or red (R)

[c] List of references: 1. (Oh et al., 2004); 2. (Oh et al., 2006); 3. (Huq et al., 2004); 4. (Leivar et al., 2008b; Shin et al., 2009); 5. (Ni et al., 1998); 6. (Bauer et al., 2004); 7. (Al-Sady et al., 2006); 8. (Kim et al., 2003); 9. (Zhong et al., 2012); 10. (Huq and Quail, 2002); 11. (Lorrain et al., 2008); 12. (Franklin et al., 2011); 13. (Lorrain et al., 2008; Hornitschek et al., 2009); 14. (Sun et al., 2012); 15. (Franklin et al., 2011); 16. (Kumar et al., 2012); 17. (Choi and Oh, 2016); 18. (Pedmale et al., 2016); 19. (Lee and Thomashow, 2012); 20. (Shen et al., 2007); 21. (Khanna et al., 2004); 22. (Fujimori et al., 2004); 23. (Khanna et al., 2007); 24. (Zhang et al., 2015); 25. (Penfield et al., 2010); 26. (Leivar et al., 2008a); 27. (Li et al., 2012a); 28. (Kidokoro et al., 2009); 29. (Luo et al., 2014); 30. (Salter et al., 2003); 31. (Nakamura et al., 2007); 32. (Li et al., 2012b); 33. (Todaka et al., 2012); 34. (Zhou et al., 2014); 35. (He et al., 2016).

[d] Skotomorphogenesis includes agravitropic growth of hypocotyl, apical hook formation, and the inhibition of cotyledon opening

[e] Auxin biosynthesis, hypocotyl growth, flowering time regulation under high temperature

5.3. Involvement of PIFs in seedling skotomorphogenesis and de-etiolation

In the absence of light-activated phytochromes, PIFs accumulate in the nucleus and bind to *cis*-elements type E-box (CANNTG), more specifically to G-box (CACGTG) or PIF-binding E-box (PBE; CACATG), to regulate genes involved in skotomorphogenesis (Zhang et al., 2013). PIF target genes were shown to be regulated by one or more PIFs, showing that PIFs can have distinct and overlapping functions (Table 1) (Leivar et al., 2008b; Zhang et al., 2013). The study of *pif*-quadruple mutant (*pifq*) lacking four PIFs (*PIF1*, *PIF3*, *PIF4*, and *PIF5*) has been critical to unveil the overlapping function of PIFs and better understand the role of PIFs in skotomorphogenesis. The *pifq* mutant, grown under dark, phenocopies WT plants grown under red light, displaying shorter hypocotyls, disrupted hypocotyl gravitropism and open cotyledons (Leivar et al., 2009; Shin et al., 2009). Interestingly, 80% of the genes that are misregulated in *pifq* mutant grown under dark are regulated by red light in the wild type (Leivar et al., 2009; Shin et al., 2009). These results show the importance of light in gene regulation, more specifically the crucial role of PIFs promoting skotomorphogenesis. Thus, the characterization of PIFs function and regulation mechanisms is important to better understand the effects of light on plant growth. Under dark, the Arabidopsis mutants, *constitutively photomorphogenic 1* (*cop1*) and the suppressor of phytochrome A -105 (SPA) triple mutant *spa1spa2spa3* (Leivar et al., 2008b) show a photomorphogenesis phenotype. Both mutants show lower levels of PIF3 (Bauer et al., 2004; Leivar et al., 2008b) and possible other PIFs, indicating that COP1 and SPA act together to stabilize PIF proteins, which could in part explain this phenotype. Moreover, COP1 decreases the activity of the bZIP TF long hypocotyl 5 (HY5), a potent PIF antagonist (Toledo-Ortiz et al., 2014). HY5 binds to DNA *cis*-elements, such as G-box and compete with PIFs for gene regulation (Lee et al., 2007). COP1, as an E3 ubiquitin ligase, can phosphorylate HY5 and decrease its ability to bind DNA (Hardtke et al., 2000). Therefore, the fluctuation of HY5 and PIF protein levels along the photoperiod is crucial to regulate gene expression, such as those of the chlorophyll biosynthetic

pathway (Toledo-Ortiz et al., 2014). Moreover, PIF3 was shown to induce the expression of other members of the PIF superfamily, HFR1 and PIL1 (Zhang et al., 2013; Soy et al., 2016) directly. PIL1 is involved in the circadian rhythm regulated growth, showing higher expression in short days at the end of the dark period, when the highest hypocotyl elongation rate is observed (Soy et al., 2016). PIL1 and HFR1 acts in a negative feedback loop to control PIFs activity since they heterodimerize with PIFs (PIL1 interact with PIF1, PIF3, PIF4 and PIF5; HFR1 interact with PIF4 and PIF5) and inhibit their capacity to regulate gene expression (Hornitschek et al., 2009; Luo et al., 2014). Luo and collaborators proposed a mechanism for this regulation. Under dark, COP1 interacts with PIL1 and HFR1 promoting their degradation and stabilization of PIFs, but under light, phytochromes inhibit the interaction COP1-PIL1 and the regulation of PIF targets is decreased (Luo et al., 2014).

After light perception through photoreceptors, the plant de-etiolation process is initiated. Red light activates phytochromes, which are translocated to the nucleus and interact with PIFs. This interaction leads to PIF degradation by the 26S proteasome. At the same time, the CCT domain of the blue light absorbing cry1 interacts with SPA1 and blocks the formation of the complex COP1-SPA1 (Liu et al., 2011a). Consequently, HY5 is not phosphorylated and promotes photomorphogenesis. This mechanism is not that simple, and both, blue and red light signaling pathways can share common processes, as cry1 CCT domain is able to interact with COP1 and phyB (Fig. 3) (Yang et al., 2001). Moreover, it was recently shown that cry1 and cry2 interact with PIFs (PIF4 and PIF5) indicating that PIFs can also be regulated by blue light (Pedmale et al., 2016). In addition to this quick light response, it was also reported that after 8h of exposure to red light, phyB levels decrease to half and continue decreasing after longer periods of exposure (Jang et al., 2010). Interestingly, PIFs and COP1 play an active role in this regulation, since *cop1-4* and *pif* single and double mutants show higher levels of phyB. Jang and collaborators proposed a mechanism of action for this interaction in which COP1 binds to phyB N-terminal and PIFs stabilize this interaction, as a consequence, phyB is degraded. Moreover, despite the fact that

COP1 interact with PIFs, they are not ubiquitinated by COP1, supporting the idea that COP1 participates in PIF stabilization (Jang et al., 2010).

5.4. Perception of internal and external signals by PIFs

Arabidopsis *pifq* mutants show induction of photomorphogenesis in the dark. Transcriptomic studies have shown that the PIF-quartet regulates expression of genes involved in hormone signaling, such as brassinosteroids (BR) and gibberellins (GA) response, Auxin biosynthesis and signaling, ethylene biosynthesis, jasmonic acid metabolism, Cytokinin breakdown and, ABA biosynthesis (Leivar and Monte, 2014). Moreover, PIFs were shown to interact with BR, auxin, and GA signaling proteins and together regulate plant growth (Lucas et al., 2008; Oh et al., 2012; Oh et al., 2014). These observations suggest a close relationship between PIFs and different phytohormones. Therefore PIFs have emerged as important regulators of plant development due to their ability to integrate environmental and internal signals to regulate cell elongation and photomorphogenesis. Several excellent reviews have covered this topic (Leivar and Quail, 2011; Wang et al., 2011; Leivar and Monte, 2014; Salehin et al., 2015; Chaiwanon et al., 2016) and here we will discuss how PIFs are involved in the crosstalk between light and different hormones (auxin, brassinosteroids, gibberellins and jasmonic acid) and also, light and temperature.

5.4.1. Internal signaling (hormones)

Auxin

Auxin response factors (ARFs) are transcription factors that act downstream the auxin signaling to regulate plant growth, such as root and shoot development (Salehin et al., 2015). In the absence of auxins, ARF are bound to their repressors, the F-box proteins auxin/indole 3-acetic acid (Aux/IAA), and therefore not active. When the auxin level increases, auxins bind to the transport inhibitor response1 (TIR1) F-box protein that interacts with aux/IAA. TIR1 is part of an E3 ligase complex, the SCF^{TIR1}, which polyubiquitylates and targets Aux/IAA for degradation. In the absence of Aux/IAA, ARFs are free to regulate growth

(Korasick et al., 2014) (Fig. 3). The crosstalk light/auxin is shown by the Arabidopsis *arf7* mutant, which displays reduced auxin sensitivity in hypocotyl growth and impaired hypocotyl phototropic growth towards blue light (Okushima et al., 2005). This light response was proposed to be mediated by PIFs, as *pif4* and *pif5* single and double mutants showed increased hypocotyl curvature (Sun et al., 2013). In fact, PIF4 directly binds to the G-box of auxin signaling repressors *IAA19* and *IAA29* to activate their expression and both IAA19 and IAA29 interact with ARF7 to repress hypocotyl phototropic response (Sun et al., 2013). On the other hand, PIFs can form heterodimers with ARFs but not all combinations are possible, PIF4 interacts with ARF6 and ARF8 but not with ARF1 or ARF7 (Oh et al., 2014). The analysis of ARF6 ChIP-seq showed high overlap with PIF4 shade-induced genes (Kohnen et al., 2016), showing that PIF4 can act as agonists of auxin signaling.

Brassinosteroids

The brassinazole-resistant 1 (BZR1) and BZR2 transcription factors are the main brassinosteroid (BR) signaling regulators inducing skotomorphogenesis (Wang et al., 2011). The BR signal pathway is complex and involves several kinases and phosphatases to regulate signal transduction, which has been thoroughly reviewed (Wang et al., 2011; Belkhadir and Jaillais, 2015). In the absence of BR, the BR membrane receptor kinase, brassinosteroid-insensitive 1 (BRI1) is inactive and bound to its inhibitor, the BRI1 kinase inhibitor 1 (BRK1). In addition, the brassinosteroid-insensitive 2 (BIN2) is active and interact with BZR1, which is phosphorylated. This phosphorylation promotes the interaction with 14-3-3 proteins that transport BZR1 to the cytoplasm to be degraded via the proteasome (Fig.3).

In the presence of BR, BRI1 dissociates from BRK1 and interact with BRI1-associated receptor kinase 1. Thus, BRI1 is activated by phosphorylation and in turn, phosphorylates BR-signaling kinase 1 (BSK1). This kinase activates the phosphatase BRI1-supressor 1 (BSU1) that dephosphorylates BIN2, thus promoting its degradation by the proteasome. Therefore, BZR1 is rapidly

dephosphorylated by protein phosphatase 2 A (PP2A) and interacts with BR-responsive elements (BRRE; CGTG(T/C)G). Besides the activation of BZR1, PP2A also dephosphorylates BRI1 to switch off BR signaling. Interestingly, BR signaling increases PIF4 protein stability through the inactivation of BIN2 (Wang et al., 2011) (Fig.3).

RNA-seq data of BZR1 and PIFs mutants subjected to brassinosteroid treatment showed that approximately 59% of the genes regulated by brassinosteroids in *bzr1-D* are also regulated in *pifq*. Also, *pifq* mutants showed to be less sensitive to exogenous brassinosteroid application as compared to WT, suggesting that the loss of *PIFs* compromise BR response. Moreover, ChIP-Seq experiments identified that 51.7% of the target genes of PIF4 are also target genes of BZR1 (Oh et al., 2012). The analysis of the promoter of those genes showed that for the majority of them BZR1 and PIF4 bind to nearby or same *cis*-elements. In fact, the same authors show that BZR1 interact with PIF4 and that both co-occupy the same G-box *cis*-element (CACGTG) (Oh et al., 2012). Furthermore, BZR1 and PIF4 directly interact with each other and show synergistic and interdependent relationship in promoting gene expression and etiolation. Genetic analyses demonstrated that both BZR1 and PIF4 are required for cell elongation responses not only to dark but also to high temperature (Oh et al., 2012).

ChIP-seq data of dark-grown seedlings showed that 42% of the PIF4 and BZR1 target genes overlap with ARF6, including many genes with known functions in cell elongation (*EXP8, BIM1, BEE1/3, PREs, HAT2, IBH1, HFR1, PAR1/2,* and *EXO*) and auxin response (*PINs* and *SAURs*) (Oh et al., 2014). The same authors showed that the three TFs bind to the same G-box *cis*-element present in the promoter of these genes. Therefore, it is proposed the formation of a complex between BZR1, PIF4, and ARF6 to regulate gene expression (Fig. 3).

Overall, these results show that BZR1, PIF4, and ARF6 regulate distinct genes but also act synergistically to promote cell elongation and etiolation.

Gibberellins

Gibberellins (GAs) are very important plant growth regulators. Analysis of mutants for the GA biosynthesis, perception and signaling have been fundamental to unveil GA functions. For instance, the *ga1-3* mutant (*GA1* encodes an enzyme of GA biosynthesis) shows shorter hypocotyls as compared to WT, but when supplemented with GA recovers WT hypocotyl length (Cowling and Harberd, 1999). The effect of GA on hypocotyl growth is light dependent, as at dark GA has a minor effect on hypocotyl growth (Cowling and Harberd, 1999). In contrast, the *gai* mutant (GAI is a DELLA protein involved in GA signaling) do not respond to GA. *Gai* mutant line is GA-insensitive and show shorted hypocotyl, especially under light. Altogether, these results show that GA response is controlled at biosynthesis and signaling level.

The hypocotyl length of WT and *ga1-3* mutant lines grown under light is similar but under dark *ga1-3* lines show shorter hypocotyl (Achard et al., 2007). These results show that the GA synthesis is more important for the hypocotyl growth under dark. In Arabidopsis, five DELLA proteins (GAI, RGA, RGA-Like1 (RGL1), RGL2, and RGL3) were identified (Hirsch and Oldroyd, 2009). The quadruple-DELLA mutant line (lacks GAI, RGA, RGL1, and RGL2) generated in WT and *ga1-3* background showed the same hypocotyl length as WT under dark (Achard et al., 2007). These results show that DELLAs repress hypocotyl growth under dark. On the other hand, both quadruple-DELLA mutant lines grown under light show taller hypocotyls than WT. These results suggest that light inhibit hypocotyl growth through DELLA proteins (Achard et al., 2007).

GA pathway is activated by the interaction of GA with its receptor, GID1. GA-GID1 interact with DELLA proteins and promote the association with the E3 ubiquitin ligase complex SCF^{SLY} to promote DELLA degradation by the proteasome (McGinnis et al., 2003). In the absence of GA, DELLA proteins physically interact with various transcription factors and inhibit their functions. In fact, it was shown that the DELLA protein RGA interacts with several transcription factors to block their action. RGA interacts with ARF6, 7 and 8 (not to ARF1) (Oh et al., 2014), with PIF4 and PIF3 (Feng et al., 2008; Lucas et al.,

2008) and BZR1 (Bai et al., 2012), thus inhibiting cell elongation and hypocotyl growth.

Jasmonic Acid

The plant hormone jasmonic acid (JA) regulates plant growth, but in opposition to auxins, BR and GA, JA inhibits growth to promote defense (Yang et al., 2012). In the presence of JA, the E3 ubiquitin ligase coronatine insensitive (COI) is activated and promotes the degradation of the jasmonate ZIM-domain (JAZ) proteins. This releases the MYC2 transcription factor that represses growth related genes and induces the expression of defense-related genes (Wasternack and Hause, 2013). Consistent with this observation, Arabidopsis *coi* mutants, as well as a *JAZ9* overexpressing line, exhibit longer hypocotyls as compared to WT. This regulation is conserved in rice, as the rice *coi* mutant exhibit elongated plant height, internode and cell lengths, as well as decreased sensitivity to JA (Yang et al., 2012).

In Arabidopsis, thirteen *JAZ* genes were identified and the *jaz* quintuple (*jazQ*) mutant, which lacks *JAZ1/3/4/9/10*, exhibits a constitutive JA response. *JazQ* mutant showed increased resistance to herbivores, increased sensitivity to exogenous JA, short root phenotype and lower rosette size and dry weight. Interestingly, when PIF4 is overexpressed in the *jazQ* background, plant growth is partially recovered without compromising defense (Campos et al., 2016), suggesting that PIF4 could act downstream of JAZ to regulate plant growth. Consistent with these observations, the *pifq* mutant no longer responds to JA-induced growth inhibition under constant white light and, the overexpression of PIF3 partially overcome JA-induced growth inhibition (Yang et al., 2012). However, under dark conditions *pifq* mutant seedlings showed JA-induced grown inhibition (Zheng et al., 2017). These studies show that light is important for JA-induced growth inhibition and that other factors, besides PIFs, are regulating this process. As it was shown for *jazQphyB* mutants that show full recovery of rosette size and dry weight as compared to *jazQ* (Campos et al., 2016).

Exogenous application of JA stabilizes DELLAs, by delaying the GA-mediated DELLA degradation (Yang et al., 2012). Therefore, DELLAs interact with PIFs and repress growth. Consistent with this, the rice DELLA mutant, *slr1* mutant, is insensitive to JA-induced growth inhibition (Yang et al., 2012). These results show a clear crosstalk between GA and JA pathways. DELLAs and MYC2 compete for JAZ-binding (Hou et al., 2010) and regulate growth. In the absence of GA, DELLAs interact with JAZ and MYC2 is released to promote JA response (growth inhibition). In contrast, in the absence of JA, JAZ binds to DELLAs and PIFs promote growth (Yang et al., 2012). In addition, PIFs were shown to repress the expression of *JAZ9* (Leivar and Monte, 2014), possibly as a feedback response to attenuate PIFs response (Fig. 3). Thus, a molecular cascade involving the COI1–JAZ–DELLA–PIF signaling module was proposed (Yang et al., 2012). This represents a switch strategy that plants use to control normal growth or defense using the crosstalk between GA and JA signaling.

Recently, a new model was proposed trying to explain how JA regulates growth under dark (Zheng et al., 2017). The authors show that JA inhibits hypocotyl growth of WT and *pifq* mutants grown under dark, but not *cop1-4* and *cop1-6* mutant seedlings, suggesting that COP1 is required for JA-induced hypocotyl growth inhibition. Moreover, RNA-seq analysis confirms that JA affects the expression of one-third of light-responsive genes and that around 60% are also regulated by COP1. Concomitantly, the authors show that JA reduces the physical interaction between COP1 and SPA1, therefore reducing COP1 activity and nuclear localization (Zheng et al., 2017). The mechanism by which JA regulate COP1 is still not clear, but the authors suggest that MYC2 suppresses COP1 activity to stabilize COP1-targeted transcription factors, such as HY5. In fact, JA promotes HY5 protein accumulation while *coi1* and *myc2* mutants showed reduced accumulation of HY5. This HY5 accumulation is not dependent of COP1, suggesting that COP1 acts downstream of COI1 and MYC2. Taken together, the authors suggest that JA releases MYC2 from its repressor (JAZ) via SCF^{COI1}. MYC2 represses COP1 activity and stabilizes HY5 inhibiting hypocotyl elongation (Zheng et al., 2017).

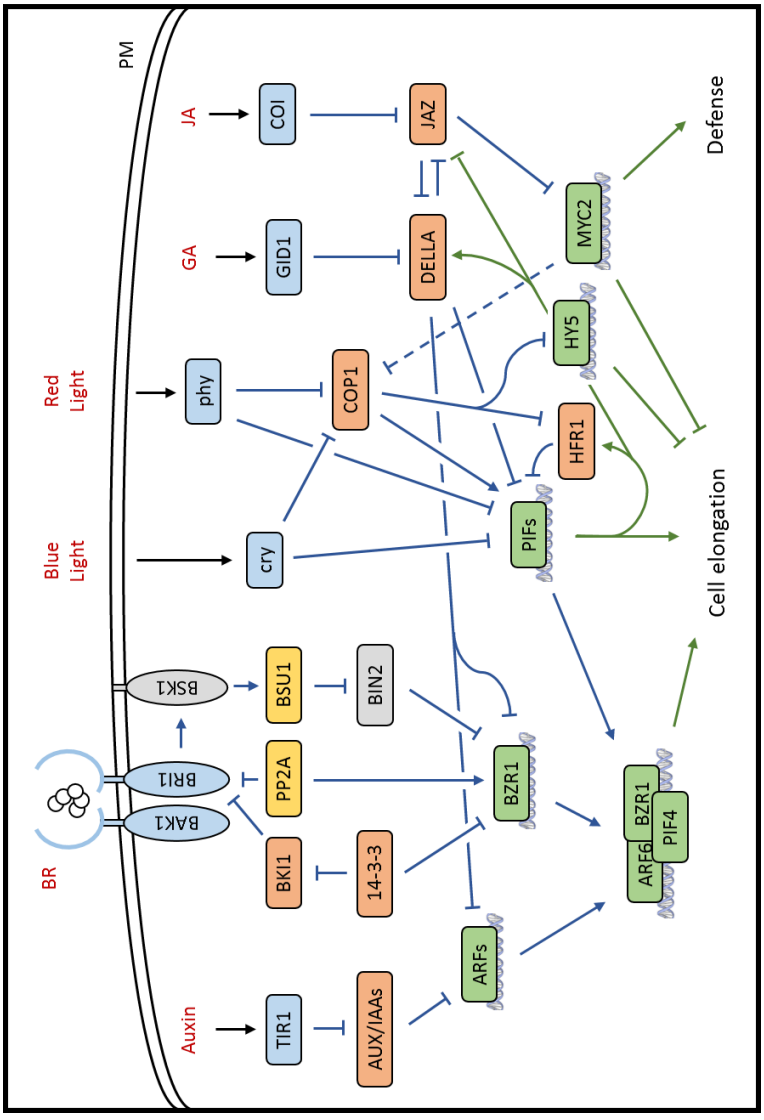

Figure 3. Light and hormonal network to control cell elongation and defense. Light and hormonal signals (red text) are perceived by membrane (ellipse) or cytoplasmic (rectangles) receptors (blue). A cascade of interactions and posttranscriptional mechanisms (blue lines) is initiated in which key regulators (orange rectangles) control the ability of transcription factors (green rectangles) to regulate gene expression (green lines) and promote cell elongation or defense. Yellow: phosphatases, gray: kinases.

5.4.2. Light-temperature crosstalk

Temperature and light are important external factors regulating plant development. Phytochrome single or multiple mutants show lower germination rate under cold (7ºC) or warm (28ºC) temperatures as compared to WT (Heschel et al., 2007). PhyA and PhyE promote germination at cold and warm temperatures, respectively, while phyB influences germination across a range of germination temperatures with a nonadditive contribution to the role of phyA and phyE (Heschel et al., 2007; Franklin, 2009).

Arabidopsis plants grown under the same light conditions but at different temperatures (16ºC, 22ºC, and 28ºC) show differences in development and architecture (Franklin, 2009). Arabidopsis seedlings, grown at 22ºC, display the greatest leaf area and biomass while at 16ºC display dwarf and compact rosette, a typical phenotype of response to low temperatures (Franklin, 2009). At low temperatures, stress-responsive genes such as the Dehydration Responsive Element Binding1/C-Repeat Binding Factor (DREB1/CBF) are induced (Liu et al., 1998; Figueiredo et al., 2012). The overexpression of DREB1/CBF genes increase cold tolerance but slows down growth (Achard et al., 2008). In contrast, Arabidopsis plants grown at 28ºC display petiole elongation, leaf hyponasty and reduced leaf area (Franklin, 2009), a phenotype that resembles the shade avoidance response (Keller et al., 2011).

Warm temperature

Warm temperature (28ºC) induces plant elongation and development, a phenotype that resembles shade avoidance response (Franklin, 2009; Stavang et al., 2009).

PIF4 gene expression is induced by warmer temperatures in a linear correlation (Stavang et al., 2009; Kumar et al., 2012; Jeong and Choi, 2013) and *pif4* mutants grown at warm temperatures do not display the warm temperature phenotype (Koini et al., 2009). Nevertheless, *pif4* mutants are not defective in temperature perception since the expression of warm temperature responsive genes, such as the heat shock protein 70 (HSP70) is observed (Franklin et al.,

2011). The warm temperature response is still not completely understood, however, auxins, GA and BR act positively in this regulation as they do for shade avoidance response (Stavang et al., 2009). In fact, PIF4 induces the expression of auxin biosynthetic genes (*YUCCA8, TAA1,* and *CYP79B2*) (Sun et al., 2012) and small auxin up RNA (*SAUR*) genes (Franklin et al., 2011). In addition, *SAUR19* overexpression in *pif4* background rescue the warm temperature phenotype. Altogether, these results indicate that PIF4 plays a major role in response to warm temperature through regulation of auxin levels.

Low temperature

The Arabidopsis *AtDREB1B* plays an important role in plant responses to abiotic stress. The *AtDREB1B* is highly induced by cold stress and its overexpression increases plant cold tolerance (Jaglo-Ottosen et al., 1998). The cold-induced *AtDREB1B* expression is higher in the light as compared to subjected night (period of time when the plants are subjected to light instead of the normal photoperiodic night time) showing that the induction of *AtDREB1B* is regulated by circadian rhythm regulatory elements (Fowler et al., 2005). Under control environmental conditions and 12h photoperiod, *AtDREB1B* has a peak of expression at 8h after light (ZT 8h) and reaches basal levels during the night, rising again in the next light period (Kidokoro et al., 2009). WT plants exposed to continuous light show repression of *AtDREB1B* expression during the subjected night (Fowler et al., 2005; Kidokoro et al., 2009). However, the *pif7* mutant does not show *AtDREB1B* repression during the subjected night, showing that PIF7 is the major negative regulator of *AtDREB1B* in subjected night (Kidokoro et al., 2009). The authors suggest that PIF7 is circadian regulated and not degraded under light conditions which explain the *AtDREB1B* expression pattern.

AtDREB1B is also differentially regulated by the length of the day. Short day (SD: 8 h light) photoperiod show higher cold tolerance as compared to long days (LD: 16 h light) photoperiod (Lee and Thomashow, 2012). The cold tolerance is correlated with the increased level of *AtDREB1B* under SD. *AtDREB1B* expression is regulated by phytochrome B and PIF4 and PIF7 since, under LD,

the *phyB* and the *pif4/pif7* mutants have much higher levels of *AtDREB1* as compared to WT. These results show that PIF4 and PIF7 repress *AtDREB1B* expression and consequently decrease cold tolerance under LD (Lee and Thomashow, 2012). In contrast, it was shown that the rice *phyB* mutant and the rice PIF-like 16 (OsPIL16) overexpressing lines show increased *OsDREB1B* expression and consequent increased cold tolerance. The authors showed that both mutants have higher cell membrane integrity and lower lipid peroxidation. These results show that in rice, OsDREB1B increases cold tolerance through cell membrane stabilization. An increased expression of *OsDREB1B* in the OsPIL16 overexpressing lines increased cold tolerance but inhibit their growth (He et al., 2016), confirming the role of DREBs as inhibitor of growth (Ito et al., 2006). *AtDREB1B* overexpressing plants showed an increased level of both RGA (DELLA) protein and *GA2ox3* (GA deactivation) gene expression consistent with the dwarf phenotype displayed (Achard et al., 2008). Therefore, the authors proposed that *AtDREB1B* inhibit growth through induction of GA deactivation gene (GA2ox) level and accumulation of DELLA proteins. DELLA proteins interact with PIFs inhibiting growth. Consistent with this mechanism, the addition of GA reverts the dwarf phenotype of *AtDREB1B* plants.

6. The rice Phytochrome-Interactor Factors-like

Six PIF-like (OsPIL11-16) were identified in rice, which shows high similarity with the Arabidopsis PIFs (Nakamura et al., 2007). The overexpression (OsPIL11-15) in Arabidopsis induces hypocotyl growth under photoperiodic conditions. These results show that rice PILs have the functional ability to regulate light signaling pathway in a way similar that of AtPIFs (Nakamura et al., 2007). Similar to Arabidopsis, the rice PILs are constituted by the active phytochrome binding motif and the bHLH domain. However, the interaction with phytochromes is poorly studied. OsPIL13 and OsPIL16 were tested for interaction with phytochrome B, but only OsPIL16 showed interaction (Todaka et al., 2012; He et al., 2016). These results suggest that OsPIL13 could be more stable under light or be regulated by another rice phytochrome. Similarly to Arabidopsis, all

the rice *PILs* show higher expression in leaf (mature and young) suggesting that rice and Arabidopsis PILs might have similar functions (Jeong and Choi, 2013). In addition, rice *PILs* gene expression shown to be regulated after 3h of abiotic stress (Jeong and Choi, 2013). For instance, drought and 200mM NaCl induce *OsPIL11* and *OsPIL15* and repress *OsPIL13*, while cold (4ºC) and heat (42ºC) repress *OsPIL13* and *OsPIL14*, respectively (Todaka et al., 2012; Jeong and Choi, 2013). Moreover, JA and salicylic acid slightly increase *OsPIL11* gene expression while ABA represses OsPIL11 expression after 6h of exposure (Li et al., 2012b).

Using tobacco plants overexpressing *OsPIL11*, it was shown that OsPIL11 inhibit hypocotyl elongation and promote cotyledon and leave growth only under red light (not at dark or far-red) (Li et al., 2012b). The growth inhibition observed in tobacco contrasts with the growth enhancement observed in Arabidopsis (Nakamura et al., 2007; Li et al., 2012b). The observed differences are attributed to the growth conditions (constant red light and 8h light/16h dark, respectively) and light fluence. However, the possibility that OsPILs show different functions in Arabidopsis, tobacco, and rice cannot be discarded. These results show the importance of the study of OsPILs functions in rice. In fact, *OsPIL15* overexpressing lines showed exaggerated shorter above-ground under dark (Zhou et al., 2014). Interestingly, this phenotype is not observed at constant red or far-red light, suggesting that red and far-red can relieve the above-ground growth inhibition observed under dark (Zhou et al., 2014). The expression of *OsPIL15* at red and far-red is still higher in overexpressing lines which do not explain the light-relieve phenotype. Therefore the authors suggest that red and far-red promotes the growth of *OsPIL15* overexpressing lines, likely through regulation of OsPIL15 protein level. Moreover, microarray analysis using the aboveground parts of *OsPIL15* overexpressing lines grown under dark, showed that OsPIL15 downregulates lipid transport, auxin-mediated signaling pathway, cell wall organization genes (Zhou et al., 2014). These results show that Arabidopsis and rice PILs can have different functions, at least under dark conditions.

OsPIL13 overexpressing transgenic rice plants are taller than WT under drought (Todaka et al., 2012). This phenotype is due to the internode cell

elongation which is consistent with the observed higher expression of OsPIL13 in the first internode portion (Todaka et al., 2012). Moreover, microarrays analysis of the internode portion shows that OsPIL13 transgenic plants have an induction of GO term genes associated with cell wall development, including expansins and cellulose synthases genes.

The analysis of another *OsPIL* overexpressing line, the *OsPIL16*-OX line, revealed that an direct association with cold tolerance (He et al., 2016). OsPIL16 enhance cold tolerance by upregulating *OsDREB1* expression. This upregulation is due to the direct binding of OsPIL16 to the *cis*-elements (N-box, G-box and/or PBE) present in the promoter of all rice DREBs, as a consequence OsPIL16-OX seedlings are shorter but more tolerant to cold (He et al., 2016). The biological function of the other rice PILs is poorly characterized, especially the OsPIL12 and OsPIL14 to which no transgenic rice plants have been reported. The information presented in this section is summarized in Table 1.

7. Thesis outline and research objectives

In Arabidopsis, PIFs have been considered important transcription factors that modulate plant growth and development and have the ability to interconnect external and internal signals. In rice, PIF/PILs are poorly characterized. Before the beginning of this work, OsPIF14 was identified as binding to the promoter of *OsDREB1B*, and it was shown that OsPIF14 interacts preferentially with the active form of phytochrome B. The main goal of this work was to understand how OsPIF14 regulates *OsDREB1B* expression, as well as to characterize the function of OsPIF14 in plant growth and development. To address this goal, we analyzed in detail the binding of OsPIF14 to the promoter of *OsDREB1B* and the consequences on *OsDREB1B* expression. In addition, we produced RNAi::*OsPIF14* transgenic rice plants to characterize the biological function of OsPIF14 and to identify direct and indirect targets of OsPIF14 in the transition from dark to light.

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Chapter 2

Rice Phytochrome-Interacting Factor protein OsPIF14 represses OsDREB1B gene expression through an extended N-box and interacts preferentially with the active form of Phytochrome B

Author contribution

This PhD thesis is a continuation of the PhD work entitled Novel Transcription Factors Regulating the Expression of the Rice Gene *OsDREB1B* developed by Duarte Figueiredo at GPlantS Unit. In this chapter it is described the identification and initial characterization of the rice (*Oryza sativa* L.*)* Phytochrome-Interacting Factor 14 (OsPIF14). This work was published at *Biochimica et Biophysica Acta - gene regulatory mechanisms* in December of 2015 (Cordeiro et al., 2015) and both André and Duarte contributed equally for its development. Duarte performed the identification of OsPIF14 by yeast one-hybrid, analyzed *OsPIF14* gene expression pattern under several abiotic stress conditions and studied the interaction between OsPIF14 and the rice phytochromes. André characterized OsPIF14 as a bHLH group B protein, studied in detail the interaction between OsPIF14 and the *cis*-element present in the *OsDREB1B* promoter, assessed the transcriptional activity of OsPIF14 and analyzed the diurnal expression of *OsDREB1B*.

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Abstract

DREB1/CBF genes, known as major regulators of plant stress responses, are rapidly and transiently induced by low temperatures. Using a Yeast one Hybrid screening, we identified a putative Phytochrome-Interacting bHLH Factor (OsPIF14), as binding to the *OsDREB1B* promoter. bHLH proteins are able to bind to hexameric E-box (CANNTG) or N-box (CACG(A/C)G) motifs, depending on transcriptional activity. We have shown that OsPIF14 binds to the *OsDREB1B* promoter through two N-boxes and that the flanking regions of the hexameric core are essential for protein-DNA interaction and stability. We also showed that OsPIF14 down-regulates *OsDREB1B* gene expression in rice protoplasts, corroborating the OsPIF14 repressor activity observed in the transactivation assays using Arabidopsis protoplasts. In addition, we showed that OsPIF14 is indeed a Phytochrome Interacting Factor, which preferentially binds to the active form (Pfr) of rice phytochrome B. This raises the possibility that OsPIF14 activity might be modulated by light. However, we did not observe any regulation of the *OsDREB1B* gene expression by light under control conditions. Moreover, *OsPIF14* gene expression was shown to be modulated by different treatments, such as drought, salt, cold and ABA. Interestingly, *OsPIF14* showed also a specific cold-induced alternative splicing. All together, these results suggest the possibility that OsPIF14 is involved in cross-talk between light and stress signaling through interaction with the *OsDREB1B* promoter. Although in the absence of stress, *OsDREB1B* gene expression was not regulated by light, given previous reports, it remains possible that OsPIF14 has a role in light modulation of stress responses.

1. Introduction

Plant growth and development are extremely influenced by environmental conditions. Abiotic stresses such as cold, drought and salinity are responsible for major losses in crop yield worldwide. In response to these environmental factors, plants have evolved mechanisms in order to cope with extreme conditions, such as the production of osmoprotectants and regulatory proteins involved in signaling pathways (Saibo et al., 2009; Hirayama and Shinozaki, 2010). Among the latter, transcription factors (TFs) play a very important role in response to these stresses, since they can regulate the expression of many genes by binding to specific *cis*-acting elements in the promoter regions. A single TF can therefore have a major effect in the response to a specific stimulus.

The Dehydration-Responsive Element-Binding 1/C-Repeat-Binding Factor (DREB1/CBF) TFs belong to the AP2/ERF family and have been described as being rapidly and transiently induced by low temperature (Gilmour et al., 1998; Liu et al., 1998; Dubouzet et al., 2003). When present in the cell nucleus, DREB1 proteins bind to a conserved *cis*-motif, the Dehydration-Responsive Element/C-Repeat (DRE/CRT), present in the promoter region of stress-inducible genes, thus regulating their transcription (Gilmour et al., 1998; Liu et al., 1998). DREB1 proteins were initially identified in Arabidopsis (Stockinger et al., 1997; Liu et al., 1998), but homologues have been identified in several other plants, such as rice (Dubouzet et al., 2003), maize (Qin et al., 2004), *Eucalyptus* (El Kayal et al., 2006), grape (Xiao et al., 2006), cotton (Shan et al., 2007) and almond (Barros et al., 2012), which is illustrative of their relevance in plant stress responses. In rice, OsDREB1A to OsDREB1G have been identified as homologues of the Arabidopsis DREB1/CBFs (Dubouzet et al., 2003; Chen et al., 2008; Wang et al., 2008). Our work focuses on the regulation of *OsDREB1B*, that was initially described as highly and specifically induced in response to cold (Dubouzet et al., 2003). More recently, this gene has been shown to respond to other stresses, such as drought, oxidative and mechanical stress as well as to ABA and salicylic acid (Gutha and Reddy, 2008; Figueiredo et al., 2012). The over-expression of this gene in rice provided increased tolerance to cold, drought and salinity(Ito et al.,

2006), whereas in tobacco it led to increased tolerance to oxidative and freezing stress, as well as to infection by tobacco streak virus (Gutha and Reddy, 2008).

Temperature and light signaling have been previously described to cross-talk (Franklin, 2009), and phytochrome signaling in particular was described as a regulator of DREB1/CBF expression in Arabidopsis (Kim et al., 2002; Franklin and Whitelam, 2007; Kidokoro et al., 2009). Phytochromes are photosensitive chromoproteins that can reversibly interconvert between two different forms: the inactive red-light absorbing Pr and the active far-red light absorbing Pfr (Franklin and Quail, 2010). In Arabidopsis there are five genes that code for phytochromes (phyA to phyE), whereas in rice there are three members (phyA to phyC), which function as the only photoreceptors to perceive red and far-red light (Takano et al., 2009). Upon activation by red light, the Pfr active form of phytochromes migrates into the nucleus, where it interacts with TFs of the basic helix-loop-helix family (bHLH), referred to as Phytochrome Interacting Factors (PIFs (Franklin and Quail, 2010)). This interaction usually results in a proteasome-dependent degradation of the PIFs, modulating the expression of genes regulated by PIFs. This regulatory mechanism was observed for example for PIF1 (Shen et al., 2005), PIF3 (Al-Sady et al., 2006) and PIF5 (Shen et al., 2007), but it does not seem to be the case of the more recently identified PIF7, because even though it co-localizes with phyB in nuclear speckles after a red light pulse, this protein appears to be light-stable (Leivar et al., 2008a). Additionally, a set of putative PIFs has also been described in rice (Nakamura et al., 2007), but so far the interaction between these proteins and the rice phytochromes is yet to be shown, as well as their stability under light/dark conditions.

The animal bHLH proteins are typically classified into six major groups (group A to group F) depending on its basic domain and consequent DNA *cis*-element binding. Animal group A is associated with bHLH activators, which recognize E-box sequences (CAGCTG or CACCTG). Animal group B seems to be the closest to the bHLH ancestor and is also found in plants and yeast. The basic domain of group B bHLHs are characterized for having an arginine at position 13 (R13), which is crucial to bind to hexameric DNA sequences with a

guanine at position 4 (e.g. G-box - CACGTG). Additionally, a leucine zipper can be found only in this group, however it is not present in all group B bHLH proteins. Animal group C represents the bHLH-PAS proteins, which are characterized by a PAS domain and binding to ACGTG or GCGTG core motifs, but they are not found in plants. Group D animal bHLHs are characterized by a lack of the basic domain, thus being unable to bind DNA. Group E animal bHLHs bind to N-boxes (e.g. CACGCG or CACGAG), which are *cis*-elements associated with bHLHs with repressor activity. Additionally, these bHLHs have a proline in their basic domain and a WRPW sequence in their C-termini. Moreover, since group E factors also have R13 in their basic domain these proteins can bind to group B *cis*-responsive elements and the other way around, however with less affinity. Animal group F bHLHs are called COE-bHLH and are not found in plants. Additionally, in 2010 plant bHLH proteins were clustered in 26 subfamilies based on phylogenetic analyses. For better characterization of bHLH proteins read (Ohsako et al., 1994; Atchley and Fitch, 1997; Fisher and Caudy, 1998; Ledent and Vervoort, 2001; Pires and Dolan, 2010).

In our work, we focused on the transcriptional regulation of *OsDREB1B*. We have previously identified seven Zn Finger TFs as binding to the promoter of *OsDREB1B*, in a Yeast One-Hybrid (Y1H) screening (Figueiredo et al., 2012). Here, we report the identification and characterization of another TF binding to the promoter of *OsDREB1B*. This new TF was previously reported as Phytochrome Interacting factor 3 – Like 14 (OsPIL14) (Nakamura et al., 2007), which belongs to the bHLH protein family and is predicted to be a putative PIF. Here, we have shown that OsPIL14 interacts with the photoactivated form of phyB and therefore named it OsPIF14. We have also studied *OsPIF14* gene response to different abiotic stresses, its transcriptional activity, and characterized the OsPIF14 interaction with the respective cis-element present in the *OsDREB1B* promoter.

2. Materials and Methods

2.1. Cold–induced cDNA expression library

The cDNA expression library was prepared as previously described (Figueiredo et al., 2012). Briefly, eight-day-old rice seedlings (*Oryza sativa* L. cv. Nipponbare) grown at 28ºC and 12h/12h photoperiod, were subjected to a 5ºC treatment. Whole seedlings were sampled after 2h, 5h, and 24h of cold treatment and the RNA extracted was used to construct the cDNA library following the manufacturer instructions (HybriZAP-2.1 XR Library Construction Kit (Stratagene)).

2.2. Yeast One-Hybrid

The *OsDREB1B* promoter fragment used as bait for the Yeast One-Hybrid screening ranged from -488bp to -3bp counting from the ATG start codon and was isolated by PCR using the primers 5'-ATGCGGCCGCTCGGAGTAACACTCGTGCAG-3' and 5'-GGACTAGTTGACTCTCTCTGGTTCACTTCG-3' (underlined sequences represent adaptors with restriction enzyme sites). This fragment was cloned as a *Spe*I-*Not*I fragment in the pHIS3/pINT1 vector system (Meijer et al., 1998; Ouwerkerk and Meijer, 2001) and integrated into yeast strain Y187 (Clontech). This bait strain was then transformed with the rice cold-induced cDNA expression library. Over one million yeast colonies were screened in CM-His$^-$ medium supplemented with 5mM 3-amino-1,2,4-triazole, as described (Ouwerkerk and Meijer, 2001). The plasmids from the yeast clones that actively grew on selective medium were extracted and the cDNA insert sequenced. These sequences were used to search for homology in the rice genome, using the BLAST algorithm. Plasmids containing genes encoding transcription factors were re-transformed into the respective bait strain, to confirm activation of the *HIS3* reporter gene.

To divide *OsDREB1B* promoter (-488 to -3bp from ATG) in two different baits we isolated both promoter sequences by PCR combining the primers described below and the new pair of primers 5'-GGACTAGTTGCTGCTGCTACTCCAGCTT-3' and 5'-

ATGCGGCCGCCCAAAAACCCAACAGAAACC-3'. Fragments were cloned as described below.

For the direct Y1H, we used the identified Y1H clone or the full coding sequence of the *OsPIF14* gene, depending on the situation. The full coding sequence of the *OsPIF14* gene was cloned into vector pAD-WT (Stratagene), by replacement of the coding region of the wild-type lambda cI, fragment C, downstream of the GAL4 activation domain, using *Eco*RI and *Pst*I. The yeast bait strains harboring the *OsDREB1B* promoter fragments ranging from -1945 to -388bp have been described elsewhere (Figueiredo et al., 2012).

2.3. Abiotic Stress Treatments

Rice seedlings were grown hydroponically in nutritive medium (Yoshida et al., 1976) at 28ºC, 700µmol fotons.m^{-2}.s^{-1}, 70% humidity and 12h/12h photoperiod for 14 days. The seedlings were then transferred to stress conditions 4h after the start of the light period. At the same time, control seedlings were transferred to fresh nutritive medium (mock control). Temperature treatments were performed by transferring the seedlings to growth chambers at the desired temperature in pre-cooled, or pre-warmed, medium. For salt and ABA treatments, seedlings were transferred to nutritive media supplemented with 200mM NaCl or 100µM ABA, respectively. Drought treatment was performed by maintaining the seedlings over dry absorbent paper. All other conditions were maintained throughout the assays. For semi-quantitative RT-PCR analysis, time points consisting of ten plants were sampled (roots and shoots separately). *Arabidopsis thaliana* ecotype Col-0 seeds were vernalized for 4 days at 4ºC in the dark and then germinated at 22ºC on MS plates (MS basal salts, 0.05% MES buffer, 1% sucrose, pH 5.7, 0.6% agar) in a 16h/8h photoperiod. For the cold treatment assay, 10-day-old seedlings were transferred to 5ºC 4h after the start of the light period, and kept there for 1 or 4 hours. Ten whole seedlings were collected at each time point.

2.4. Semi-quantitative RT-PCR and RT-qPCR

Total RNA from both rice and Arabidopsis seedlings was extracted using the RNeasy Plant Mini kit (Qiagen). For semi-quantitative RT-PCR analysis, first strand cDNA was synthesized from 1µg total RNA, using an oligo-dT primer and the SuperscriptII reverse transcriptase (Invitrogen) following the manufacturer's instructions. The cDNA was then amplified by PCR using gene-specific primers (See Table S1). For rice, *ACTIN1* (Os03g50885) was used as an internal control for all experiments, except for shoots in the drought assay and roots at 10ºC, where *EUKARYOTIC ELONGATION FACTOR 1-α* (Os03g08060) and *UBIQUITIN-CONJUGATING ENZYME E2* (UBC2; Os02g42314) were used, respectively. Besides a biological replicate, measurements were performed in triplicate for each time point and efficiency curves were prepared in duplicate.

For quantitative PCR analysis, RNA extraction was conducted as described above. First strand cDNA synthesis was performed using 4µg total RNA with an oligo-dT primer using Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to the manufacturer's instructions. The cDNA was then amplified by PCR using gene-specific primers (See Table S2). Ubiquitin-conjugating enzyme E2 was used as internal control. Real Time PCR was done in a Lightcycler 480 (Roche), using Lightcycler 480 Master I Mix (Roche). Determinations were performed in triplicate for each time point and efficiency curves in duplicate. Relative expression levels were determined by Roche's E-method using the Lightcycler 480 software.

Total RNA from rice protoplasts was extracted using the Direct-zol kit from Zymo. 500ng of RNA were used to synthesize cDNA using oligo-dT primer and the Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. The quantitative PCR analysis was performed as described above.

2.5. Rice protoplasts transformation

Rice protoplasts were prepared from etiolated wild-type seedlings (7 DAG), using an protocol previously described by our group (Lourenço et al., 2013). The protoplasts were transformed with 10ug of OsPIF14::HA, cloned into the pHBT95

plasmid to construct the 35S::OsPIF14::HA cassette. Water transformed protoplasts were used as control. After transformation protoplasts were incubated in 24 well plate under dark, at room temperature for 18h.

2.6. Transactivation assay

The effector plasmid was built by recombining the full coding sequences of gene *OsPIF14* into vector pH7WG2 (VIB-Ghent University), to be under the control of the full CaMV35S promoter. The reporter plasmid used was the pLUCm35GUS (Figueiredo et al., 2012). The *OsDREB1B* promoter region used in the Y1H was cloned upstream of the minimal 35S promoter, as a *Sal*I fragment, to drive the expression of the *GUS* gene. The reporter plasmid was confirmed by restriction analysis and sequencing.

Arabidopsis protoplasts were prepared as described (Anthony et al., 2004). For each independent transformation, 5µg of reporter plasmid and 10µg of effector plasmid were used. Each transformation was performed in triplicate. Cells were incubated for 24h at 22ºC in the dark and then collected at 450g for 1min in a swing-out rotor. Cell lysis and determination of GUS and Luciferase levels was performed as described (Figueiredo et al., 2012). Readings were performed in triplicate. Activation of gene expression was calculated as a GUS/LUC ratio.

2.7. Yeast-two hybrid assay

The full coding sequence of *OsPIF14* was cloned into vector pAD-WT, as described in the Y1H section. The sequences coding for the C-terminal non-photoactive regions of rice phytochromes A, B and C were cloned in vector pDONR221 (Invitrogen), according to the manufacturer's instructions. The cDNA sequences that were used encoded a.a. 620-1129 for *OsphyA* (Os03g0719800), 654-1172 for *OsphyB* (Os03g0309200) and 620-1138 for *OsphyC* (Os03g0752100). These sequences were then recombined into vector pBD-GW. This vector was obtained by cloning a Gateway (GW) cassette into plasmid pBD-GAL4 Cam (Stratagene). Sense orientation and translational fusion between GAL4-BD and PHY encoding genes were confirmed by restriction digestion and sequencing, respectively.

The bait and prey plasmids were transformed into yeast strain AH109 (Stratagene), as described (Ouwerkerk and Meijer, 2001). Yeast colonies were plated on CM-leu-trp-his media and growing colonies were confirmed by PCR.

2.8. Bimolecular Fluorescence Complementation

The *OsPIF14* coding region was cloned into the vector YFP^N43, to be in fusion with the N-terminal portion of the Yellow Fluorescent Protein (YFP). The non-photoactive C-terminal portions of the three rice phytochromes were cloned in vector YFP^C43, to be in fusion with the C-terminal portion of the YFP. Cloning in YFN^C43 and YFC^N43 vectors was done according to Gateway technology (Invitrogen). To use as negative control in the interaction assays with OsPIF14, the Arabidopsis SNF1 kinase homolog 10 (Akin10) was tested in fusion with the N-terminal portion of the YFP. These plasmids, together with a construct harboring the silencing suppressor HcPro (Wydro et al., 2006), were transformed into *Agrobacterium thumefaciens* strain LBA4404. Agro-infiltration of tobacco leaves was performed as described by Wydro *et al.* (2006), with modifications. Briefly, *Agrobacterium* cultures harbouring the constructs were grown overnight in LB medium supplemented with 150µM acetosyringone. The bacteria were centrifuged and the pellets ressuspended in 10mM $MgCl_2$ 150µM acetosyringone and incubated for 2h at RT. The *Agrobacterium* strains were diluted and combined to a maximum total OD_{600} of 0.5 (0.2 OD from each of the YFP reporter vectors and 0.1 OD from HcPro) to infiltrate *Nicotiana benthamiana* leaves. After incubation in the dark for 2 days, the abaxial epidermis of the leaves was detached after gluing on a microscope slide with medical adhesive (Hollister). The samples were observed with a confocal microscope (Leica SP5).

2.9. In vitro co-immunoprecipitation assay

The vectors for this assay were prepared as following: the OsPIF14:GAD was constructed by removing the Arabidopsis *PIF7* coding region from plasmid PIF7:GAD (Leivar et al., 2008a) and replacing it with the *OsPIF14* coding region, using *Nde*I and *Bam*HI. Vectors for *in vitro* expression of the rice phytochromes were obtained by recombining their full coding sequences from pDONR221 into

pDEST17 (Invitrogen). Control constructs GAD:PIF3 and GAD were previously described (Ni et al., 1998). Recombinant proteins were produced *in vitro* using the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of [^{35}S]Methionine. Preparation of bait and prey was performed as described by Khanna *et al.* (2004). Light treatments were performed by exposing the samples to 4min of red light (660nm) or 4min of red light followed by 4min of far-red light (750nm). Binding and washes were carried out as described (Khanna et al., 2004), and samples run on 10% SDS-PAGE gels. Gels were dried and signals obtained using a STORM 860 PhosphorImager (Molecular Dynamics).

2.10. Expression and purification of recombinant thioredoxin-tagged OsPIF14

OsPIF14 coding sequence was recombined into pET32a (Novagen, USA), in fusion with a thioredoxin tag. pET32a/OsPIF14 was used to transform Rosetta pLysS for protein production. Cells were grown to an OD_{600} 0.6 and protein expression was induced with 100 μM IPTG and allowed to occur for 4h at 28ºC. Cells were harvested by centrifugation (3.500xg, 20 min, 4ºC). The bacterial pellet was then resuspended in 20 mM sodium phosphate pH 7.5, 500 mM NaCl, 10 mM imidazole, 250 μM $MgCl_2$, 1.25 mM PMSF, 1x Complete Protease Inhibitor (Roche) and 8 μg/ml DNase. Cells were lysed by enzymatic digestion (Lysozyme 50mg/ml) for 1hour at 4ºC with agitation. The lysate was centrifuged for 1 hour at 18.000xg and 4ºC. The supernatant was filtered with a 0.45 μm filter and soluble thioredoxin-OsPIF14 protein was purified using the HiTrap IMAC FF system (GE Healthcare). Column-bond protein was eluted with 20 mM sodium phosphate pH 7.5, 500 mM NaCl and 250 mM imidazole. Eluted protein was further purified by size exclusion in 20 mM sodium phosphate pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% glycerol using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) and samples were stored at -80ºC. The thioredoxin tag was produced similarly as described above.

2.11. Electrophoretic Mobility Shift Assay (EMSA)

DNA probes were generated by annealing oligonucleotide pairs, in a PCR machine as described in (Serra et al., 2013) and the annealing temperatures (Table S3). The OsPIF14-DNA binding reaction was performed in a volume of 10 μL, which contained 10 mM HEPES pH 7.9, 40 mM KCl, 1 mM EDTA pH8, 50 fmol of labeled probe, 1 mM DTT, 50 ng herring sperm DNA, 15 μg BSA and 500 ng of purified thioredoxin::OsPIF14 or 500 ng of purified thioredoxin. The reactions were incubated for 1 hour on ice and loaded onto native 5% polyacrylamide gel (37.5:1). Electrophoresis was run as described by (Serra et al., 2013).

3. Results

3.1. OsPIF14 is a novel bHLH TF that binds to the promoter of OsDREB1B

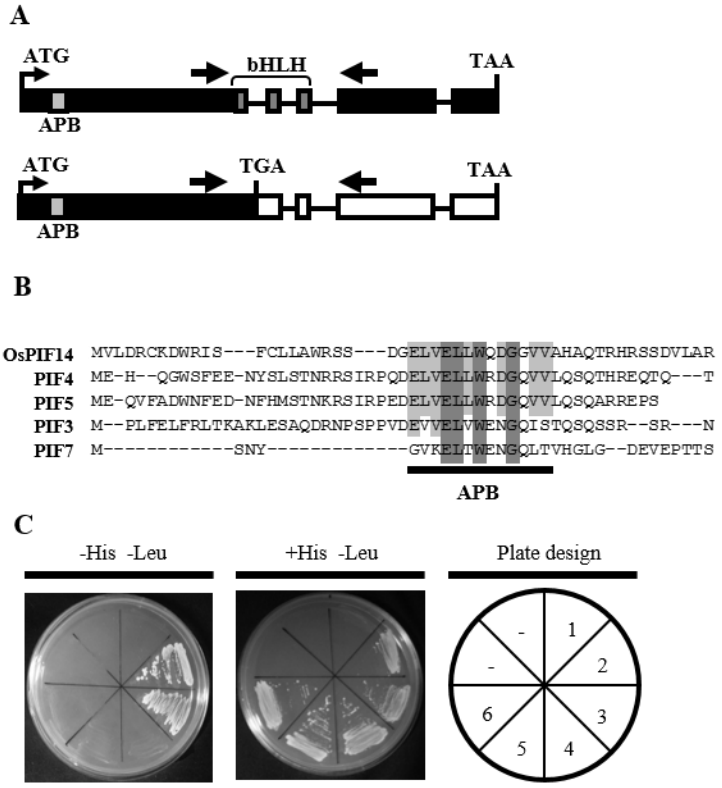
In order to identify TFs that regulate the expression of *OsDREB1B*, we screened a cold-induced rice cDNA expression library, using as bait four overlapping fragments covering 1945bp upstream the *OsDREB1B* start codon. In addition to the seven Zinc Finger TFs previously identified as binding to *OsDREB1B* (Figueiredo et al., 2012), we identified a rice putative Phytochrome Interacting Factor (PIF) binding to the fragment -488bp to -3bp before ATG. Figure 1C shows that PIF binds specifically to this fragment of the *OsDREB1B* promoter. This TF belongs to the bHLH family and had been previously named Phytochrome Interacting factor 3-Like 14 (OsPIL14) by Nakamura *et al.* (2007). In 2010, this rice PIL was clustered within the plant bHLH subfamily VII (a+b). These proteins show a phytochrome interaction region at the N-terminal and a domain of unknown function on C-terminal (Pires and Dolan, 2010). The sequence of this gene was differently predicted in two public databases (GenBank reference Os07g0143200 and Rice Genome Annotation Project 2013, reference Os07g05010) and also different from the sequence we isolated. We identified a 1,245bp coding sequence encoding a 414 amino acid-long protein (JN400276), with a predicted molecular weight of 44.49 kDa and a pI of 4.714. This protein has a putative phytochrome B interacting region (active phytochrome binding; APB) at the N-terminal of the protein and a conserved bHLH DNA binding domain, extending from amino acid 222 to 275 (Fig. 1A and 1B). This gene was found to have four introns, two of which within the bHLH coding region. When comparing its predicted APB domain with that of some of the Arabidopsis PIFs (Fig. 1B), several amino acid residues are conserved between all the proteins, but the rice PIL seems to have the highest degree of identity to Arabidopsis PIF4 and PIF5 (85% APB homology; Fig.1B). Since, in this manuscript (Figure 8), we show that OsPIL14 indeed interacts with the photoactivated form of phytochrome B, from now on we will call it OsPIF14. This protein clustered with both AtPIF4

and AtPIF5, together with a rice putative PIF (PIL13) (Nakamura et al., 2007; Carretero-Paulet et al., 2010).

In order to confirm the binding of OsPIF14 to the promoter of *OsDREB1B*, we re-transformed the yeast bait strain with the plasmid containing the cDNA clone that we identified in the Y1H screen (Fig. 1C). Moreover, the full length transcript was isolated and cloned in a plasmid in order to be in a translational fusion with the GAL4-Activation Domain (GAD). This construct was also transformed into the yeast bait strain. In both cases, we could see an activation of the *HIS3* reporter. We also tested the interaction of the full length OsPIF14 with other regions of the *OsDREB1B* promoter. As seen in Fig. 1C, the protein only interacted with the promoter region ranging from -488 to -3bp, counting from the ATG start codon. Using direct Y1H, we further tested whether OsPIF14 could also bind to the promoter of *OsDREB1A*, the other cold-responsive *DREB1/CBF* gene in rice. In this case, we could not observe an activation of the reporter *HIS3* for any of the bait strains (Fig. S1) indicating that the interaction between OsPIF14 and the -488 to -3bp *OsDREB1B* promoter is specific.

Figure 1. Characterization of the rice phytochrome-interacting factor 14. A, Schematic representation of the OsPIF14 transcript present under control conditions (top) and the alternative splice form that appears under cold stress (bottom). APB - Active Phytochrome Binding motif (light grey box); bHLH - basic Helix Loop Helix domain (dark grey). Thick arrows represent RT-PCR primer locations. Black boxes represent translated exons, white boxes represent non-translated exons and lines represent introns. B, Alignment of the N-terminal a.a. sequence of OsPIF14 with the same region of the Arabidopsis PIF3, PIF4, PIF5, and PIF7, showing the conserved APB domain. Dark shaded boxes show a.a. residues conserved in all the protein sequences, whereas light shaded boxes show residues conserved between OsPIF14 and some of the Arabidopsis PIFs within the APB domain. C, Yeast One-Hybrid assay testing the interaction of OsPIF14 with the promoter of OsDREB1B. Protein coded by the cDNA clone that we identified in the Y1H screen (OsPIF14-Y1H), was tested as well as the full length protein (OsPIF14-FL). The OsPIF14-FL protein was then

tested for interactions against several fragments of the OsDREB1B promoter. On the left is the selection plate, showing positive protein-DNA interactions. In the middle panel the yeast growth in medium supplemented with histidine is shown. On the right panel the plate design is shown. The table to the right details the yeast bait strains and the constructs used in each portion of the plates.



Label	*OsDREB1B* promoter region (from ATG)	Protein in translational fusion with GAL4-AD
1	-488 to -3bp	-
2	-488 to -3bp	OsPIF14-Y1H
3	-488 to -3bp	OsPIF14-FL
4	-1028 to -388bp	OsPIF14-FL
5	-1527 to -961bp	OsPIF14-FL
6	-1945 to -1447bp	OsPIF14-FL

3.2. OsPIF14 binds to OsDREB1B promoter through an extended version of N-BOX

It is described that bHLH TFs can bind to E-box (CANNTG) or N-box (CACG(C/A)G) depending on their transcriptional activity (Fisher and Caudy, 1998). In order to identify the specific DNA sequence to which OsPIF14 binds, we analyzed the *OsDREB1B* promoter bait where OsPIF14 was identified, searching for E-box or N-box *cis*-elements using the Geneious program. *In silico* results revealed the presence of two N-boxes (CACGCG), within the 288bp upstream of the start codon of *OsDREB1B* that we called N-box 1 and N-box 2 (Fig. 2A). To confirm that OsPIF14 binds to these *cis*-elements, we divided the bait fragment (-488bp to -3bp from ATG) into two fragments (fragment 1: -488bp to -222bp and fragment 2: -288bp to -3bp from ATG) and constructed two yeast baits for direct Y1H. Both yeast bait strains were re-transformed with the plasmid containing the OsPIF14 cDNA clone that we identified in the Y1H screen. The results of direct Y1H, showed that OsPIF14 only binds to fragment 2 (Fig. S2). In addition, we designed 18 overlapping small fragments (double-stranded oligonucleotides with 30bp each) spanning fragment 2 (Fig. 2A) and used them as probes in Electrophoretic mobility shift assays (EMSA) carried out with the recombinant Trx::OsPIF14 protein. We identified three small fragments (2.7, 2.8 and 2.11) in which Trx::OsPIF14 binds (Fig. 2B and 2C). Fragments 2.7 and 2.8 share the same N-box domain (N-box 1; N1) and Trx::OsPIF14 is able to bind to both sequences. The Trx alone do not bind to any of these probes (Fig. S3A). Hence, we can conclude that Trx::OsPIF14 binds within the 2.7/2.8 overlapping DNA sequence (Fig. 2D, square box). Trx::OsPIF14 also binds to N-box 2 (N2) in fragment 2.11, but the interaction seems to be much weaker than that observed for N1. In order to test whether OsPIF14 actually binds to the N-box, we designed a mutated probe for N-box 1 (N1_m2), in which the middle Cytosine and Adenine were changed to an Adenine and Thymine, respectively (Fig. 3C). This double point mutation completely abolished the protein-DNA interaction, showing that Trx::OsPIF14 in fact binds to the N-box and that both nucleotides are crucial for DNA binding (Fig 3A).

Despite N-box 1 and N-box 2 sharing the same six core nucleotides CACGCG (Fig. 2D), EMSA results showed different signal intensities (Fig. 3A), which could be due to differences in the flanking region (Fig 2D). Comparing the two bases from 5' and 3' flanking regions of N-box 1 and N-box 2, they only differ in one Cytosine in the 5' region (Fig. 2D). In order to investigate the importance of this Cytosine for protein-DNA interaction we did point mutations in both probes (Fig. 3C and D). We first added the lacking 5' Cytosine to N-box 2 (N2_m1) and removed the corresponding Cytosine from N-box 1 (N1_m1). When the 5' Cytosine is added (N2_m1) the signal intensity of the mutated probe is similar to the wild type version of N-box 1 (Fig. 3A). On the other hand, when the 5' Cytosine is removed from N-box 1 (N1_m1) the signal intensity decreases reaching the level of N-box 2 wild type (Fig. 3A). These results show that this Cytosine is not essential, but plays an important role in Trx::OsPIF14-DNA binding strength. In general, *cis*-elements are composed of an even number of base-pairs. In order to evaluate whether the corresponding nucleotide in the 3' region of the *cis*-element is also important, we made a point mutation (N1_m3, Fig. 3C). When we changed this nucleotide from a Guanine to an Adenine, the EMSA signal is much weaker compared to that obtained with the WT probe (N1_wt), thus showing the importance of this nucleotide for the protein-DNA interaction. In order to evaluate whether the subsequent nucleotides were also important for this interaction, we mutated them in the 5' and 3' region, individually and simultaneously (N1_m4, N1_m5, N1_m6). The results revealed that these nucleotides are not important for OsPIF14-DNA interaction (Fig. 3B). We can then conclude that OsPIF14 binds to both N-box 1 and N-box 2 of *OsDREB1B* promoter, though it prefers the extended version (CCACGCGG).

3.3. OsPIF14 has properties of a bHLH Group B protein

In order to further characterize the OsPIF14 bHLH domain, we aligned the basic domain of bHLH proteins belonging to group A, B and E (Fig. 4), as those three groups are the most similar to OsPIF14. Group B is characterized by an arginine at position 13 (R13), which is also present in OsPIF14 (Fig. 4). This R13

confers the capability of binding to *cis*-elements that have a Guanine at position 4 (G4; Fig 3 C/D), a feature shared between N-box and G-box (Ohsako et al., 1994). As we showed before, OsPIF14 is able to bind to the N-box motif (CACGCG), however we do not know if OsPIF14 is able to bind to the G-box *cis*-element (CACGTG). In order to test that, we changed the N-box to a G-box in which a Cytosine at position 5 was changed to a Thymine in N-box 1 (Fig. 3C, N1_m7).

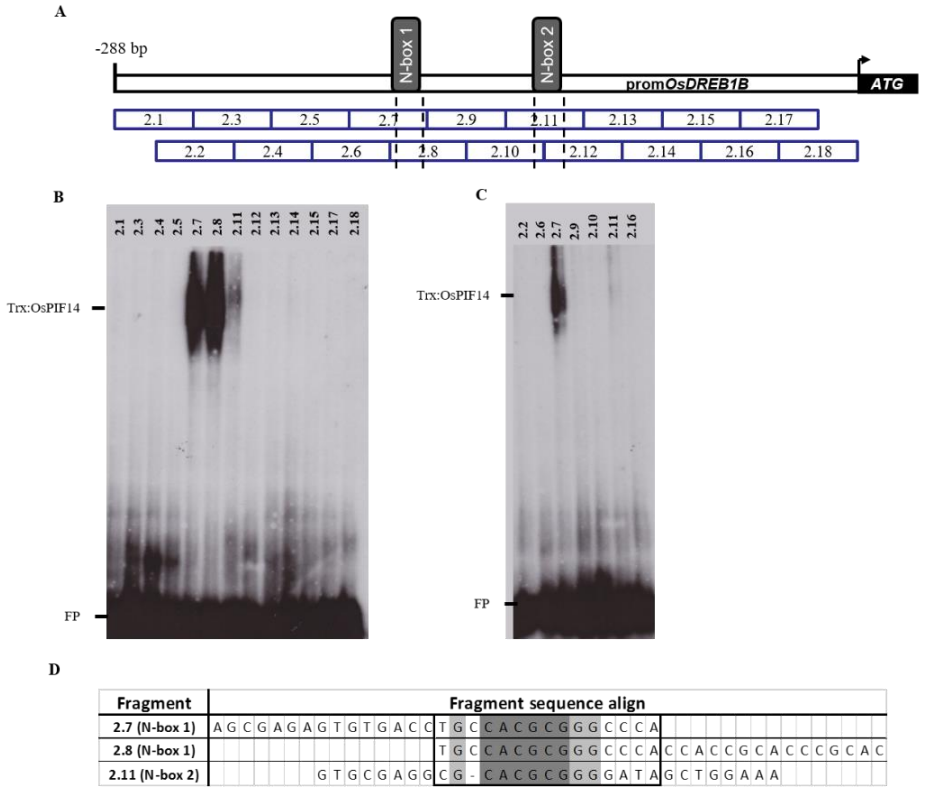
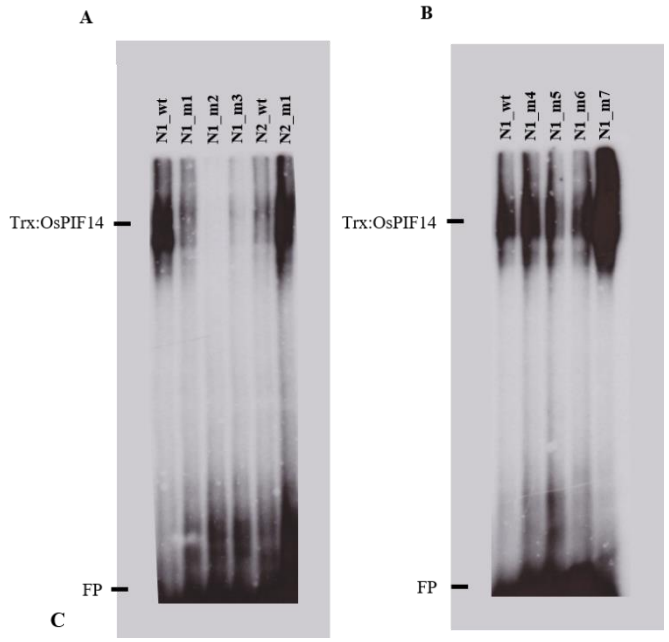


Figure 2. OsPIF14 binds to both *OsDREB1B* promoter N-box (N-box 1 and 2). A, Schematic representation of the eighteen probes used in Electrophoretic Mobility Shift Assay (EMSA) to screen *OsDREB1B* promoter. Each probe has 30 bases length in which 15 bases overlap with next probe. N-box 1 and 2 squares represent the sequence (CACGCG). Probes 2.7, 2.8 and 2.11 have intact N-box, while 2.12 lacks the first base of N-box, as represented by dotted lines. Probe 2.7 and 2.8 share the same N-box. B and C, EMSA results of *OsDREB1B* promoter probes screen showing that OsPIF14 binds to both N-box of *OsDREB1B* promoter with different intensities. FP, free probe. D, alignment of the three probes to which OsPIF14 binds in EMSA. Square represents the important bases for OsPIF14-DNA binding. Dark shaded bases

represents the N-box. Light shaded bases represents the common bases between the three probes.



C

N-box 1										
Position	-1	0	1	2	3	4	5	6	7	8
N1_wt	G	C	C	A	C	G	C	G	G	G
N1_m1	G	-	C	A	C	G	C	G	G	G
N1_m2	G	C	C	A	A	T	C	G	G	G
N1_m3	G	C	C	A	C	G	C	G	A	G
N1_m4	A	C	C	A	C	G	C	G	G	G
N1_m5	A	C	C	A	C	G	C	G	G	A
N1_m6	G	C	C	A	C	G	C	G	G	A
N1_m7	G	C	C	A	C	G	T	G	G	G

D

N-box 2										
Position	-1	0	1	2	3	4	5	6	7	8
N2_wt	G	-	C	A	C	G	C	G	G	G
N2_m1	G	C	C	A	C	G	C	G	G	G

Figure 3. Flanking region of N-box plays an important role in OsPIF14-DNA binding. A, point mutation on N-box (N1_m2) and first flanking base (N1_m1, N1_m3, N2_m1). B, single or double point mutation on second flanking base (N1_m4, N1_m6, N1_m5, respectively) and point mutation to change N-box to G-box (N1_m7). C and D, represents N-box 1 and 2 (light shaded bases) and the two flanking bases of the wild type (wt) and mutated (m) probes used in EMSA. Red marked bases represents the point mutations (substitutions), (-) represents base deletion or absence (for N1_m1 and N2_wt, respectively). Full probe sequence is represented in Supplemental Table S3. FP, Free Probe

The EMSA results clearly showed that OsPIF14 is able to bind to G-box (Fig. 3B) and the EMSA signal was stronger for the G-box than for the N-box. In addition, it is reported that bHLH proteins from group B are not able to bind to *cis*-elements of group A because they lack the G4 (e.g. CACCTG) (Ohsako et al., 1994). To test this, we used a mutated G-box probe, changing the G4 to a Cytosine (G-box MUT). In this case we observed that OsPIF14 loses its ability to bind to DNA confirming that Guanine at position 4 is important for DNA binding (Fig. S3B).

	Basic Region															
Positon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Group A	R	R	E	I	A	N	S	N	E	R	R	R	M	Q	S	AP-4
	R	R	Q	A	N	N	A	R	E	R	I	R	I	R	D	da
	S	V	I	R	R	N	A	R	E	R	N	R	V	K	Q	ac
Group B	K	R	R	T	H	N	V	L	E	R	Q	R	R	N	E	c-myc
	K	R	K	N	H	N	F	L	E	R	K	R	R	N	D	L-myc
	R	R	A	Q	H	N	E	V	E	R	R	R	R	D	K	USF
	A	**A**	**E**	**V**	**H**	**N**	**L**	**S**	**E**	**R**	**R**	**R**	**R**	**D**	**R**	**OsPIF14**
Group E	R	R	S	N	K	P	I	M	E	K	R	R	R	A	R	hairy
	R	K	V	M	K	P	L	L	E	R	K	R	R	A	R	E(spl) m7
	R	K	S	S	K	P	I	M	E	K	R	R	R	A	R	HES-1

Figure 4. OsPIF14 belongs to Group B bHLH. Alignment of the basic domain of the most related bHLH groups (Group A, B and E). Shaded bases represents the similarities between OsPIF14 and other bHLH groups. Adapted from Ohsako et. al. 1994.

3.4. OsPIF14 is a repressor of OsDREB1B

Drosophila bHLH TFs that bind to the N-box motif have been characterized as repressors (Fisher and Caudy, 1998). To investigate whether OsPIF14 is a transcriptional repressor or activator, we have performed a transactivation assay in Arabidopsis protoplasts. The full length OsPIF14 cDNA clone was used as the effector, while the *GUS* gene driven by the same *OsDREB1B* promoter fragment, used as bait in the Y1H screening, was used as reporter (Fig. 5A). Fig. 5B shows that co-transformation of OsPIF14 with the reporter vector resulted in a statistically significant, albeit modest, decrease in GUS activity, as compared to the reporter vector alone. This decrease was not observed when the reporter did

not contain the promoter fragment of *OsDREB1B*, indicating that the presence of this sequence is necessary for the binding of OsPIF14 and consequent repression of transcription. The OsPIF14 transcriptional repression activity and its binding to *OsDREB1B* promoter was also tested *in vivo* in rice protoplasts. The rice protoplasts were transformed with the full *OsPIF14* CDS sequence driven by the 35S and the expression of endogenous *OsDREB1B* was analyzed after 18h of dark incubation (Fig. 5D). The results showed that OsPIF14 over-expression leads to an *OsDREB1B* down regulation (as compared to non-transformed protoplasts), indicating that OsPIF14 binds to *OsDREB1B* promoter, thus repressing its gene expression. These results confirmed that OsPIF14 is indeed a repressor and provides further evidence for *in vivo* binding to the *OsDREB1B* promoter.

3.5. OsPIF14 transcript is regulated by alternative splicing under cold stress

To understand how *OsPIF14* influences the plant responses to cold, and whether it is also involved in the responses to other stresses, we have investigated *OsPIF14* expression under different abiotic stress conditions. Two week-old rice seedlings were subjected to cold, salt, drought and ABA treatments over a period of 24h (Fig. 6A). The cold-induced gene expression of *DREB1/CBF*s in Arabidopsis was previously shown to be gated by the circadian clock, and to be more pronounced when stress is applied 4h after the start of the light period (Fowler et al., 2005). We decided to start our assays at that time of the day since we expect to observe higher differences in Os*DREB1B* regulation after treatment imposition.

The gene expression studies showed that under control conditions the *OsPIF14* transcript level slightly increases in shoots during late afternoon and the night period (5h and 10h in Fig. 6A, respectively), indicating a light/dark responsive behavior, as previously described (Nakamura et al., 2007). In rice seedlings subjected to different treatments, the *OsPIF14* gene expression was repressed after 20min under salt and drought and 1h after ABA treatment, in both roots and shoots.

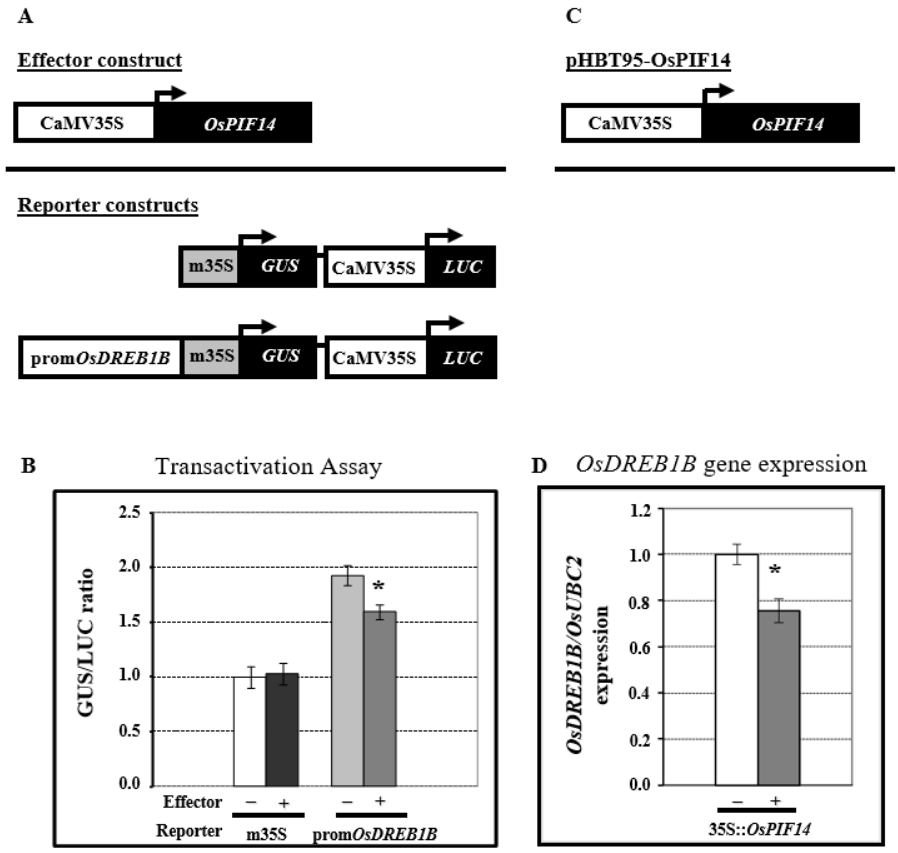


Figure 5. Analysis of the OsPIF14 transcriptional activity. A, Constructs used for Arabidopsis protoplast transformation (pCambia 1391z). Effector construct used corresponds to the OsPIF14 coding region under the control of the full CaMV 35S promoter. Reporter constructs contain the GUS gene driven by the minimal CaMV 35S promoter (control vector) or under the minimal CaMV 35S promoter plus the fragment of the OsDREB1B promoter used as bait in the Y1H screening. The LUC gene under the control of the full CaMV 35S promoter was used to normalize GUS expression levels. B, Transactivation analysis of OsPIF14 as a GUS/LUC activity ratio. Data from a representative experiment is shown. Values shown are multiples of the GUS/LUC ratio obtained with the control vector without effector. Data represents mean +/- SD (N=3). C, Construct used for rice protoplast transformation (pHBT95). OsPIF14 corresponds to the full OsPIF14 CDS sequence. D, expression of endogenous OsDREB1B after 18h of dark incubation. Results were normalized to 1 and represent the mean of two independent experiments +/- SD (N=5). * - Differences statistically significant (t-test, $p<0.05$).

The transcript was then up-regulated some hours afterwards: under salt stress this up-regulation occurred 2h after stress, both in roots and shoots; in the case of drought, the same pattern was observed for roots, but was not so pronounced in shoots; and, in the case of ABA treatment, the up-regulation only occurred in shoots and only 10h after treatment imposition.

The transcriptional regulation of *OsPIF14* under cold was unique, in the sense that it was the only stress inducing the appearance of an upper mRNA band, both in shoots and roots (Fig. 6A). After cloning and sequencing, we found that the second band was an alternative splicing form of the transcript (Fig. 1A). Under cold conditions, the alternative splicing of the *OsPIF14* transcript leads to the retention of the first intron, and consequent formation of a premature stop codon. Since the stop codon is positioned more than 50 nucleotides upstream of the next exon-exon junction, we predict that this splicing form would be down-regulated by nonsense-mediated mRNA decay (NMD) (Lewis et al., 2003). On top of that, the putative protein product lacks a complete bHLH domain, thus it is expected to be transcriptionally inactive if produced. We named this splicing form *OsPIF14β*, whereas the splicing form under control conditions will be referred to as *OsPIF14α*. Interestingly, the gene expression pattern of both splicing forms seems to be slightly different when rice seedlings were subjected to different temperatures (Fig. 6A). At 5ºC, transcripts of the *β*-form were detected 40min after stress onset, increasing along the treatment, whereas the *α*-form started to decline after 1 to 2h. In seedlings subjected to 10ºC, the transcript level of the *β*-form appeared 20min after stress onset, reaching a peak at 5h and declining to non-detectable levels afterwards. The *α*-form did not show significant alterations along the 10ºC treatment. The expression pattern of both forms was similar in both roots and shoots. To determine whether this alternative splicing event was specific to low temperature, or if it occurred in temperature shifts in general, we analyzed the *OsPIF14* gene expression after transferring rice plants to different temperatures for 5h (Fig. 6B). We could only detect the presence of the alternative splicing form *OsPIF14β*, when the plants were transferred to 10ºC and not to

15ºC, 28ºC, 36ºC or 45ºC. This indicates that this splicing event is specific to low temperatures.

Given that PIF7 was described as a regulator of *DREB1/CBF* expression in Arabidopsis (Kidokoro et al., 2009), we wanted to know whether, under cold conditions, any of the Arabidopsis *PIF* transcripts would also undergo alternative splicing. Thus, 10-day-old Arabidopsis seedlings were subjected to 5ºC for 4h and the gene expression of *PIF1, 3, 4, 5, 6* and *7* was analyzed by semi-quantitative RT-PCR, using intron-spanning primers (Fig. S4A). Interestingly, this analysis identified alternative splicing forms for *PIF3, 6* and *7* both under control and cold conditions. The *PIF6* splicing forms observed had been previously reported (Penfield et al., 2010). For *PIF3* and *PIF7*, the splicing events corresponded to the retention of introns in the vicinity of the bHLH coding region (Fig. S4B), leading to the formation of premature stop codons, similarly to what happens with *OsPIF14*.

3.6. OsPIF14 interacts with the active form of OsphyB

In Arabidopsis it is known that phytochromes, upon activation by red light, migrate into the nucleus, where they interact with PIFs. This interaction usually results in a proteasome-dependent degradation of the PIFs, modulating the expression of genes regulated by PIFs. The interaction between rice phytochromes (A, B, and C) and putative PIFs had not yet been shown in rice. Thus, we have analyzed whether OsPIF14 interacted with any of the rice phytochromes. Initially, we performed a Yeast Two-Hybrid assay (Y2H), using the coding region of *OsPIF14* as prey, and as baits the C-terminal non-photoactive coding regions of the three rice phytochromes, respectively (A, B and C; Fig. 7A). From the yeast growth rate in histidine-lacking medium (Fig. 7B), our results indicated that OsPIF14 interacts with OsPHYB C-terminal domain in a stronger manner than it does with the comparable OsPHYA or OsPHYC domains. To further confirm these results, a Bimolecular Fluorescence Complementation assay (BiFC) was performed (Fig. S5). YFP fluorescence signals could be detected in the nucleus of *Nicotiana benthamiana* leaf cells co-infiltrated with the OsPIF14::YFP^N and

OsPHYB::YFP^{C} fusion proteins (Fig. S5B). No fluorescence was detected when leaves were co-infiltrated with OsPIF14::YFP^{N} plus either OsPHYA::YFP^{C} or OsPHYC::YFP^{C}.

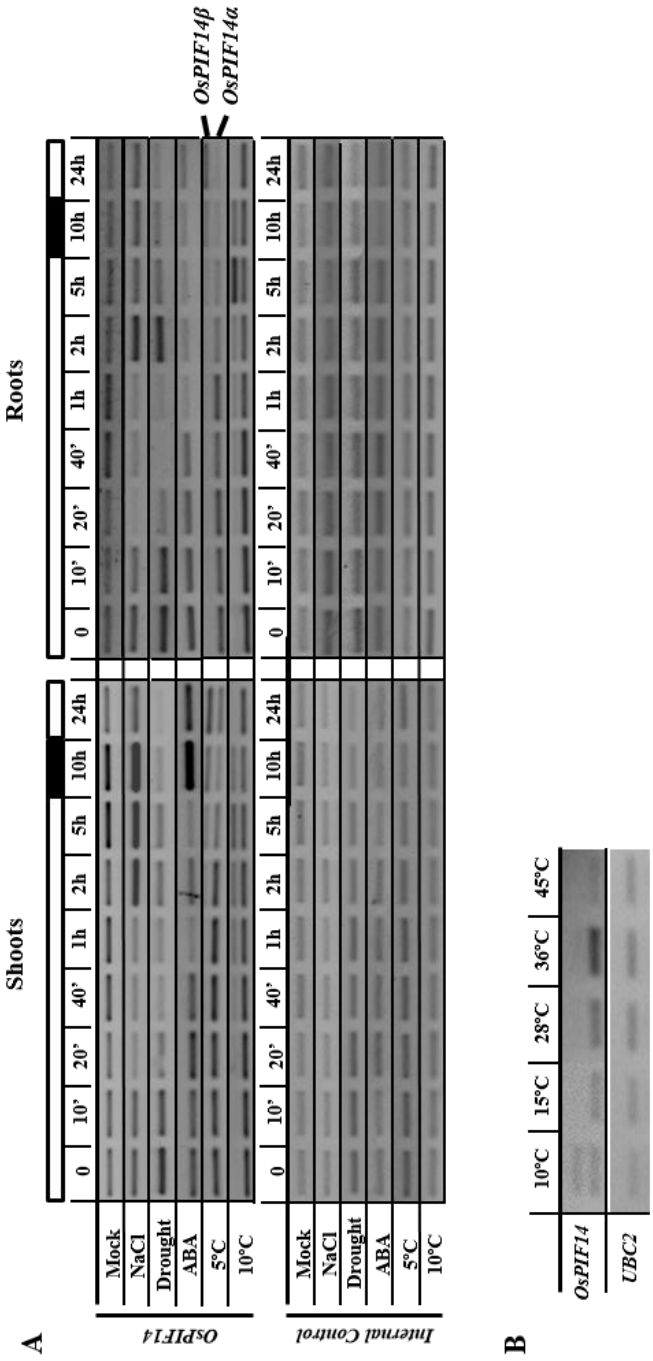


Figure 6. Expression profile of *OsPIF14* in two week-old rice seedlings under different treatments. The plants were grown in a 12h:12h photoperiod and all the treatments started 4h after the beginning of the light period. A, *OsPIF14* gene expression pattern under different abiotic stress conditions. The black and white bars on top indicate the time of the day at which the time-points were taken (light or dark). Roots and shoots were analyzed separately. Two alternative splice forms identified under cold stress are indicated on the right. B, Analysis of the alternative splicing of the *OsPIF14* transcript after exposing the plants to different temperatures for 4h. Whole plants were assayed.

The Y2H and BiFC results support the hypothesis that OsPIF14 can bind to the C-terminal domain of OsPHYB. In order to test whether this binding was dependent on the active or inactive form of the full-length phytochrome, we expressed OsPIF14 and the full-length OsphyB *in vitro* and analyzed their interaction using a co-immunoprecipitation assay. As shown in Fig. 8B, OsPIF14 interacts preferably with the active Pfr form of OsphyB. The positive interaction between OsphyB-Pfr and Arabidopsis PIF3 was used as a positive control for the interaction(Ni et al., 1998). Although we observed that OsPIF14 binds to the active form of OsphyB and that OsPIF14 represses *OsDREB1B* expression, we could not observe a significant regulation of *OsDREB1B* expression by light under normal, non-stress conditions (Fig. S6).

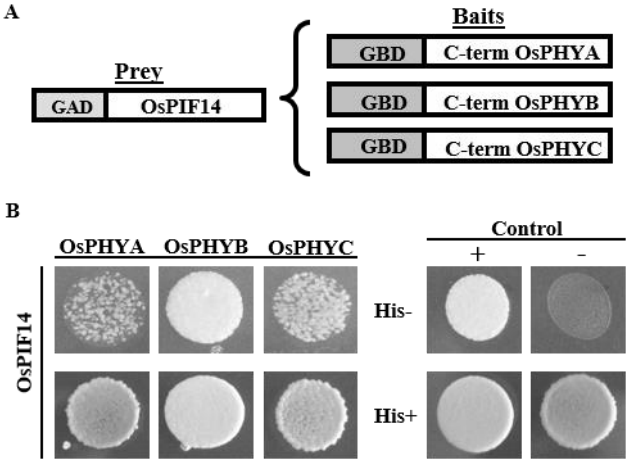


Figure 7. Analysis of the interaction between OsPIF14 and the three rice phytochromes, using a Yeast Two-Hybrid assay. A, Protein constructs used in the Y2H assay. OsPIF14 was used as prey, in a translational fusion with the GAL4 AD. The C-terminal non-photoactive regions of rice PHYA, B and C were fused with the GAL4 BD to be used as baits. B, Analysis of protein-protein interactions in yeast growing in histidine-lacking medium. Positive control used was the interaction between pAD-WT and pBD-WT, and negative control was the absence of interaction between GAD:OsPIF14 and pBD-WT. Bottom panel shows yeast growth in histidine-supplemented medium.

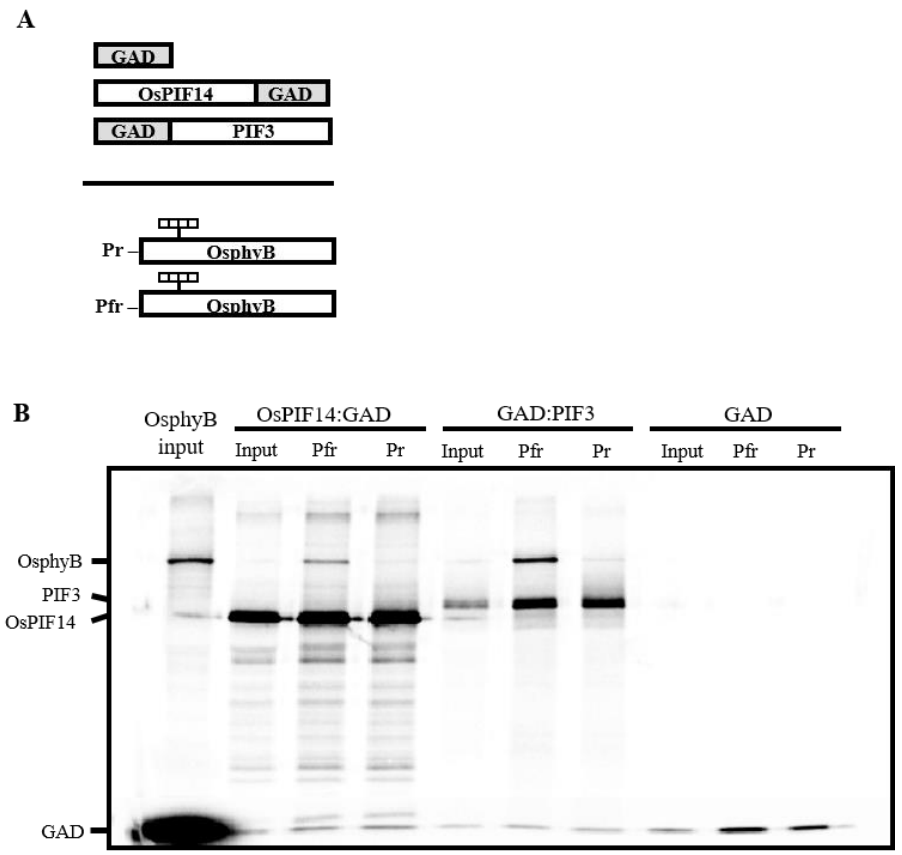


Figure 8. Analysis of the interaction between OsPIF14 and the Pr and Pfr forms of OsphyB, using a co-immunoprecipitation assay. A, Schematic representation of the protein constructs used for the assay. OsPIF14 was used as bait, in a translational fusion with the GAL4 AD. The GAL4 AD vector was used as a negative control for the interaction, whereas a fusion of GAD with the Arabidopsis PIF3 was used as the positive control. The Pr and Pfr forms of OsphyB were used as prey. B, SDS-PAGE separation of pellet fractions for each interaction and inputs for the proteins used in the assay. Data from a representative experiment is shown.

4. Discussion

The DREB1/CBF regulon has long been known to play an important role in the cold stress response in plants. Furthermore, it has already been reported that, in Arabidopsis, *DREB1* genes are light regulated, having their expression dependent on light quality (red/far-red ratio) (Kim et al., 2002; Franklin and Whitelam, 2007). PIF7 was described as a possible link between light and cold signaling in Arabidopsis, since it binds to the promoter of *DREB1C*, and the *pif7* null mutant was shown to have altered transcript levels of *DREB1B* and *DREB1C* (Kidokoro et al., 2009). In our work, using a Y1H system, we have identified OsPIF14 as binding to the promoter of *OsDREB1B*. We assessed the role of OsPIF14 in the regulation of *OsDREB1B* and its possible involvement in the link between light and cold signaling in rice. Even though PIF7 was described as a regulator of *DREB1C* and *DREB1B* in Arabidopsis (Kidokoro et al., 2009), the OsPIF14 APB domain shows the least degree of identity to PIF7, whereas PIF4 and PIF5 show the highest identity (85%), in agreement with what was previously reported by Nakamura, 2007. Our Y1H assays show that OsPIF14 binds specifically to the *OsDREB1B* promoter region ranging from -288 to -3bp, from the ATG start codon and it does not bind to the promoter of *OsDREB1A*. This means that OsPIF14 does not bind to the promoter of all members of *DREB1/CBF*s. The same was observed in Arabidopsis for PIF7 (Kidokoro et al., 2009). In order to better understand *OsDREB1B* regulation by OsPIF14, we have analyzed in detail the interaction of OsPIF14 with the *cis*-element present in the *OsDREB1B* promoter. Previous studies have reported that the flanking region of *cis*-elements play an important role in the activity of TFs, specially the bases closest to the *cis*-element (Fisher and Goding, 1992). Here, we showed that OsPIF14 has higher affinity to an extended version of the N-box (CCACGCGG). These results illustrate the complexity of bHLH-DNA binding which acts as a mechanism of specificity. Since OsPIF14 binds to the N-boxes present in the *OsDREB1B* promoter, and given that these boxes are associated with repressors, we examined whether OsPIF14 acts as a repressor (Fisher and Caudy, 1998). We observed that OsPIF14 represses the expression of the reporter gene driven by the

OsDREB1B promoter in Arabidopsis protoplasts and also the endogenous *OsDREB1B* in rice protoplasts. These results are in agreement with those describing Hairy, a bHLH TF from *Drosophila* that binds to an N-box, acts as a repressor, and also prefers to have a Cytosine at 5' flanking region of *cis*-element (Ohsako et al., 1994). Actually, it was previously reported that a rice PIL15 acts as a repressor of genes involved in auxin pathway and cell wall organization or biogenesis (Zhou et al., 2014). Arabidopsis PIF7 was also described as a repressor of *CBF2/DREB1C* (Kidokoro et al., 2009). However, it is interesting to note that several Arabidopsis PIFs, including PIF7, have also been described as activators of transcription (Huq et al., 2004; Al-Sady et al., 2008; Leivar et al., 2008a). This may indicate that transcriptional activity of these proteins depends on promoter context, as recently suggested (Leivar and Quail, 2011), and/or additional interacting proteins. Our transactivation studies in Arabidopsis protoplasts showed an activation of the reporter gene even without the effector (35S::OsPIF14). This induction is most probably due to the fact that *OsDREB1B* promoter is induced by mechanical stress (Figueiredo et al., 2012), and the process of preparing Arabidopsis protoplasts surely triggers a stress response.

The fact that *OsDREB1B* promoter has two N-boxes and OsPIF14 recognizes them with different binding strength suggests a complex regulation of *OsDREB1B* repression. In order to further characterize the interaction OsPIF14-DNA, we have analyzed the basic region of OsPIF14. It has been reported that Arginine at position 13 (R13) within basic DNA binding region is crucial for binding to *cis*-elements with Guanine at position 4 (G4) (e.g. N-box (CACGCG) or G-box (CACGTG)), which is a mechanism to ensure that repressors do not bind to activator *cis*-elements (e.g. CACCTG) (Ohsako et al., 1994). Here, we show the presence of R13 within the OsPIF14 basic domain and observe that OsPIF14 binds to *cis*-elements with G4, whose interaction is lost when the Guanine is changed to a Cytosine. All together these results suggest that G4 is important for OsPIF14-DNA binding affinity. Moreover, our EMSA results show that OsPIF14 is able to bind both N-box and G-box sequences. In this context, these results allowed us to characterize OsPIF14 as a bHLH belonging to group B. OsPIF14,

showed higher binding affinity to G-box than to N-box, as described for group B proteins. We also showed that the presence of a 5'Cytosine and a 3'Guanine flanking the N-box increases OsPIF14 ability to bind to the N-box core motif. This suggests that OsPIF14 may bind to a large variety of gene promoters with different binding strengths according not only to *cis*-element core motif but also to the flanking regions.

OsPIF14 was shown to be regulated at the transcriptional level under several abiotic stress conditions. In response to salt, drought, and ABA treatments, the transcript levels are down-regulated within 20min to 1h and, in most cases, the levels are recovered after some hours. This suggests that *OsPIF14* could have a potential role in rice responses to different abiotic stresses, through the regulation of *OsDREB1B* and/or possibly other downstream genes. In fact, it was already demonstrated that over-expression of maize PIF3 (ZmPIF3) confers more tolerance to dehydration and salt to rice plants (Gao et al., 2015). The increased tolerance is due to activation of stress responsive genes, like *OsDREB2A*. In addition, phytochrome signaling has already been implicated in the response to salt stress in *Mesembryanthemum crystallinum* (Cockburn et al., 1996), to drought in tomato plants (Biehler et al., 1997), and to ABA metabolism in *Nicotiana plumbaginifolia* (Kraepiel et al., 1994).

In contrast to the above-mentioned treatments, the effect of phytochrome signaling in general, and of PIFs in particular, in the Arabidopsis responses to cold has been well described (Kim et al., 2002; Franklin and Whitelam, 2007; Franklin, 2009; Kidokoro et al., 2009). Previously, we showed that under mild cold conditions (10ºC) *OsDREB1B* stays up-regulated during the first 10h, reaching control levels after 24h, while under severe cold conditions (5ºC) *OsDREB1B* stays induced even after 24h of stress imposition (Figueiredo et al., 2012). Those results indicate that at 5ºC the down-regulation of *OsDREB1B* over time is compromised. Here, we observed that low temperatures induce the formation of an alternative splice form of the *OsPIF14* transcript. The *α*–form, which is transcriptional active and the *β*–form, which corresponds to the transcriptionally inactive form since it lacks the complete bHLH domain. In addition, we predict

that this *β*–form is down-regulated by NMD due to its premature stop codon. Interestingly, the expression patterns of both splice forms, *α* and *β*, were different when rice seedlings were subjected to 10ºC or 5ºC. In response to a mild stress (10ºC), the expression of the *α*–form is not significantly altered, whereas the *β*–form is only expressed during the first 10hours of cold. After 24 hours of cold (10ºC) only the *α*–form is present, which correlates with the low *OsDREB1B* levels due to the repressor activity of OsPIF14. When rice seedlings were subjected to a more severe stress (5ºC), the constitutive *α*–form seems to be replaced by the *β*–form along the treatment, especially at 24h. We hypothesize that after 24h at 5ºC the low levels of *α*–form are not enough to down regulate *OsDREB1B*. These results suggest a differential response of *OsPIF14* to low temperatures, depending on how severe the stress is. Moreover, we observed that this splicing event only occurs when the plants are transferred to cold conditions, and not to warmer temperatures, which indicates that *OsPIF14β* must play a role in the plant responses specific to cold.

We also demonstrated that the Arabidopsis *PIF3, 6* and *7* also show alternative splice forms both under control and cold conditions, while the *OsPIF14* splice forms are cold specific. *PIF6* had already been shown to have two alternative splice forms with different roles in Arabidopsis seed dormancy (Penfield et al., 2010). These results, together with ours, indicate that alternative splicing in *PIFs* may be a common mechanism for the regulation of PIF protein levels in the cells, in response to environmental conditions or certain developmental stages. We cannot rule out other putative splice forms for the genes tested, eventually not detected in our assays. It is yet to be shown whether these *PIF* alternative splice forms, so far identified, code for proteins that maintain the ability to bind phytochromes, since they have an intact APB domain. If so, this mechanism of regulation may be more than a simple way to modulate PIF protein levels, it might also regulate phytochrome signaling itself. Arabidopsis PIF3 was previously described as controlling hypocotyl cell elongation through its binding to phyB, modulating the abundance of this photoreceptor, independently of being able to bind DNA (Al-Sady et al., 2008). PIF4, PIF5 and PIF7 have also been reported to

modulate phyB levels, in a process involving the proteasome pathway (Khanna et al., 2007; Leivar et al., 2008a). This was further confirmed by the finding that PIF3 modulate phyB levels by enhancing its *in vivo* poly-ubiquitination through the recruitment of LRB [Light-Response Bric-a-Brack/Tramtrack/Broad (BTB)] E3 ubiquitin ligases to the PIF3-phyB complex (Ni et al., 2014). Moreover, for PIF4 and 5 it was already demonstrated that COP1 is also important in this *in vitro* degradation process (Jang et al., 2010; Ni et al., 2013). A question thus arises on whether the *OsPIF14β* transcript, that has a longer 3'UTR when compared to the *α*-form, is eliminated by nonsense-mediated mRNA decay (Kertész et al., 2006) or results in a shorter, truncated protein. If the *OsPIF14β* transcript is translated into a functional protein, it is possible that it has a role in the regulation of phytochrome protein levels in rice, modulating the light signaling pathway, for example under cold conditions.

In Arabidopsis, several PIFs have been shown to interact with the active forms of the phytochromes (Ni et al., 1998; Khanna et al., 2004; Shen et al., 2007; Leivar et al., 2008a). However, to the best of our knowledge, no reports have yet been published on the interaction between PIFs and phytochromes in other species, including rice. Our co-immunoprecipitation results show that OsPIF14 preferably binds to the active form of OsphyB. Nevertheless, the Y2H assay, performed using the C-terminal non-photoactive forms of the rice phytochromes, also indicated that this terminal portion of the phytochrome B appears to be sufficient for the binding. Similar results had already been published for PIF3 in Arabidopsis (Ni et al., 1998; Ni et al., 1999; Zhu et al., 2000). Interestingly, the Y2H assays also show that OsPIF14 can bind weakly to the C-terminal domains of the rice phytochromes A and C. However, we were not able to validate this interaction either by BiFC or co-immunoprecipitation assays. It is also interesting to note that the heterologous Arabidopsis PIF3 binds to OsphyB more efficiently than OsPIF14 does. The OsPIF14-OsphyB interaction might result in the sequestration of OsPIF14 or its targeting for degradation. In Arabidopsis, PIF7, which was implicated in cold stress responses (Kidokoro et al., 2009), was described as light stable even though it interacts with the active form of phyB (Leivar et al., 2008a).

It is possible, therefore, that OsPIF14 is functionally more similar to PIF7, even though its APB sequence is more similar to PIF4 and 5.

In our work, we have shown that the *OsPIF14* transcript is regulated by low temperature and, in addition, it has been reported that phyB levels are dependent on PIFs in Arabidopsis (Khanna et al., 2007; Leivar et al., 2008a; Jang et al., 2010; Ni et al., 2013; Ni et al., 2014). Thus, it may be that the regulation of PIFs by temperature has an effect on phytochrome levels, modulating their downstream signaling at different temperatures.

Overall our data provide evidence that both light and cold-temperature signaling pathways may converge on the *OsDREB1B* regulon of rice, likely via different mechanisms. The action of OsPIF14 may be regulated through its preferential interaction with the red light active form of OsphyB, which in turn could target OsPIF14 for degradation or, alternatively, sequester it and abrogate its function (as a repressor of *OsDREB1B* gene expression). It will be important to determine whether OsPIF14-OsphyB interaction prevents OsPIF14 from binding the *OsDREB1B* promoter. OsPIF14 binds to the *OsDREB1B* promoter N-boxes thereby acting as a repressor, but the regulation of *OsDREB1B* may be also modulated through alternative splicing of the OsPIF14 transcript. Moreover, we cannot rule out that other TFs repress the *OsDREB1B* expression, which under different light and temperature conditions is certainly regulated by additional TFs that are yet to be identified.

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7. Supplemental Data

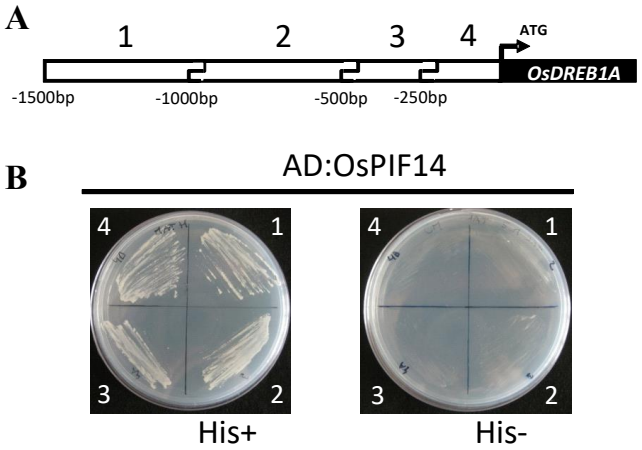
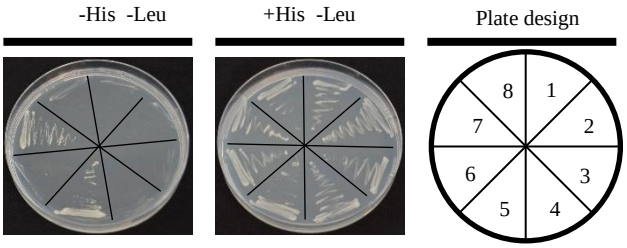


Figure S1. Yeast One-Hybrid assay to test the interaction of OsPIF14 with the promoter region of *OsDREB1A*. A, The first 1500bp of the promoter of *OsDREB1A* were divided into four overlapping fragments, as depicted, to be used as baits. B, These baits were tested for interactions with AD:OsPIF14. Each number on the plate corresponds to one bait strain. No interactions were observed for any of the promoter fragments tested.



Label	*OsDREB1B* promoter region (from ATG)	Protein in translational fusion with GAL4-AD
1	-1028 to -388bp	-
2	-488 to -3bp	-
3	-488 to -222bp	-
4	-288 to -3bp	-
5	-288 to -3bp	OsPIF14-Y1H
6	-488 to -222bp	OsPIF14-Y1H
7	-488 to -3bp	OsPIF14-Y1H
8	-1028 to -388bp	OsPIF14-Y1H

Figure S2. Yeast One-Hybrid assay to narrow down the region of *OsDREB1B* promoter in which OsPIF14 binds. The clone identified in Y1H screen (OsPIF14-Y1H) and the empty vector (-, pACTII) were used for direct Y1H against different *OsDREB1B* promoter regions as represented in the table at the bottom. On the left is the selection plate, showing positive protein-DNA interactions. In the middle panel the yeast growth in medium supplemented with histidine is shown. On the right panel is shown the plate design.

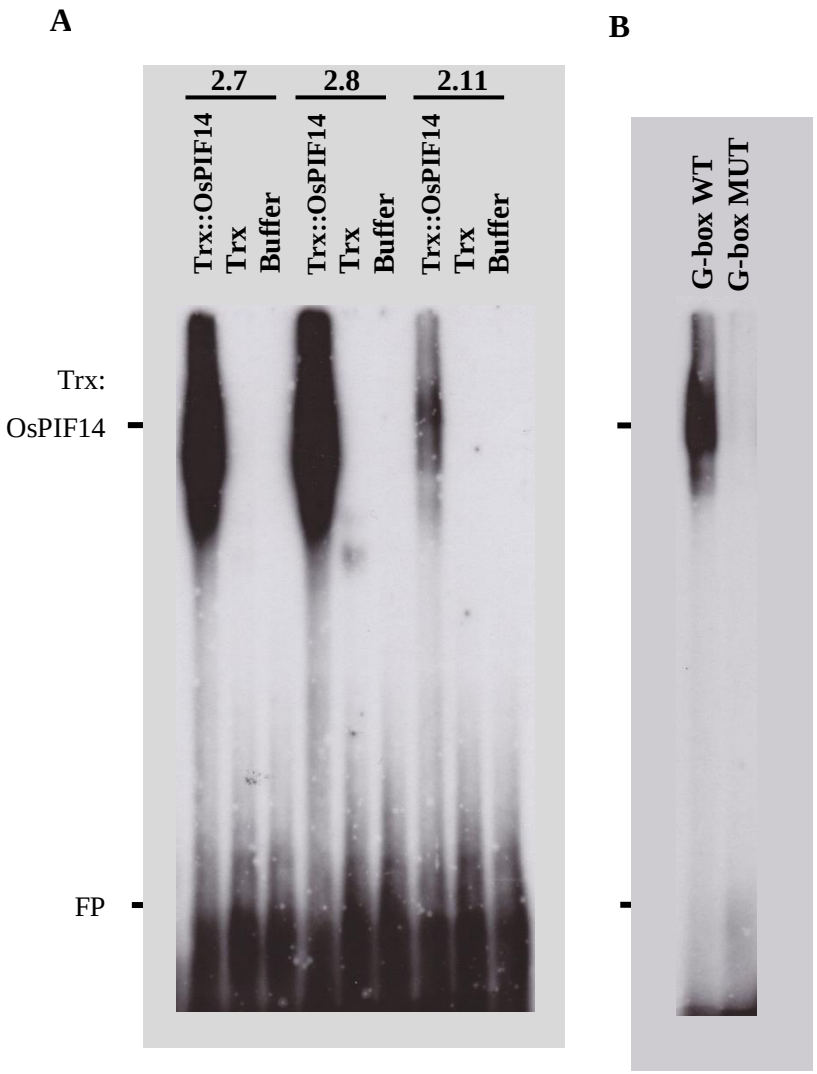


Figure S3. OsPIF14 binds to N-box and G-box motifs. A, EMSA controls for the three probes where the recombinant Trx:OsPIF14 protein binds. B, Guanine at position 4 within *cis*-element is important for OsPIF14 binding. G-box WT represents the motif (CAC<u>G</u>TG) and G-box MUT represents the motif (CAC<u>C</u>TG). Thioredoxin:OsPIF14 was used to perform this assay. FP, Free probe.

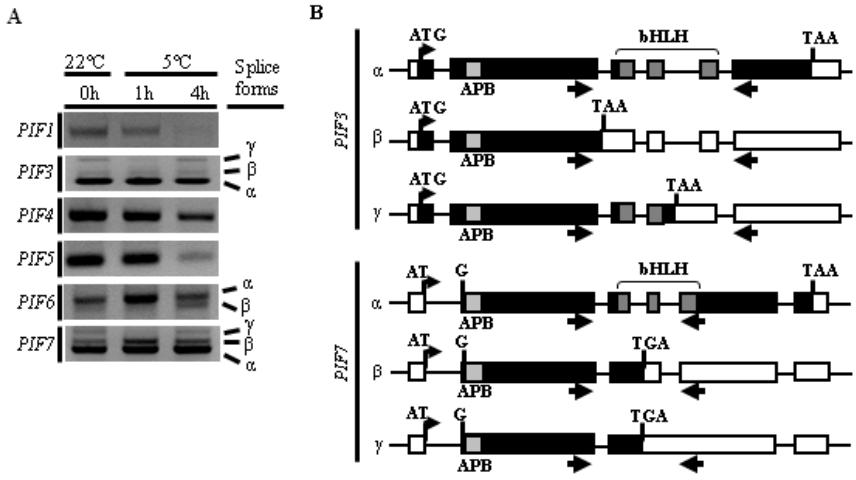


Figure S4. Expression pattern and alternative splice forms of the *PIF* genes in Arabidopsis seedlings subjected to low temperature conditions. A, Analysis of the Arabidopsis *PIF* transcript levels by semi-quantitative RT-PCR in cold-treated plants. Indication of bands corresponding to alternative splice forms for *PIF3*, *PIF6* and *PIF7* can be found on the right-hand side column. B, Schematic representation of the different alternative splice forms identified for *PIF3* and *PIF7*. APB - Active Phytochrome Binding motif (light grey box); bHLH - basic Helix Loop Helix domain (dark grey). Thick arrows represent RT-PCR primer locations. Black boxes represent translated exons, white boxes represent non-translated exons and lines represent introns.

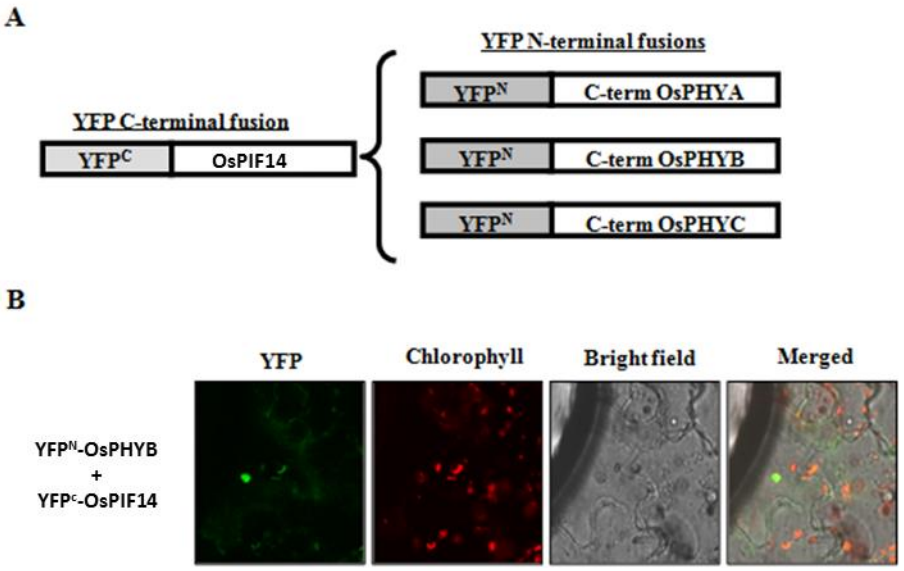


Figure S5. Analysis of the interaction between OsPIF14 and different rice phytochromes, using a BiFC system in detached tobacco leaf epidermis. A, Protein constructs used for the assay. The C-terminal region of YFP was expressed in fusion with OsPIF14 and the N-terminal region of YFP was expressed in fusion with the C-terminal non-photoactive domains of the rice phytochromes. B, YFP fluorescence obtained under 520nm emission for the interaction of OsPIF14 with OsPHYB, followed by chlorophyll fluorescence, bright field and overlay images of the agro-infiltrated *N. benthamiana* leaves. No YFP fluorescence was observed for the interactions between OsPIF14 and rice OsPHYA or C or for the negative control, in which a fusion of YFPN with *Arabidopsis* Akin10 was used.

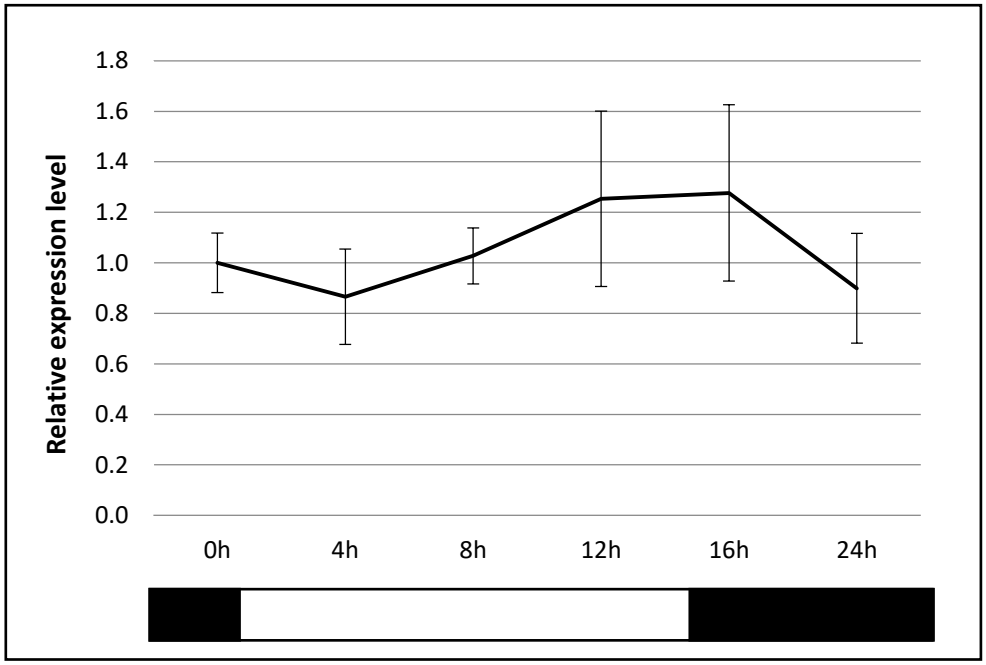


Figure S6. *OsDREB1B* gene expression pattern determined by qRT-PCR in 14-day-old rice seedlings grown at 28ºC, 12h/12h photoperiod and maintained in the same temperature conditions during the assay. Black boxes represent dark period and white boxes represent light period. The expression of UBC2 was used as an internal control. Relative expression levels were normalized to expression at time zero. Data represents mean +/- SD (N=3).

Table S1 – Primers pairs used for semi-quantitative RT-PCR

Gene	Primer Sequence 5'-3'
OsPIF14	CAGCAGTTCTGTGACGAGGT
	TAGAGCCGAGAACGAATGCT
PIF1	GAGGCTGAGAGGGGATTTTA
	ATGAACTTCAGCAGCACGAG
PIF3	CCATCCGAAAGTCCTTCACT
	CTCGATGGCTTCATCTAGCA
PIF4	ACCGACCGTAAGAGAAAACG
	CCATCCACATCACTTGAAGC
PIF5	AGGTTTGACCTCAACCGATG
	GGGAAACATCATCGGACTTG
PIF6	CATGGATTTGTATGAGGCAGAG
	TCTGTTACCCATCGTCATCA
PIF7	CCAATATGTCTTGGGCGTCT
	GTGGCAAGTTGGCTCTTAGG
ACTIN1	GTCGCACTTCATGATGGAGTTG
	CATGCTATCCCTCATCTCGAC
eEF1α	ACCCTCCTCTTGGTCGTTTT
	AAATACCCGCATTCCACAAC
UBC2	CAAAATTTTCCACCCGAATG
	ATCACATGAATCAGCCATGC

Table S2 – Primers used for quantitative RT-qPCR

Gene	Primer Sequence 5'-3'
OsDREB1B	CCAGAAATTGGGGGAAAAA
	GGAATCACAAAAGGAGGGAGA
UBC2	TTGCATTCTCTATTCCTGAGCA
	CAGGCAAATCTCACCTGTCTT

Table S3 – *OsDREB1B* promoter probes used for EMSA

Probe	Primer Sequence 5'-3'	Annealing temperature
2.1	ACGTGCCAAAAACCCAACAGAAACCACAGAAAACGG	58ºC
	ATCACCGTTTTCTGTGGTTTCTGTTGGGTTTTTGGC	
2.2	ACGTGAAACCACAGAAAACGCCGTGAAGCTGCCTGG	63ºC
	ATCACCAGGCAGCTTCACGGCGTTTTCTGTGGTTTC	
2.3	ACGTGCCGTGAAGCTGCCTGCAAGCTGGAGTAGCAG	65ºC
	ATCACTGCTACTCCAGCTTGCAGGCAGCTTCACGGC	
2.4	ACGTGCAAGCTGGAGTAGCAGCAGCAGCGGGCG	67ºC
	ATCACGCCCGCTGCTGCTGCTGCTACTCCAGCTTGC	
2.5	ACGTGGCAGCAGCAGCGGGCAGTGAGACTGAAGACG	65ºC
	ATCACGTCTTCAGTCTCACTGCCCGCTGCTGCTGCC	
2.6	ACGTGAGTGAGACTGAAGACAGCGAGAGTGTGAGCG	63ºC
	ATCACGCTCACACTCTCGCTGTCTTCAGTCTCACTC	
2.7	ACGTGAGCGAGAGTGTGAGCTGCCACGCGGGCCCAG	67ºC
	ATCACTGGGCCCGCGTGGCAGCTCACACTCTCGCTC	
2.8	ACGTGTGCCACGCGGGCCCACCACCGCACCCGCACG	72ºC
	ATCACGTGCGGGTGCGGTGGTGGGCCCGCGTGGCAC	
2.9	ACGTGCCACCGCACCCGCACGCTGTTCCCTTTCCAG	67ºC
	ATCACTGGAAAGGGAACAGCGTGCGGGTGCGGTGGC	
2.10	ACGTGGCTGTTCCCTTTCCATTTCCAGCTATCCCCG	63ºC
	ATCACGGGGATAGCTGGAAATGGAAAGGGAACAGCC	
2.11	ACGTGTTTCCAGCTATCCCCGCGTGCGCCTCGCACG	67ºC
	ATCACGTGCGAGGCGCACGCGGGGATAGCTGGAAAC	

Table S3 (continuation)

2.12	ACGTGGCGTGCGCCTCGCACCGAGTAACGACCACCG	67ºC
	ATCACGGTGGTCGTTACTCGGTGCGAGGCGCACGCC	
2.13	ACGTGCGAGTAACGACCACCACTTACCTATAAATAG	58ºC
	ATCACTATTTATAGGTAAGTGGTGGTCGTTACTCGC	
2.14	ACGTGACTTACCTATAAATATTCTCCATCTCCAACG	56ºC
	ATCACGTTGGAGATGGAGAATATTTATAGGTAAGTC	
2.15	ACGTGTTCTCCATCTCCAACTCCAAGTCTCCAACCG	62ºC
	ATCACGGTTGGAGACTTGGAGTTGGAGATGGAGAAC	
2.16	ACGTGTCCAAGTCTCCAACCTCAGCTCAGCTCAAGG	63ºC
	ATCACCTTGAGCTGAGCTGAGGTTGGAGACTTGGAC	
2.17	ACGTGTCAGCTCAGCTCAAGCTCGCCGGCGAAGTGG	65ºC
	ATCACCACTTCGCCGGCGAGCTTGAGCTGAGCTGAC	
2.18	ACGTGCTCGCCGGCGAAGTGAACCAGAGAGAGTCAG	65ºC
	ATCACTGACTCTCTCTGGTTCACTTCGCCGGCGAGC	

Chapter 3

Oryza sativa Phytochrome-Interacting Factor 14 (OsPIF14) is involved in rice root curling

Table of Content – Chapter 3

Abstract

Phytochrome-Interacting Factor 14 (OsPIF14) is a bHLH that may mediate light signaling through its interaction with phytochrome B. Here, we show that OsPIF14 is the most expressed *OsPIL* in whole rice seedling and that etiolated seedlings silencing *OsPIF14* (RNAi::*OsPIF14*) show higher percentage of root curling than WT seedlings. Given that phytochromes and jasmonic acid (JA) are known to regulate rice root curling, we hypothesized that OsPIF14 could be the link between phytochromes and JA, more specifically in the regulation of JA biosynthesis and/or signaling. Here, we show that OsPIF14 binds to the promoter of an important JA biosynthesis pathway gene, the *Allene Oxide Synthase 1* (*AOS1*). However, the etiolated RNAi::*OsPIF14* seedlings did not show differences in *OsAOS1* expression compared to WT. In addition, the transcript level of three JA signaling repressors, *jasmonate zim-domain 6* (*JAZ6*), *JAZ11* and *JAZ12*), was analyzed, and two of them showed to be downregulated in RNAi::*OsPIF14* as compared to WT. These results open the possibility that OsPIF14 is regulating root curling through JA biosynthesis and/or signaling. However, the mechanism is still not fully understood and further studies must be performed to better understand the role of *OsPIF14* in rice root curling.

1. Introduction

Plants, as autotrophic organisms, rely on leaves and roots for their growth and development. Roots are particularly important for the uptake of water and nutrients, but also to anchor the plant in the soil. After germination, roots start to explore the environment in order to establish appropriated root architecture. During this process, roots adjust their growth according to external stimuli, such as gravity, temperature, humidity, mechanical barrier, light and hormone signaling.

Studies of rice seminal root growth performed in agar plates show two distinct phenotypes, root waving and root curling. The observed root phenotype is dependent on the agar concentration. Under low agar concentration seminal root tip waves, most probably to facilitate medium penetration (Wang et al., 2011a), however at high agar concentration or after sensing a mechanical barrier, seminal root tip curls (Wang et al., 2011b; Lourenço et al., 2015). Furthermore, it has been shown that root wave and curl phenotypes are specific for each rice variety (Wang et al., 2011a). For instance, wavy root phenotype was observed in *indica* rice varieties, such as TCN1, but not in *japonica* varieties, such as Nipponbare. Furthermore, Wang *et al*. did not observe Nipponbare root curling (Wang et al., 2011a), however, Lourenço *et al.* showed that seminal root of Nipponbare plants is able to curl in response to a mechanical barrier (Lourenço et al., 2015). Nevertheless, these results indicate that the mechanisms behind root wave and root curl can be different, although both are light dependent (Wang et al., 2011b). Experiments of Wang *et al.* in which they covered the shoot of one batch of rice seedlings and covered the roots of the other batch and expose the seedlings to light showed that root waving was only observed in the roots that were exposed to light, indicating that roots perceive light and this phenotype is not due to a signal from shoots (Wang et al., 2011a).

Plants have evolved different photoreceptors that are able to perceive specific wavelengths. Cryptochromes and phototropins are the blue light/UV-A receptors while phytochromes are the red/far-red light photoreceptors. In rice (*Oryza sativa* L. cv. Nipponbare), three phytochromes were identified (phyA, phyB and phyC)

and shown to be present in both shoots and roots (Takano et al., 2005; Shimizu et al., 2009). Phytochromes act as red/far-red switch receptors that upon red light exposure interact with Phytochrome-Interacting Factors (PIFs), inhibiting their activity through sequestration or degradation via 26S proteasome (Al-Sady et al., 2006; Shen et al., 2007; Leivar et al., 2008a). Rice has six Phytochrome-Interacting Factor 3 – like (PILs; OsPIL11 to OsPIL16) (Nakamura et al., 2007). Phytochrome B interacts with PIL14 and PIL16 but the implications of that interaction is unknown. As solo red/far-red light photoreceptors, phytochromes have unique and also overlapping functions. For instance, red and far-red inhibition of seminal root elongation are independent processes. OsphyA is the key player for inhibition of root elongation under far-red light, while under red light both OsphyA and OsphyB are involved in that inhibition (Shimizu et al., 2009).

Inhibition of Nipponbare seminal root elongation was shown to be dependent on light intensity, exposure period and seedling developmental stage. For instance, the third and fourth days (48h-72h) after germination is when seminal root elongation is more inhibited. Moreover, 24h of light exposure even with low intensity is able to inhibit seminal root elongation (Shimizu et al., 2009). The mechanisms behind light inhibition of seminal root elongation is not yet fully understood, however, light seems to play an important role in the inhibition of seminal root elongation and promotion of root curling, through phytochromes (Shimizu et al., 2009). Root curling is a consequence of the continuous cell elongation on the convex side of the root, which originate a curling in the tip of the root. It is now known that there are other factors, such as jasmonic acid (JA), promoting root curling (Jiang et al., 2007; Shimizu et al., 2009; Lourenço et al., 2015).

JA biosynthesis begins in chloroplasts with the cleavage of linoleic acid from chloroplasts membrane. After two oxidation steps and one cyclisation catalyzed by 13- lipoxygenase (13-LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), respectively, the bioactive 12-oxo-phytodienoic acid (12-OPDA) is transported to peroxisome. There, 12-OPDA is converted in 8-Oxopentenyl-

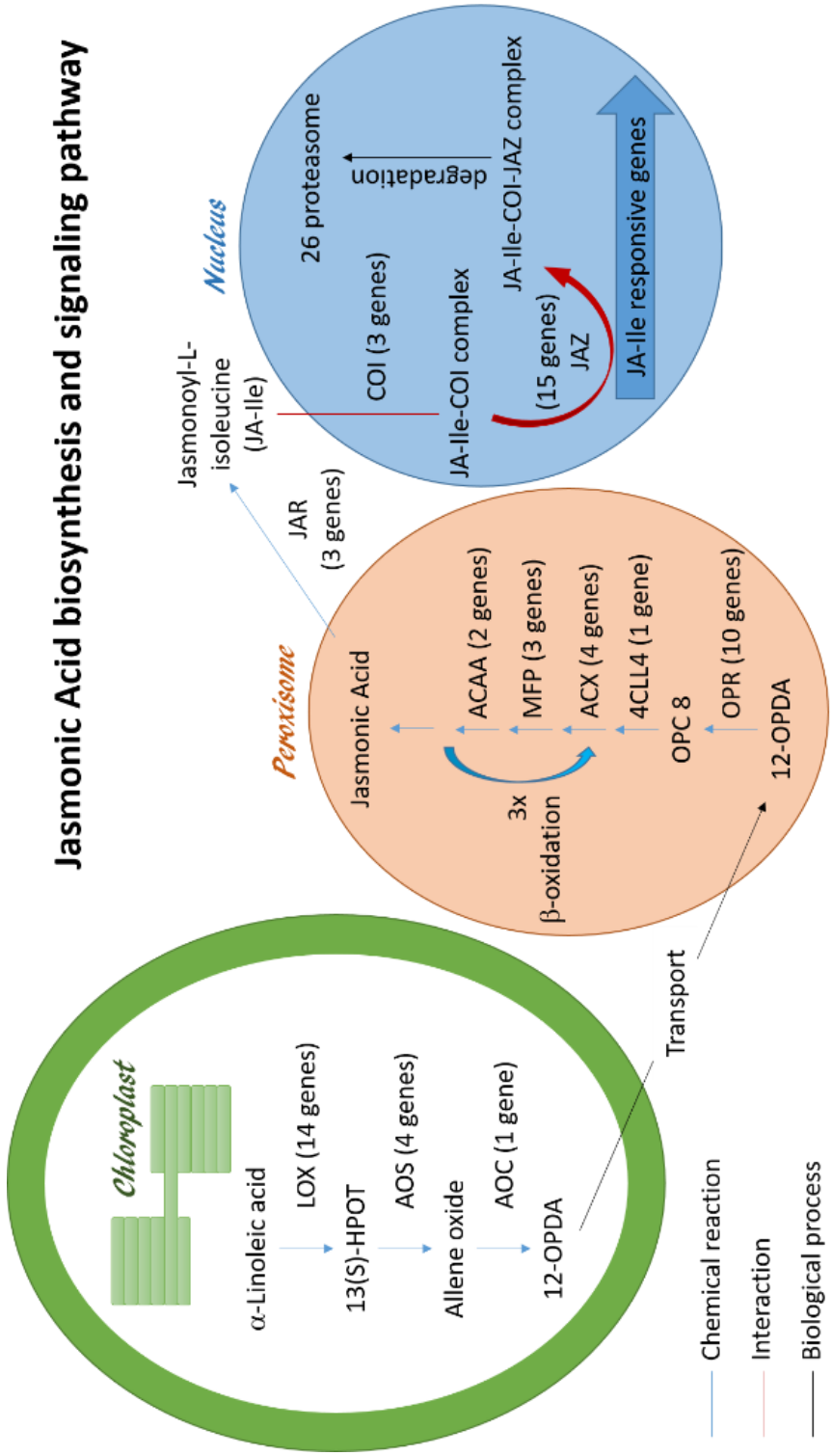

Jasmonic Acid biosynthesis and signaling pathway
Chloroplast
α-Linoleic acid
LOX (14 genes)
13(S)-HPOT
AOS (4 genes)
Allene oxide
AOC (1 gene)
12-OPDA
Transport
Peroxisome
12-OPDA
OPR (10 genes)
OPC 8
4CLL4 (1 gene)
ACX (4 genes)
MFP (3 genes)
ACAA (2 genes)
3x
β-oxidation
Jasmonic Acid
JAR
(3 genes)
Jasmonoyl-L-
isoleucine
(JA-Ile)
Nucleus
JA-Ile-COI complex
COI (3 genes)
26 proteasome
degradation
JA-Ile-COI-JAZ complex
(15 genes)
JAZ
JA-Ile responsive genes
Chemical reaction
Interaction
Biological process

Figure 1 – Schematic representation of jasmonic acid (JA) biosynthesis and signaling pathway. Biosynthesis occurs in chloroplast and peroxisome. α-linoleic acid is converted into 12-OPDA by LOX, AOS and AOC. 12-OPDA is then transported to peroxisome and converted in JA by OPR and three cycles of β-oxidation. Signaling starts in cytoplasm where JA is conjugated with isoleucine (Ile) by JAR regenerating the active signaling molecule (JA-Ile). JA-Ile is transported to nucleus and interact with COI. The JA-Ile-COI complex interact with JAZ and the latter is degraded by the 26 proteasome. Thus, the repression of JA-Ile responsive genes is released and plant is able to react accordingly. In the scheme, it is represented the number of genes of each protein that was used in the *in silico* analysis. For further explanation refer to text. Abbreviations: LOX: Lipoxigenase; AOS: Allene Oxyde Synthase; AOC: Allene Oxyde Cyclase; 12-oxo-phytodienoic acid (12-OPDA); OPR: OPDA reductase; 4CLL4: 4-coumarate-CoA ligase-like 4; ACX: Acyl-CoA oxidase; MFP: b-oxidation multifamily protein; ACAA: Acetyl-CoAacyltransferase; JAR: Jasmonate resistant; COI: Coronatine insensitive; JAZ: Jasmonate zim-domain.

cyclopentyl-octanoate (OPC 8) by OPDA reductase (OPR) family. One molecule of acyl-CoA is bound to OPC 8 by 4-coumarate-CoA ligase-like 4 (4CLL4) and after three cycles of β-oxidation, performed by acyl-CoA oxidase (ACX), β-oxidation multifamily protein (MFP) and acetyl-CoAacyltranferase (ACAA), JA is synthesized and released to cytoplasm (Fig. 1 and reviewed in (Wasternack and Hause, 2013; Dhakarey et al., 2016)). Studies using rice phytochrome mutants unveiled that the expression of several JA biosynthesis genes are regulated by light through phytochromes (Haga and Iino, 2004; Xie et al., 2011). For instance, rice triple mutant, *OsphyAphyBphyC* showed reduced expression of *OsAOS2* and *OsLOX2* (Xie et al., 2011). While, *OsphyA* single rice mutant showed lower expression of *OsAOS1* under far-red light and no differences under red light compared to WT seedlings (Haga and Iino, 2004).

JA is conjugated with isoleucine (Ile), by the enzyme jasmonate resistant (JAR), to produce the bioactive molecule (JA-Ile) and initiate the downstream signal cascade. JA-Ile is translocated into the nucleus where is recognized by its receptor coronatine insensitive (COI), which is an E3-ubiquitin ligase. The complex JA-Ile-COI is able to interact with jasmonate zim-domain (JAZ) proteins

targeting the latter to proteasome degradation. The degradation of JAZ proteins release the JA repression, thus allowing the transcriptional regulation of the JA-Ile responsive genes (Fig.1 and reviewed in (Wasternack and Hause, 2013; Dhakarey et al., 2016)). Rice has fifteen JAZ proteins (Ye et al., 2009), which are involved in the regulation of several processes, such as floral development (Cai et al., 2014), plant defense (Yamada et al., 2012), salinity stress (Wu et al., 2015), stem growth and grain size (Hakata et al., 2012) and also root growth (Thireault et al., 2015). The importance of JA signaling for rice root curling has been clearly shown. For instance, rice plants silencing E3-ubiquitin ligase *OsHOS1* do not show root curling (Lourenço et al., 2015). These lines are less sensitive to JA and only show root curing phenotype under high concentration of JA. Another mutant with altered JA perception, the root meander curling (*OsRMC*) silencing line, show smaller roots and higher sensitivity to JA as compared to WT (Jiang et al., 2007). The mechanism behind these two phenotypes is not yet fully understood, however it is clear that JA perception/signaling plays an important role in rice root curling.

In this chapter, we show that *OsPIF14* is the *OsPIL* with the highest transcript level in the whole seedling. To study its function, we generated *OsPIF14* silencing (RNAi::*OsPIF14*) lines. When grown under constant dark, RNAi::*OsPIF14* seedlings show a higher percentage of root curling as compared to WT. Since root curling has been associated with JA biosynthesis and/or signaling, we decided to analyze how OsPIF14 could be involved in JA biosynthesis and/or signaling. We show that OsPIF14 binds to *OsAOS1* promoter and is able to repress *OsAOS1* expression in rice protoplasts. However, etiolated RNAi::*OsPIF14* plants do not show differences in *OsAOS1* expression as compared to WT seedlings. Moreover, we analyzed the expression of JA signaling genes, and show that *OsJAZ11* and *OsJAZ12* are downregulated in etiolated RNAi::*OsPIF14* seedlings as compared to WT. Despite the fact that we still do not know how OsPIF14 is regulating rice root curling, we propose that OsPIF14 might be involved in the light- and JA-mediated root curling.

2. Materials and Methods

2.1. Plant Material

In this work we used wild type (*Oryza sativa* L. cv. Nipponbare) and generated transgenic RNAi::*OsPIF14* rice plants in the Nipponbare background.

2.2. RNAi::OsPIF14 rice transgenic plants

2.2.1. Preparation of the RNA interference (RNAi) construct

RNAi construct was prepared as previously described by (Lourenço et al., 2013) with few adaptations. GATEWAY-based (Invitrogen, USA) pANDA vector (Miki and Shimamoto, 2004) was used to prepare the RNA interference construct used in this work. A 300 bp region (primers Fw: GGG GAC A AG TTT GTA CAA AAA AGC AGG CTC GTA TCA TTT TTT TGC GCT TAA and Rv: GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA TCC CTA TAC ACT GCT TAT CA; underlined regions are the attB regions) from *OsPIF14* (LOC_Os07g05010) 3'UTR sequence was used to prepare the RNAi genetic construct. The RNAi::*OsPIF14* fragment was cloned into the pDONR221 and, after sequencing confirmation, transferred to the pANDA vector. The correct insertion of the *OsPIF14* fragment was confirmed by PCR using the reverse primer of the construct and the PANDA GUS linker reverse primer (CCA CGT AAG TCC GCA TCT TC) and introduced in *Agrobacterium tumefaciens* strain (EHA105).

2.2.2. Production and selection of transgenic RNAi::OsPIF14 rice plants

To produce transgenic rice plants, we used *Oryza sativa* L. cv. Nipponbare as background following the protocol described by (Hiei and Komari, 2008). Briefly, embryogenic callus tissues were selected and co-cultivated with Agrobacterium EHA105-RNAi::*OsPIF14* strain. The selection process was performed under continuous light and co-cultivated *calli* were selected with 50mg/L of Hygromycin B (Duchefa, The Netherlands). Hygromycin-resistant calli were then transferred to regeneration medium supplemented with 20mg/L Hyg. The

resistant plantlets were potted in containers with soil mixture (2:2:1, v/v/v, soil:peat:vermiculite) and placed in the glasshouse. The putative transgenic plants were analyzed by PCR for the presence of the T-DNA insert. Leaf samples from putative transgenic rice plants were collected, and genomic DNA (gDNA) was immediately extracted using QuickExtract^{TM} Plant DNA Extraction Solution (epicentre) following the manufacturer's instructions. The efficiency of gDNA extraction was assessed by PCR using primers specific for OsUBC2 (Fw: CAA AAT TTT CCA CCC GAA TG and Rv: ATC ACA TGA ATC AGC CAT GC). The transgenic RNAi::*OsPIF14* plants were confirmed by PCR using a combination between the specific primer for pANDA GUS linker and the primer specific for RNAi construct sequence. Only the plants with positive PCR amplification for both sequences were selected as RNAi::*OsPIF14* positive transgenic plants. These were then allowed to self-pollinate to get the T_1 progeny.

2.3. Rice seedling growth

Non-dehusked seeds were surface sterilized with ethanol (70%, v/v) for 1 min with shaking. Seeds were washed twice with sterile water and then incubated in 2% (v/v) sodium hypochlorite solution for 30 min with agitation. Seeds were then washed 8 times with sterile water and germinated in water at 28ºC, in the dark for 3 days.

For rice protoplast isolation, germinated seeds were grown in glass tubes in Yoshida medium (Yoshida et al., 1976) for 7-10 days in the dark at 28ºC.

For the analysis of *OsPILs* gene expression, RNAi::*OsPIF14* and WT germinated seeds were grown in glass tubes containing Yoshida medium supplemented with 0 mg/L or 20 mg/L Hygromycin, respectively, for 10 days at 28ºC, 16h/8h photoperiod. Seven homogenous seedling with no signs of lesions were collected and frozen in liquid nitrogen.

For JA-responsive gene analysis and root phenotype, after surface sterilization, seeds were placed directly in sterile glass tubes containing water and incubated under 16h/8h photoperiod, 28ºC/26ºC (day/night), irradiance of 500

μmolm^{-2}s^{-1} or constant dark 28ºC/26ºC for 4 days. A pool of ten homogeneous seedlings was collected and frozen in liquid nitrogen.

2.4. Rice protoplasts isolation and transformation

To overexpress *OsPIF14* in rice protoplasts the vector pHBT95::OsPIF14::HA was used. OsPIF14::HA was constructed by cloning the coding region of *OsPIF14* (primer Fw: AGTCGGATCCATGGTTCTTGATCGATGTAA and Rv: GCTTGCGGCCGCTTTTATGGTCCCATCAGA; underlined region represent the *BamH*I and *Not*I restriction site, respectively) in pE2C vector (Dubin et al., 2008). Then, OsPIF14::HA was cloned in pHBT95 (Yoo et al., 2007) using the following primers (Fw: AGTCGGATCCATGGTTCTTGATCGATGTAA and Rv: GCTTCTGCAGTCGAGAGTACTGCTAGCGGC; underlined region represent the *BamH*I and *Pst*I restriction site, respectively) and the construct pHBT95::OsPIF14::HA was confirmed by sequencing.

Rice protoplasts were prepared using a protocol previously described by our group (Lourenço et al., 2013). Briefly, cell wall was enzymatically digested and protoplasts were collected by centrifugation. The number of protoplasts was quantified by counting using a haemocytometer. 3x10^5 protoplasts were used per each transformation and transformed with 10ug of pHBT95::OsPIF14::HA using polyethylene glycol. Water transformed protoplasts were used as control. After transformation, protoplasts were incubated in 24 well plate under dark, at room temperature. After 16h of incubation, protoplasts were centrifuged (5min, 300 x *g* at RT), supernatant was discarded, and protoplasts frozen in liquid nitrogen.

2.5. RNA extraction

Whole seedling, without seed, was grinded until fine powder and total RNA was extracted using the Direct-zol RNA Miniprep kit (Zymo Research, USA). In the case of protoplasts two transformations (6x10^5 protoplasts) were combined and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions.

2.6. Reverse transcription-quantitative PCR (RT-qPCR) analysis

cDNA first strand was synthesized from 1 µg or 2 µg of total RNA (for JA-responsive genes expression or *OsPILs* expression, respectively) using oligo-dT primers and Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Switzerland).

In the case of protoplasts, cDNA first strand was synthesized from 0.1 to 0.5 µg of total RNA using oligo-dT primers and SuperscriptIII reverse transcriptase kit (Invitrogen), following the manufacturer's instructions.

Reverse Transcription-quantitative PCR (RT-qPCR) reactions were performed using the Light Cycler 480 system (Roche, Switzerland) and the SYBR Green I Master mix (Roche, Switzerland). For seedlings, the equivalent to 50 ng of total RNA was used *per* reaction, while for protoplasts 15ng to 28ng of total RNA was used *per* reaction. The CT values were calculated from three technical replicates and normalized against the CT values of *OsUBC2*, used as internal control. The method to calculate the relative expression of transcripts was based on the relative quantification of kinetic PCR efficiency correction using the $2^{-\Delta\Delta CT}$ comparison method (Livak and Schmittgen, 2001). The specific primers used in this analysis are described in Table S1.

2.7. Rice root phenotypic analysis

Root curling phenotype was scored at four days after imbibition seedlings grown in glass tubes containing water (as described above). The presence or absence of root curling was visually scored in seedlings with roots longer than 1cm.

2.8. In silico cis-elements identification

To identify putative OsPIF14 targets involved in jasmonic acid biosynthesis and/or signaling, sixty key genes were selected based on literature and KEGG platform (Table S2). The 2000 bp sequences upstream of the translational starting site (ATG) were obtained for each gene from the rice genome annotation (IRGSP-1.0, plants.ensembl.org) using biomaRt (Durinck et al., 2009). Promoters

containing any G-box (CACGCG) or extended N-box (CACGCGG; N-box_G or CCACGCGG; C_N-box_G) were identified using fuzznuc (EMBOSS, Rice et al., 2000) and results were parsed using a custom built python script (Barros, unpublished).

2.9. Yeast One-Hybrid

The *OsAOS1* promoter fragment used as bait for direct Yeast One-Hybrid ranged from -534bp to -24bp, counting from the ATG start codon, was isolated by PCR using the primers 5'-GCGGCCGCCCTACTATTCGGAGGAGCCC-3' and 5'- ACTAGTACCTAGCTACTACTCCCCGA-3' (underlined sequences represent adaptors with restriction enzyme sites). This fragment was cloned as a *Not*I-*Xba*I fragment in the pHIS3/pINT1 vector system (Meijer et al., 1998; Ouwerkerk and Meijer, 2001) and integrated into yeast strain Y187 (Clontech). This bait strain was then transformed with pACTII-OsPIF14 (originally identified in Y1H) (Chapter 2) and grown in CM-His$^-$ medium supplemented with 5mM 3-amino-1,2,4-triazole, as described (Ouwerkerk and Meijer, 2001). The yeast bait strains F3 and F4, harboring the *OsDREB1B* promoter fragments ranging -488bp to -3bp and -1028 to -388bp from ATG, described in Chapter 2, were used as positive and negative control, respectively.

2.10. Expression and purification of recombinant OsPIF14 fused to thioredoxin

OsPIF14 coding sequence was cloned into pET32a (Novagen, USA) in fusion with a thioredoxin tag. The OsPIF14::TRX protein was produced in Rosetta pLysS and purified using HiTrap IMAC FF system (GE Healthcare) and HiLoad 16/600 Superdex 200 pg column (GE Healthcare) as described in Chapter 2. The thioredoxin protein tag was produced similarly.

2.11. Electrophoretic Mobility Shift Assay (EMSA)

DNA probes were generated by annealing oligonucleotide pairs, in a PCR machine as previously described (Serra et al., 2013). The oligonucleotide sequences and annealing temperatures used are described in Table S3. The

OsPIF14-DNA binding reaction was performed as described in Chapter 2. The reactions were incubated for 1 hour on ice and loaded into native 5% polyacrylamide gel (37.5:1). Electrophoresis was run as previously described (Serra et al., 2013).

2.12. Accession numbers

Sequence data of the genes analyzed in this chapter can be found in the Rice Database (http://rice.plantbiology.msu.edu/) under the following accession numbers: *OsPIL11* (LOC_Os12g41650), *OsPIL12* (LOC_Os03g43810), *OsPIL13* (LOC_Os03g56950), *OsPIF14* (LOC_Os07g05010), *OsPIL16* (LOC_Os05g04740), *OsAOS1* (LOC_Os03g55800), *OsJAZ6* (LOC_Os 03g28940), *OsJAZ11* (LOC_Os03g08320), *OsJAZ12* (LOC_Os10g25290), *OsUBC2* (LOC_Os02g42314). The sequence of *OsPIL15* (Os01g0286100; Q0JNI9) can be found in the UniProt Database (http://www.uniprot.org/).

3. Results

3.1. OsPIF14 transcript level is downregulated in RNAi::OsPIF14 seedlings

We have previously identified *Oryza sativa* Phytochrome-Interacting Factor 14 (OsPIF14) as a basic Helix-Loop-Helix (bHLH) transcription factor involved in light response (Cordeiro et al., 2015). In order to better characterize OsPIF14, we generated *OsPIF14* RNA interference lines (RNAi::*OsPIF14*). Rice has six Phytochrome-Interacting Factor 3 – like (PILs; OsPIL11 to OsPIL16) including OsPIF14 (Nakamura et al., 2007). In order to identify the best region to be used in the construct for silencing of OsPIF14, we aligned the cDNA of all *OsPILs*, including 5'UTR and 3'UTR (Fig. 2A). The coding region is relatively conserved among all OsPILs, however, the 3'UTR of *OsPIF14* showed to have a distinct DNA sequence. For that reason the RNA interference construct was designed for the 300bp of the *OsPIF14* 3'UTR (Fig. 2A and 2B). In order to confirm that the RNAi construct is specific for *OsPIF14,* the transcript level of all *OsPILs* was analyzed in *Oryza sativa* L. cv. Nipponbare (WT) and RNAi::*OsPIF14* seedlings. As observed in Fig. 3A, *OsPILs* are differentially expressed in WT seedlings. The gene expression for *OsPIL11* was not possible to detect and *OsPIL12* showed very low levels as compared to the internal control (0.01x OsUBC2), as previously described (Nakamura et al., 2007). On the other hand, *OsPIF14* showed the highest transcript level among the *OsPILs* genes. As shown in Fig. 3B, RNAi::*OsPIF14* mutant lines (#4 and #5) showed a downregulation of *OsPIF14* transcript level as compared to WT (36% and 50%, respectively), while no differences were observed for *OsPIL12* and *OsPIL16*. Nevertheless, *OsPIL13* and *OsPIL15* showed altered gene expression in the RNAi lines. Since the expression of both *OsPIL13* and *OsPIL15* is regulated by light (Nakamura et al., 2007), and taking into consideration that OsPIF14 may mediate light-dependent gene regulation, it is possible that the downregulation of *OsPIF14* in RNAi lines could downregulate *OsPIL13* and upregulate *OsPIL15*. These two RNAi::*OsPIF14* lines will be further characterized in this chapter.

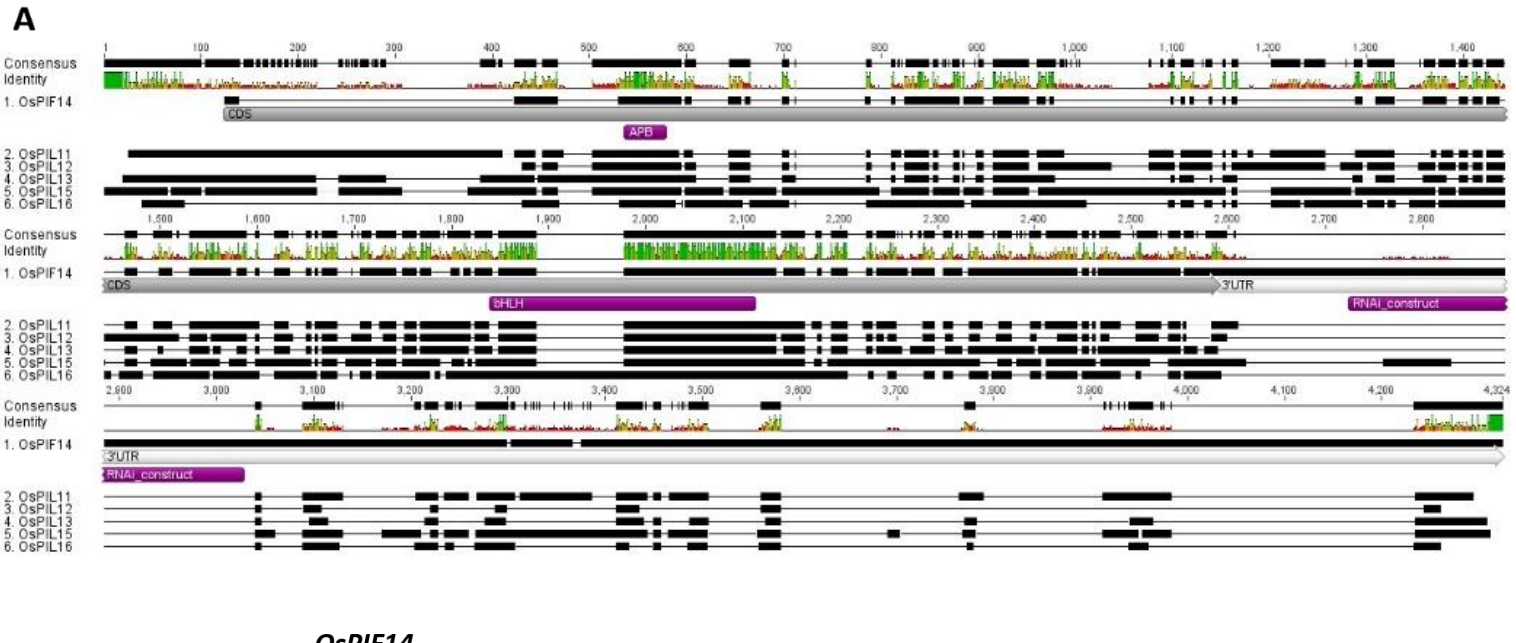


Figure 2. Schematic representation of *OsPIF14* coding sequence, 3'UTR and RNAi construct. A, Alignment of *OsPIF14* with all the other *Oryza sativa* Phytochrome-Interacting Factor-like (*OsPIL11* to *OsPIL16*). Abbreviations: A: predicted active phytochrome B binging domain; bHLH: basic Helix-Loop-Helix domain; RNAi_construct: sequence used for RNA interference. B, Schematic representation of *OsPIF14* coding region (CDS) and 3'UTR including RNA interference (RNAi) sequence localization.

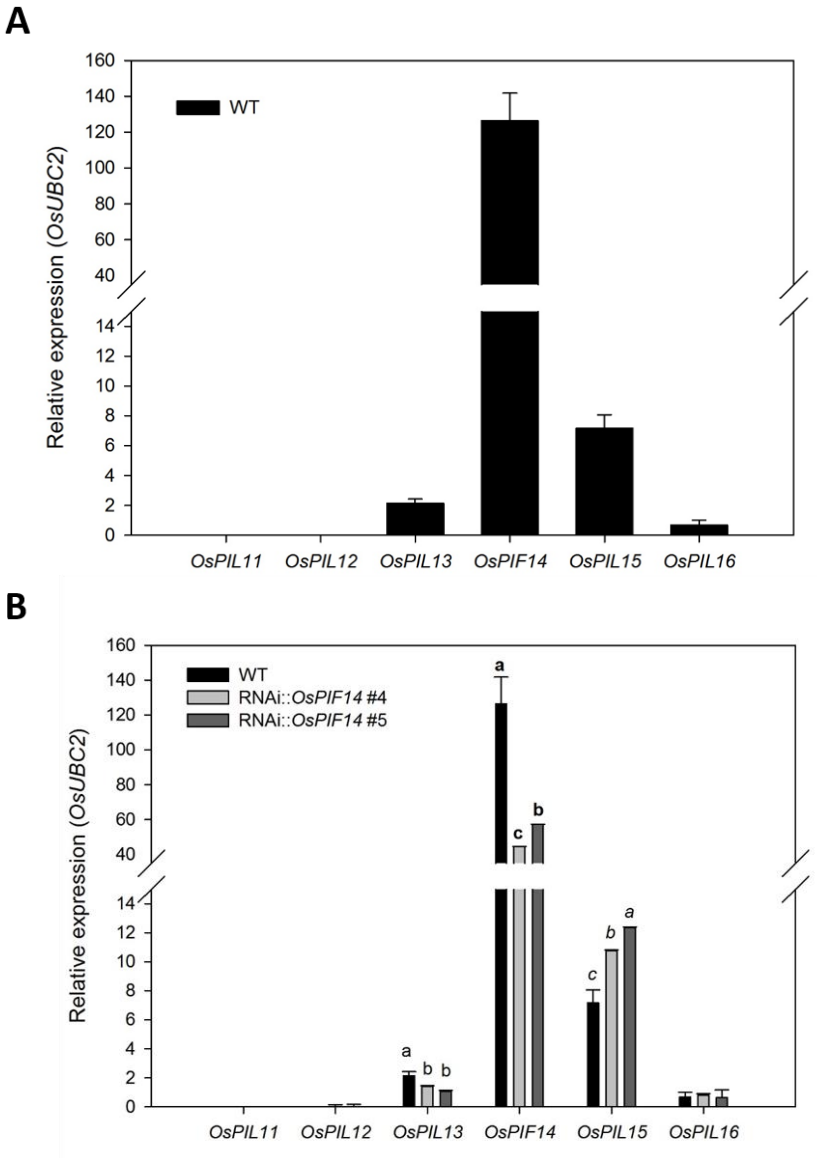


Figure 3. Expression of all OsPILs (OsPIL11 to osPIL16) in whole seedling (14 days after germination). Seedlings were grown in 12h/12h photoperiod and whole seedling was collected 2h after light onset. A, OsPIL expression in WT seedlings grown in Yoshida. B, OsPIL expression in WT and RNAi::OsPIF14 line #4 and #5, grown in Yoshida with 0 and 20 mg/L of Hygromycin, respectively (WT values are the same as shown in Fig. 3A). Expression of each gene was compared between WT and RNAi::OsPIF14 lines and letters indicate differences according to Tukey test ($p<0.05$).

3.2. Etiolated RNAi::OsPIF14 seedlings show higher seminal root curling percentage than WT

In Chapter 2, we showed that OsPIF14 interacts with phytochrome B, thus being regulated by light at protein level. In order to further characterize OsPIF14 light regulation, RNAi::*OsPIF14* and WT seedlings were grown in two light regimes: under 16h/8h or constant dark. Four days after imbibition (DAI), rice seedlings showed the characteristic phenotype and development associated with light or dark growth (Fig. 4A). Under 16h/8h, seminal root of RNAi::*OsPIF14* and WT showed similar curling percentage (Fig. 4B). Interestingly, etiolated RNAi::*OsPIF14* seedlings, grown under constant dark, showed higher seminal root curling percentage as compared to WT (Fig. 4C). Only line #4 showed to be statistically significant different from WT and this might be due to the higher silencing level of these plants.

Seminal root curling is not only regulated by light. In fact, recent studies, have reported the importance of JA in this process (Lourenço et al., 2015). Thus, we hypothesize that OsPIF14 could regulate JA biosynthesis and/or signaling.

3.3. Many JA biosynthesis and signaling pathway genes are putative targets for OsPIF14

In Chapter 2, we described OsPIF14 as a transcription factor able to bind to G-box (CACGTG) and two extended N-box (CACGCGG; N-box_G and CCACGCGG; C_N-box_G) with different strength. We showed that OsPIF14 has higher binding strength to G-box than to extended N-box *cis*-elements. In order to investigate whether OsPIF14 could regulate genes involved in JA biosynthesis or signaling, the 2000bp upstream of ATG of genes involved in JA biosynthesis or signaling pathway (Fig. 1, Table S2) were analyzed *in silico*. We identified one promoter containing both C_N-box_G and G-box, twenty one promoters only with G-box, five promoters with G-box and N-box_G and two containing only N-box_G (Fig.5A). Overall, 29 out of the 60 analyzed gene promoters (48%) contain either G-box or extended N-box in their promoters. This result indicates that almost half of the JA biosynthesis and/or signaling genes are potential direct targets of OsPIF14. Interestingly, genes encoding proteins localized in chloroplast

and nucleus showed higher percentage of potential targets of OsPIF14, 47% and 72%, respectively (Fig. 5B). In this context, the expression of genes encoding enzymes localized in chloroplast, such as LOX, AOS and AOC and/or nuclear proteins, such as COI and JAZ, are the most prone to be regulated by OsPIF14.

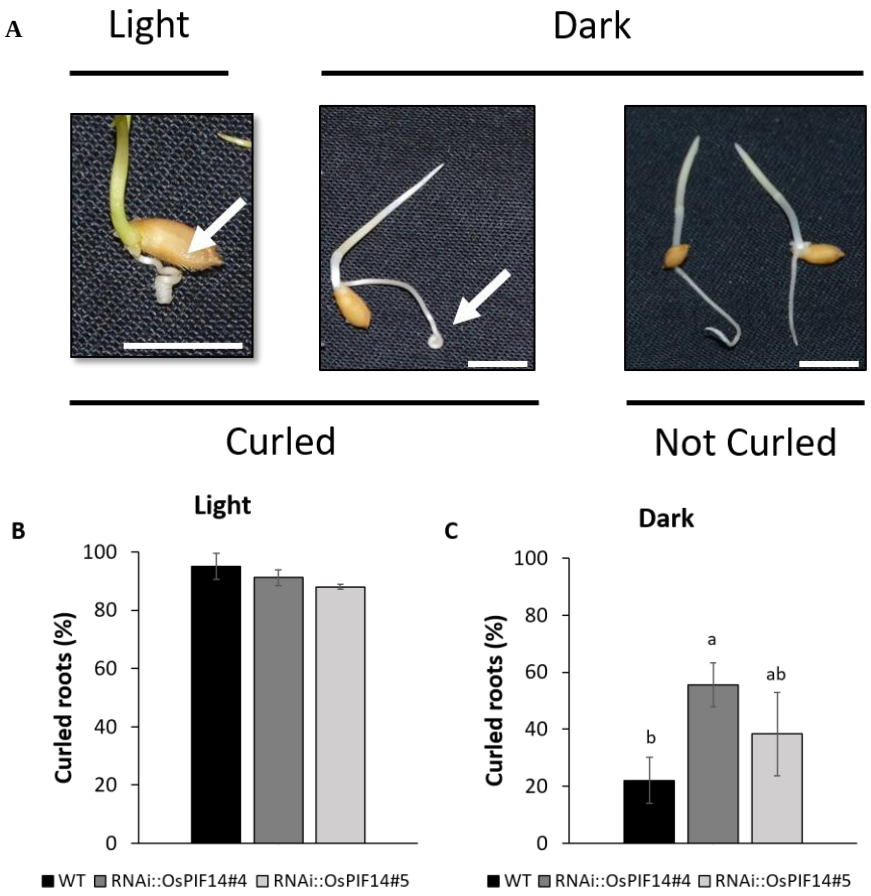
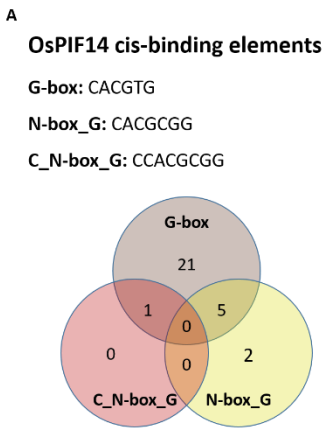


Figure 4. Seminal root curling phenotype of rice seedlings four days after imbibition (DAI). A, Representative photos of rice root phenotype. Arrows show root curling phenotype. Scale bar = 1cm. B, Percentage of root curling in four DAI rice seedlings WT and RNAi::*OsPIF14* lines (#4 and #5) grown in water at 16h/8h or constant dark in test tubes. Letters indicate differences according to Tukey test ($p<0.05$).



B

		G-box + C_N-box_G	G-box	G-box + N-box_G	N-box_G
Chloroplast 47%	LOX (14)	0	6	1	0
	AOS (4)	0	1	0	0
	AOC (1)	0	1	0	0
Peroxisome 30%	OPR (10)	0	1	0	0
	4CLL4 (1)	0	1	0	0
	ACX (4)	0	1	1	0
	MFP (3)	1	0	0	0
	ACAA (2)	0	0	0	1
	JAR (3)	0	0	1	0
Nucleus 72%	COI (3)	0	1	0	0
	JAZ (15)	0	9	2	1

Figure 5. Jasmonic acid biosynthesis and signaling genes containing G-box and or extended N-box within the 2000 bp upstream of translational starting site (ATG). A, Venn diagram representing the number of genes identified with G-box and or extended N-box. B, Number of genes, in brackets, from each family of proteins involved in jasmonic acid biosynthesis and signaling. The % symbol represent the percentage of cis-elements identified in that organelle. Venn diagram was built using the VENNY tool (http://bioinfogp.cnb.csic.es/tools/venny/)

3.4. OsPIF14 binds to a G-box present in the OsAOS1 promoter

The reaction catalyzed by AOS has been considered an important step in JA biosynthesis in potato and Arabidopsis (Harms et al., 1995; Park et al., 2002). In rice, the overexpression of *OsAOS2* was shown to increase JA content (Mei et al., 2006). Rice has four *AOS* genes (*OsAOS1* – *OsAOS4*), however, the *in silico* promoter analysis showed that only *OsAOS1* contains a putative PIF binding element (G-box) in its promoter. Given that *OsAOS1* expression is regulated by light (Haga and Iino, 2004), we hypothesize that *OsAOS1* gene expression could be directly regulated by OsPIF14.

The *in silico* analysis identified a G-box in *OsAOS1* promoter, between 393bp and 387bp upstream of the ATG (Fig. 6A). Interestingly, this position is close to the *OsDREB1B* promoter OsPIF14 binding site, localized in the first 288bp upstream of the ATG (Chapter 2). In order to confirm if OsPIF14 binds to *OsAOS1* promoter through this G-box, a Yeast One-Hybrid assay was performed using as bait the region between -534bp and -24bp upstream of the *OsAOS1* ATG (Fig.6A). The baits used in Chapter 2 were used as controls. The bait F4 of

OsDREB1B, in which OsPIF14 was identified, was used as positive control, and the bait F3 was used as negative control. All the baits were re-transformed with the plasmid containing the *OsPIF14* cDNA clone that was identified in the Y1H screen. Fig. 6B shows that OsPIF14 binds to the region of *OsAOS1* promoter where the G-box is located. This interaction was also confirmed by electrophoretic mobility shift assay (EMSA) (Fig. 6C). The recombinant OsPIF14 (Trx::OsPIF14) protein was incubated with a short *OsAOS1* promoter fragment containing the G-box *cis*-element flanked by 9 bases each side (Fig. 6A; -402bp to -378bp from ATG). In Fig. 6C, we show that Trx::OsPIF14 binds specifically to the G-box present in *AOS1* promoter, since after mutation of the core region of G-box (G-box mut) the signal drastically decreased. In order to confirm that OsPIF14 is able to bind and regulate *OsAOS1* expression *ex vivo*, rice protoplasts were transformed with a construction to overexpress OsPIF14. Fig. 6D shows that OsPIF14 slightly represses *OsAOS1* gene expression in rice protoplasts.

Altogether, these results show that OsPIF14 binds to the promoter of *OsAOS1* through a G-box *cis*-element. Moreover, the OsPIF14 seems to repress *OsAOS1* expression in rice protoplasts. Thus, we hypothesized that RNAi::*OsPIF14* seedlings should modulate *OsAOS1* transcript level.

3.5. OsAOS1 gene expression is similar in RNAi::OsPIF14 and WT etiolated seedlings

In order to investigate our hypothesis, the transcript level of *OsAOS1* was analyzed in etiolated RNAi::*OsPIF14* (#4 and #5) and WT seedlings four DAI. Despite the fact that OsPIF14 binds to *OsAOS1* promoter, the transcript level of *OsAOS1* was similar between RNAi::*OsPIF14* (#4 and #5) and WT seedlings (Fig. 7). These results suggest that the level of silencing of *OsPIF14* in RNAi seedlings is not sufficient to induce alterations in *OsAOS1* transcript level. However, these results do not explain why RNAi::*OsPIF14* show higher root curling under dark. To understand this apparent paradox, the expression of other genes involved in JA biosynthesis and signaling must be analyzed.

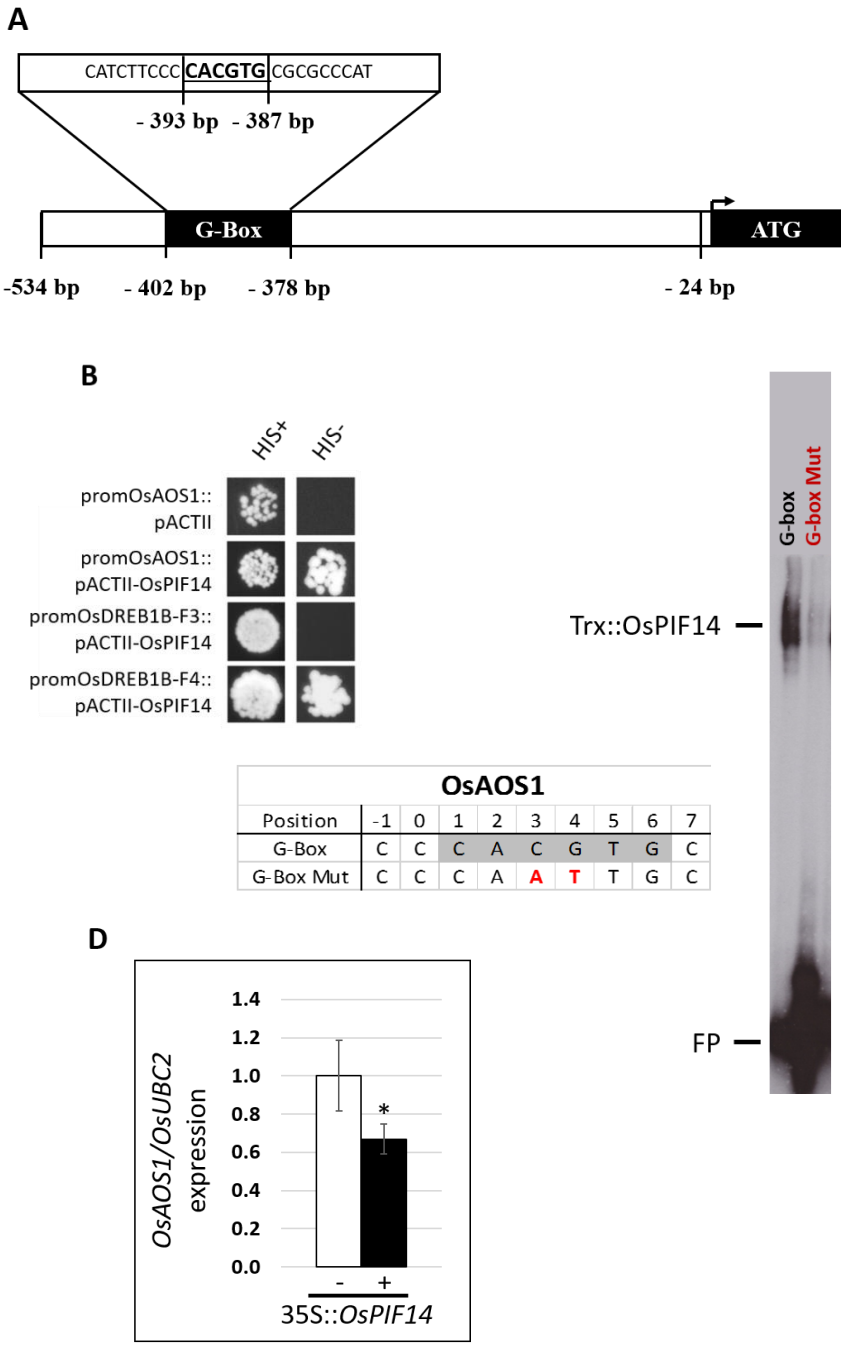


Figure 6. Analysis of OsPIF14 binding affinity and transactivation activity. A, Schematic representation of the 534bp upstream of ATG at *OsAOS1* promoter. The underlined sequence represents G-box localization in the full probe used for EMSA

(panel C). B, Yeast One-Hybrid testing the interaction between OsPIF14 and 534 bp fragment (shown in panel A) of the *OsAOS1* promoter (prom*OsAOS1*). prom*OsDREB1B-F3* was used as negative control. prom*OsDREB1B-F4* was used as positive control. C, EMSA results for *OsAOS1* G-box and G-box Mut probes showing that recombinant protein (Trx::OsPIF14) binds specifically to G-box *cis*-elements. Schematic representation of the native (G-box) or mutated (G-box Mut) G-box *cis*-element used in EMSA. G-box is represented in grey. G-box mutated bases are represented in red. FP, free probe. D, Transactivation assay in rice protoplasts. *OsAOS1* endogenous gene expression analysis performed by RT-qPCR in rice protoplasts transformed with water or *35S::OsPIF14*, after 16h of dark incubation at RT. The transcript level of *OsUBC2* was used as internal control. * Differences statistically significant (t-test, $p<0.06$).

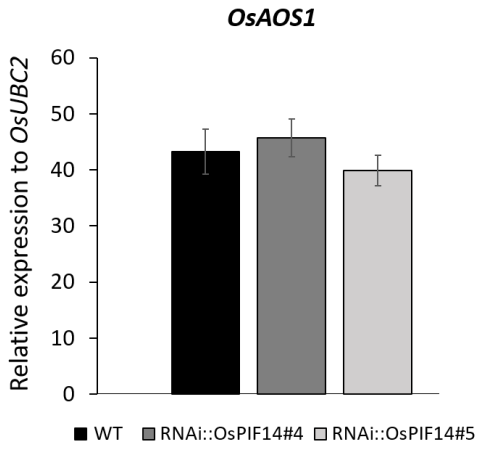


Figure 7. Gene expression analysis of OsAOS1 in four DAI wild type (WT) and RNAi::OsPIF14 lines (#4 and #5) seedlings grown under constant dark. This study was performed by RT-qPCR and the transcript level of OsUBC2 was used as internal control. No statistical differences were detected according to Tukey test ($p=0.193$).

3.6. Etiolated RNAi::OsPIF14 shows downregulation of JA signaling genes

Rice has fifteen *jasmonate zim-domain* (*OsJAZ*) genes (*OsJAZ1-OsJAZ15*) and 73% (11 out of 15) of these genes have at least one G-box in the 2000bp upstream of the ATG (Fig. 5B). From the *OsJAZs* most induced by JA, we selected three that contain a G-box in their promoter (*OsJAZ6*, *OsJAZ11* and

OsJAZ12), and analyzed their transcript level in four DAI etiolated RNAi::*OsPIF14* and WT seedlings. As shown in Fig. 8, the transcript level of *OsJAZ11* and *OsJAZ12* in RNAi::*OsPIF14* seedlings is 50% of the WT, while the *OsJAZ6* gene expression was not altered. Taking in consideration that OsPIF14 acts as a repressor of *OsDREB1B* and *OsAOS1* expression, the downregulation of *OsJAZ11* and *12* in RNAi::*OsPIF14* may indicate that these genes are not direct targets of OsPIF14. Thus, there must be other players downstream of OsPIF14 that are regulating *OsJAZs* expression in etiolated seedlings.

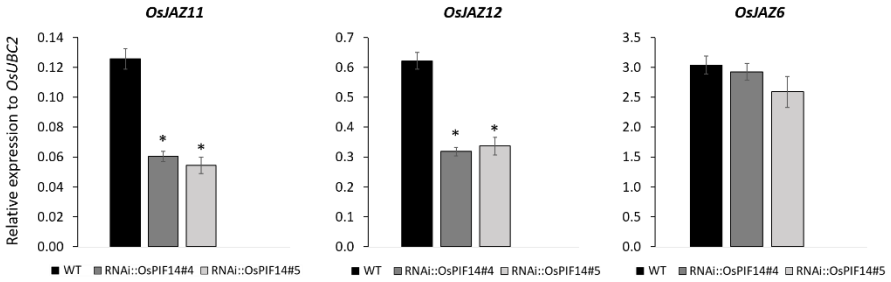


Figure 8. Gene expression analysis of OsJAZ11, OsJAZ12 and OsJAZ6 performed by RT-qPCR in four days after imbibition Wild Type (WT) and RNAi::OsPIF14 lines (#4 and #5) grown under constant dark. The transcript level of OsUBC2 was used as internal control. * Differences statistically significant according to Tukey test ($p<0.001$).

4. Discussion

Plants depend on gene transcription to respond to different stimuli, therefore, this process needs to be tightly regulated. Phytochrome-Interacting Factors are transcription factors involved in several developmental and stress responses. They are more expressed in leaves (Jeong and Choi, 2013) and some rice *OsPILs* (*OsPIL11, 12, 13* and *16*) showed a diurnal expression pattern (Nakamura et al., 2007; Todaka et al., 2012; Jeong and Choi, 2013; Zhou et al., 2014). The expression of all *OsPILs* was analyzed in whole seedling of WT and RNAi::*OsPIF14*. *OsPIF14* is the most expressed Phytochrome-Interacting Factor in WT and despite the fact that the two RNAi::*OsPIF14* lines (#4 and #5) showed a reduction of 65% and 50% of *OsPIF14* transcript, respectively, *OsPIF14* remains the most expressed of all *OsPILs* in these lines. In the specific case of *OsPIL11*, no signal was obtained in real-time PCR analysis of either WT or RNAi lines. This gene was previously shown to be low expressed in rice seedlings (Nakamura et al., 2007). Moreover, the gene expression of *OsPIL11* showed to be organ specific, showing higher expression in new leaves (Li et al., 2012). Thus, the fact that we analyzed whole seedling may explain why we did not detect *OsPIL11* transcript by PCR. We also observed that the expression of *OsPIL12* and *OsPIL16* is low and no significant differences were observed between WT and RNAi lines. These results are in accordance with what has been previously reported (Nakamura et al., 2007). On the other hand, *OsPIL13* is downregulated and *OsPIL15* is upregulated in RNAi::*OsPIF14* lines as compared to WT. In the literature there are reports showing that the transcription of *OsPIL13* and *OsPIL15* is induced and repressed by light, respectively (Nakamura et al., 2007; Todaka et al., 2012; Zhou et al., 2014). Our results suggest that OsPIF14 can be somehow involved in the regulation of *OsPIL13* and *OsPIL15* gene expression. In fact, it was already reported that some PIFs can regulate the expression of other *PIFs*. For instance, in Arabidopsis AtPIF3 binds to *AtPIL1* promoter through a G-box and induces its expression (Soy et al., 2012). In this context, the differences observed in *OsPIL13* and *OsPIL15* expression might be an effect of *OsPIF14* downregulation in RNAi lines. *OsPIL15* has two N-box on the 2000bp upstream

of the start codon, indicating that *OsPIL15* could be repressed by directly by OsPIF14. However, *OsPIL13* do not have a G-box or N-box suggesting that *OsPIL13* could be an indirect target of OsPIF14. Nevertheless, we cannot exclude the hypothesis that the fragment used for silencing *OsPIF14* can also regulate the expression of *OsPIL13* and *OsPIL15*.

Root is a crucial organ for plant survival, providing physical support and uptake of water and nutrients. Therefore, it is important to study and understand the root developmental responses to environmental cues. We observed that our etiolated RNAi::*OsPIF14* silencing lines, show higher percentage of root curling as compared to etiolated WT seedlings. In literature there are only a few reports showing the importance of bHLHs in root development and architecture. The rice mutant lines defective in root hair elongation (*Osrhl1*) showed neither differences in root architecture nor in root development, except for a very long root hair (Ding et al., 2009). On the other hand, in Arabidopsis, the overexpression of *AtPIF3* inhibits root growth under control conditions, and these plants showed to be less sensitive to root growth inhibition induced by nitric oxide (Bai et al., 2014). Interestingly, the *SPATULA (SPT)* gene, which encodes a bHLH transcription factor that evolved from the PIF family by losing an APB-like domain, making its regulation independent of phytochromes (Reymond et al., 2012), was reported to be involved in root growth (Makkena and Lamb, 2013), namely in restriction of root apical meristem size and root length. Loss of function *spt* mutants have a larger zone of cell division leading to a higher growth rate in the roots and longer primary roots (Makkena and Lamb, 2013).

Root growth and development can be modulated by hormones and light. JA promotes rice root curling (Jiang et al., 2007) and light induces JA biosynthesis (Riemann et al., 2003). In fact, there is a strong indication of the involvement of PIFs with the JA signaling pathway. The *pif* quadruple mutant is unable to repost to JA-induced growth inhibition (Yang et al., 2012), showing that PIFs mediate the JA response. Given that OsPIF14 interacts with phytochrome B, and RNAi::*OsPIF14* phenocopies the JA-induced root curling phenotype, we hypothesize that OsPIF14 could be involved in JA biosynthesis and/or signaling.

In Chapter 2, we showed that OsPIF14 binds to G-box and extended N-box and the G-box has been shown to drive plant gene expression in response to light (Giuliano et al., 1988), Therefore, we analyzed the promoter of genes involved in JA biosynthesis and signaling. Forty eight percent of the analyzed genes had a G-box in their promoter, which led us to think that JA pathway could have an enrichment in genes containing G-box in their promoter. To address that, we screen the rice genome to identify all genes that showed a G-box in the 2000bp upstream of the ATG, however, we did not observe an enrichment in JA pathway. These results show that G-box *cis*-elements are present in other light responsive cell pathways.

The *OsAOS1* was one of the identified genes with a G-box in its promoter and it is involved in growth. The rice *OsAOS1* mutant (*cpm1*), which has lower OsAOS1 activity due to a point mutation in one amino acid of OsAOS1, exhibits a long coleoptile phenotype under white light (Biswas et al., 2003). Moreover, the expression of *OsAOS1* is induced by light (Haga and Iino, 2004), indicating that it has potential to be directly regulated by OsPIF14. Thus, we hypothesized that OsPIF14 could bind to *OsAOS1* promoter G-box *cis*-element and regulate *OsAOS1* gene expression. Indeed, we showed that OsPIF14 binds to the G-box *cis*-element present in *OsAOS1* promoter and acts as repressor in transactivation assays. In this context, we expected that the RNAi::*OsPIF14* lines would have increased *OsAOS1* transcript level and consequently more JA, leading to increased percentage of root curling in response to a mechanical barrier. Surprisingly, the expression of *OsAOS1* in etiolated WT and RNAi::*OsPIF14* seedlings was similar. Some possible explanations can be pointed out. 1) Despite the fact that the RNAi lines showed downregulation of *OsPIF14* gene expression (35%-50%), the protein level of OsPIF14 that is being accumulated during four days at dark, could be enough to modulate *OsAOS1* expression. 2) Other bHLH and/or PILs can have redundant functions in the regulation of *OsAOS1* expression. It has been demonstrated that AtPIFs have redundant functions (Leivar et al., 2008b). 3) The regulation of *OsAOS1* by OsPIF14 could occur before the fourth day after imbibition since root curling phenotype is already observed at this point.

4) OsPIF14 does not have a role in *OsAOS1* dark regulation but it can be important during other developmental stages, such as flowering in which it is known that JA is important, for instance to spikelet development (Cai et al., 2014). 5) The fact that we have analyzed whole seedling, could mask small changes in *OsAOS1* transcript level in root. In fact, since JA prioritizes defense over growth (Yang et al., 2012) and we do not observe differences in seedling size and shape, we do not expect to observe high increase in *OsAOS1* expression. In addition, when we supplement etiolated WT seedling with external JA to promote root curling, the rice seedlings showed a strong growth arrestment and no root curling (data not shown) showing that JA pathway is tightly regulated. 6) We cannot rule out the hypothesis that *in planta*, the impact of OsPIF14 on *OsAOS1* expression could be minimal or even absent, since OsPIF14 has a weak transcriptional activity in protoplasts. In order to clarify this apparent contradictory regulation and understand if the observed root curling phenotype is due to the increased JA content, this hormone needs to be quantified in etiolated WT and RNAi::*OsPIF14* seedlings. Etiolated rice seedlings will be collect under dark and light-exposed for a few hours. JA biosynthesis will be induced by light and the role of OsPIF14 in that regulation will be accessed. The results will be crucial to understand if OsPIF14 is involved in JA biosynthesis.

An important step in JA response is the degradation of the constitutive JA signaling repressor, the Jasmonate Zim-domain (JAZ) protein. After JA sensing, JAZ is degraded allowing the transcription of JA-Ile-responsive genes. Interestingly, the JA signaling is regulated by a negative feedback loop in which ten *OsJAZ* are induced by JA and half show more than 10 fold induction (*OsJAZ5, 6 11, 12* and *13*) within two hours (Ye et al., 2009). Among these five *OsJAZ*, *OsJAZ6*, *OsJAZ 11*, and *OsJAZ 12* have a G-box in their promoter. When the expression of these genes was analyzed in etiolated WT and RNAi::*OsPIF14* seedlings, no differences were detected in *OsJAZ6* expression, while the expression of *OsJAZ11* and *OsJAZ12* showed to be downregulated in the RNAi::*OsPIF14* lines. In Arabidopsis, it was shown that PIFs repress the expression of *JAZ9* (Leivar and Monte, 2014). However, our results suggest that

OsJAZ11 and *OsJAZ12* are not direct targets of OsPIF14 unless OsPIF14 could be acting has an activator of *OsJAZ* expression. In fact, in Arabidopsis, it was shown that AtPIF7 has a repressor domain at C-terminal and an activation domain at N-terminal (Kidokoro et al., 2009). However, the most plausible explanation is that *OsJAZ11* and *OsJAZ12* are not directly regulated by OsPIF14 at transcriptional level and there must be an unknown regulator of *OsJAZs* downstream of OsPIF14. Moreover, in rice and Arabidopsis it was demonstrated that JAZ are able to interact with bHLH (Seo et al., 2011; Song et al., 2013). The latter, act as repressors of JA response, which add another layer of regulation between bHLH and JAZ (Song et al., 2013). Nevertheless, our results indicate that the JA signaling pathway is impaired in RNAi::*OsPIF14* lines.

The expression of *OsJAZ6*, *OsJAZ11* and *OsJAZ12* was previously analyzed in the RNAi::*OsHOS1* lines, which do not show root curling under light (Lourenço et al., 2015). In these lines, it was observed that the expression of *OsJAZ6* was similar to WT while *OsJAZ11* and *OsJAZ12* were upregulated (data not shown), which, being opposite to what we observe in RNAi::*OsPIF14* lines, correlates with the root phenotype. This regulation of *OsJAZ*, especially *OsJAZ11* and *OsJAZ12*, prompted us to suggest that *OsJAZ11* and *OsJAZ12* are important for root curling regulation, however, the expression of the other *OsJAZ* genes needs to be analyzed.

Overall, our results show that OsPIF14 is involved in rice seminal root curling, however the mechanism behind that regulation is still not yet fully understood. Here, we showed some indications that OsPIF14 can be involved in the regulation of the rice seminal root curling by modulating JA biosynthesis and/or signaling. However, to fully understand the molecular mechanisms underlying seminal root curling and the role of OsPIF14, further analyses must be carried out.

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André M. Cordeiro performed the experimental work with the following collaborations: Pedro Barros contributed for the *in silico cis*-elements identification; Isabel A. Abreu contributed for the purification of the recombinant OsPIF14 protein; Tiago Lourenço and Rita Leal contributed for Yeast One-Hybrid transformation. M. Cecília Almadanim and Tiago Lourenço for the advices regarding the production of transgenic RNAi::*OsPIF14* rice plants; Diego Almeida for the advices regarding EMSA; Tiago Lourenço also contributed for rice protoplasts isolation and rice root curling phenotypic analysis. The planning of the research work and discussion of results was carried out by André M. Cordeiro, Nelson Saibo, Isabel A. Abreu, Tiago Lourenço and M. Margarida Oliveira.

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7. Supplemental data

Table S1. Primers used for Reverse transcription-quantitative PCR (RT-qPCR) analysis

Gene	Primer sequence (5'- 3')
OsPIL11	AACACCACCAGCATGGGCGA
	ACGACGTGGCCATTGCACCA
OsPIL12	ATTATCCGTTGGGGGCCAAG
	AGTAGAAGGATCCCATACCTGA
OsPIL13	GAGGCAACCGAAGAGACCAA
	TGAACTTCAGCTGCACGAGT
OsPIF14	TAATCGCATCCGCGGGCCAA
	TGGAATGACAGCGCCAGAGAGT
OsPIL15	ATTTGCATCTTTGGCTGGTC
	ATTCGCTATGCCTTGTTGCT
OsPIL16	ACCATGCACCATCCACAACA
	GTCTCTTCTCCTCTGCGAGC
OsAOS1	GCCCGGTCATCTTATTTTCC
	ACCAGTGCAACTCCGTATCC
OsJAZ6	GGGTTCCCCTTCAGATGC
	CGGCAACCTGAGGTCCTA
OsJAZ11	CAGCAGGAAGCCAAAGTGT
	TACCGGCCATGTTGTAGCTC
OsJAZ12	GCCGGAGAAAAGCTTCG
	CTCCTCTAAACCGGGCAGA
OsUBC2	TTGCATTCTCTATTCCTGAGCA
	CAGGCAAATCTCACCTGTCTT

Table S2. Key genes of jasmonic acid biosynthesis and signaling pathway analyzed *in silico*

Gene		MSU Locus identification
Lipoxigenase (OsLOX) (Umate, 2011)		LOC_Os02g10120
		LOC_Os02g19790
		LOC_Os03g08220
		LOC_Os03g49260
		LOC_Os03g49380
		LOC_Os03g52860
		LOC_Os04g37430
		LOC_Os05g23880
		LOC_Os06g04420
		LOC_Os08g39840
		LOC_Os08g39850
		LOC_Os11g36719
		LOC_Os12g37260
		LOC_Os12g37320
Allene Oxyde Synthase (OsAOS) (Haga and Iino, 2004)	OsAOS1	LOC_Os03g55800
	OsAOS2	LOC_Os03g12500
	OsAOS3	LOC_Os02g12680
	OsAOS4	LOC_Os02g12690
Allene Oxyde Cyclase (OsAOC) (Riemann et al., 2013)		LOC_Os03g32314

Table S2 (continuation)

Gene		MSU Locus identification
OPDA reductase (OsOPR) (Tani et al., 2008)	OsOPR1	LOC_Os06g11290
	OsOPR2	LOC_Os06g11280
	OsOPR3	LOC_Os06g11260
	OsOPR4	LOC_Os06g11240
	OsOPR5	LOC_Os06g11210
	OsOPR6	LOC_Os06g11200
	OsOPR7	LOC_Os08g35740
	OsOPR8	LOC_Os02g35310
	OsOPR9	LOC_Os01g27240
	OsOPR10	LOC_Os01g27230
4-coumarate-CoA ligase-like 4 (4CLL4) (Kanehisa and Goto, 2000; Kanehisa et al., 2016)		LOC_Os03g04000
Acyl-CoA oxidase (ACX) (Kanehisa and Goto, 2000; Kanehisa et al., 2016)	ACX1	LOC_Os06g01390
	ACX2	LOC_Os11g39220
	ACX	LOC_Os01g06600
	ACX	LOC_Os05g07090

Table S2 (continuation)

Gene		MSU Locus identification
β-oxidation multifamily protein (MFP) (Kanehisa and Goto, 2000; Kanehisa et al., 2016)		LOC_Os01g24680
		LOC_Os02g17390
		LOC_Os05g29880
Acetyl-CoAacyltranferase (ACAA) (Kanehisa and Goto, 2000; Kanehisa et al., 2016)		LOC_Os02g57260
		LOC_Os10g31950
Jasmonate resistant (OsJAR) (Wakuta et al., 2011)	OsJAR1	LOC_Os05g50890
	OsJAR2	LOC_Os01g12160
	OsJAR3	LOC_Os11g08340
Coronatine insensitive (OsCOI) (Lee et al., 2013)	OsCOI1a	LOC_Os01g63420
	OsCOI1b	LOC_Os05g37690
	OsCOI2	LOC_Os03g15880

Table S2 (continuation)

Gene		MSU Locus identification
Jasmonate zim-domain (OsJAZ) (Ye et al., 2009)	OsJAZ1	LOC_Os04g55920
	OsJAZ2	LOC_Os07g05830
	OsJAZ3	LOC_Os08g33160
	OsJAZ4	LOC_Os09g23660
	OsJAZ5	LOC_Os04g32480
	OsJAZ6	LOC_Os03g28940
	OsJAZ7	LOC_Os07g42370
	OsJAZ8	LOC_Os09g26780
	OsJAZ9	LOC_Os03g08310
	OsJAZ10	LOC_Os03g08330
	OsJAZ11	LOC_Os03g08320
	OsJAZ12	LOC_Os10g25290
	OsJAZ13	LOC_Os10g25230
	OsJAZ14	LOC_Os10g25250
	OsJAZ15	LOC_Os03g27900

Table S3: Probes used for Electrophoretic Mobility Shift Assay (EMSA)

Probe	Oligonucleotide Sequence 5'-3'	Annealing temperature
G-BOX	ACGTGCATCTTCCCCACGTGCGCGCCCATG	62ºC
	ATCACATGGGCGCGCACGTGGGGAAGATGC	
G-BOX_mut	ACGTGCATCTTCCCCAATTGCGCGCCCATG	62ºC
	ATCACATGGGCGCGCAATTGGGGAAGATGC	

Chapter 4

OsPIF14 characterization: Light stability and transcriptome analysis of rice silencing lines

Table of Content – Chapter 4

Abstract

In *Arabidopsis thaliana*, Phytochrome-Interacting Factors (PIFs) accumulate under dark playing an active role in the induction of seedling growth, while under light, PIFs are degraded in a process dependent on phytochromes. In rice (*Oryza sativa* L.), the stability of the PIF proteins subjected to different light conditions was never assessed, and the function of the rice PIFs is still poorly characterized. Here, the light stability of one of the rice PIFs (OsPIF14) was evaluated *in vitro*, and our results suggest that OsPIF14 is degraded in a process dependent on OsphyB. To better understand the function of OsPIF14, the transcript profile of RNAi::*OsPIF14*, *OsphyB*, and WT rice seedlings was analyzed in the transition from dark to light, using microarrays. Our results show two major clusters, dark and light samples. In each cluster, the transcripts of RNAi::*OsPIF14* lines are more similar to WT than to *phyB*, showing that light and OsPIF14 have, respectively, the highest and lowest effect on gene regulation. A few genes were identified as differently expressed genes (DEG) in RNAi lines as compared to the other lines. All the upregulated genes show a *cis*-element in their promoter where OsPIF14 could bind. This observation is consistent with the repressor transcriptional activity previously observed for OsPIF14, suggesting that these genes could be directly regulated by OsPIF14. Moreover, the only DEG found to be downregulated in RNAi lines as compared to WT did not have any *cis*-element where OsPIF14 could bind. Altogether, our microarray data suggest that OsPIF14 may directly or indirectly regulate the expression of genes involved in several processes, such as growth, lipid metabolism, and cell wall structure and organization.

1. Introduction

Light represents a vital source of energy for plants. It is crucial to regulate many processes throughout plant life cycle, from seedling growth and development until flowering time and grain quality and yield (Liu et al., 2014). It is therefore not surprising that plants developed mechanisms to perceive light intensity, wavelength, and photoperiod. Plants can perceive light through specialized photoreceptors, which include phytochromes, cryptochromes, and phototropins, among others. Thus, understanding the mechanisms underlying plants light response will contribute to improving plant growth/development and productivity.

Phytochromes are the red/far-red light photoreceptors (Takano et al., 2009) and are constituted by a light absorbing chromophore group coupled to the phytochrome apoprotein (PHY). In the cytosol, the phytochrome chromophore (linear tetrapyrrole chromophore - phytochromobilin) is covalently bound to a cysteine in PHY N-terminus (Rockwell et al., 2006) to constitute the bioactive light receptor molecule, the phytochrome holoprotein (phy). After reconstitution, phy proteins are in their inactive form (Pr), which can absorb red light (~660nm) (Björn, 2015). After red light absorption, phy conformation changes to the active form (Pfr) and phy proteins are translocated to the nucleus (Huq et al., 2003; Fankhauser and Chen, 2008). There, phytochromes interact with Phytochrome-Interacting Factors (PIFs) (Ni et al., 1998), thus inducing PIF phosphorylation and consequent proteasome degradation (Al-Sady et al., 2006; Shen et al., 2007; Ni et al., 2013). This process can be quickly reverted by far-red light (~740nm), or more slowly by dark incubation (dark reversion), in which the phytochrome is converted back to its Pr form and consequently cannot interact with PIF (Ni et al., 1999).

In *Arabidopsis thaliana*, five phytochrome proteins (phyA to phyE) (Kircher et al., 2002) and seven PIFs (AtPIF1, 3, 4, 5, 6, 7, 8) (Leivar and Quail, 2011) were identified. All AtPIFs have an active phyB binding (APB) domain in their N-terminus (Leivar and Quail, 2011), but only AtPIF1 and AtPIF3 have an active phyA binding (APA) domain (Leivar and Quail, 2011). The presence of both

domains APB and APA is necessary for AtPIF3 red light-induced phosphorylation and degradation, since AtPIF3 degradation is only abolished when both APB and APA are mutated (Al-Sady et al., 2006). This type of cooperative phy function was also observed in AtPIF5 degradation, since only in the *phyAB* double mutant the AtPIF5 degradation after red light was reduced (Shen et al., 2007). However, the degradation of AtPIF5 by red light was only eliminated in the triple *phyABD* mutant (Shen et al., 2007). Nevertheless, phosphorylation-mediated degradation does not seem to be the only mechanism to control AtPIFs activity, since AtPIF7 is not phosphorylated and/or degraded after interaction with phytochrome B (Leivar et al., 2008; Kidokoro et al., 2009). Altogether, the currently established model of regulation of PIFs proposes that the level of PIF proteins fluctuate along with the day/night cycle, displaying residual protein levels under light and accumulating at the end of the dark period as a consequence of the lower levels of phytochrome Pfr. Under a short-day regime, the accumulation of PIF at the end of the night period was shown to be important to regulate the expression of genes containing G-box (CACGTG) *cis*-elements in their promoter and to induce hypocotyl growth (Soy et al., 2012). Whereas upon light exposure, photoactivated phy reverses this PIF activity by targeting PIFs to proteolytic degradation (Soy et al., 2012; Leivar and Monte, 2014). The use of Arabidopsis quadruple *pif* mutant (*pifq*) lacking PIF1, PIF3, PIF4, and PIF5, which under dark conditions phenocopies the morphological development of light grown wild type, was crucial to unveil new *cis*-element to which PIFs can bind, e.g. the PIF binding E-box (PBE; CACATG) (Zhang et al., 2013). Under constant dark, PIFs bind preferentially to G-box and PBE representing 73% of the PIF binding sites (Zhang et al., 2013).

In rice (*Oryza sativa* L.), three phytochrome proteins (OsphyA to phyC) (Takano et al., 2005) and six PIF-like (PILs) proteins (OsPIL11 to OsPIL16) (Nakamura et al., 2007) have been identified, but their interactions, as well as the function of these interactions, is largely unknown. Phytochromes are the only red/far-red light photoreceptors in rice, and they are involved in the regulation of seedling de-etiolation (Takano et al., 2001), inhibition of seminal root elongation

(Shimizu et al., 2009), leaf blade angle (Takano et al., 2005), flowering time (Takano et al., 2005), and fertility (Takano et al., 2009). However, OsPILs are poorly characterized. The first study towards the characterization of rice PIL function was carried out by Nakamura *et al.* in which they overexpressed several OsPILs (OsPIL11 to OsPIL15) in Arabidopsis and observed hypocotyl elongation higher than WT under short-day conditions. The authors compared these mutant plants to the Arabidopsis *phyB* mutant (*phyB-9*) and the AtPIL6-ox line, which showed similar hypocotyls length (Nakamura et al., 2007). These results demonstrated that OsPILs have the functional ability to interfere with the light signaling pathway in a way similar to that of AtPIFs. More recently, it was shown that OsPIL11 is involved in hypocotyl growth regulation. Tobacco seedlings overexpressing OsPIL11 showed shorter hypocotyl as compared to non-transformed plants under continuous red light (Li et al., 2012). Moreover, the functional characterization of OsPILs was also analyzed in rice, namely by overexpression of OsPIL13, OsPIL15, or OsPIL16. OsPIL13 was shown to promote internode elongation, and OsPIL13-ox rice lines showed to be taller at reproductive stage as compared to WT (Todaka et al., 2012). The OsPIL15-ox lines showed shorter aerial parts under dark when compared to WT plants. This phenotype was reverted upon red or far-red light exposure. The same study also revealed that OsPIL15 represses a set of genes involved in the auxin pathway and cell wall organization (Zhou et al., 2014). The OsPIL16-ox rice lines showed enhanced cold tolerance and increased *OsDREB1s* expression. Authors proposed that OsPIL16 enhances cell membrane integrity, which would increase cold tolerance (He et al., 2016). Altogether, these studies show that OsPILs play a role in seedling growth, namely by regulation of cell elongation, membrane integrity, and cell wall organization.

In this chapter, we aim at understanding how is OsPIF14 regulated by light and what are the genes regulated by OsPIF14. We show that OsPIF14 degradation is, at least partially, phyB dependent, and our transcriptomic studies suggest that OsPIF4 is involved in the regulation of genes involved in cell wall structure and/or cell elongation, and lipid metabolism.

2. Materials and Methods

2.1. Plant Material

In this work, we used wild type (*Oryza sativa* L. cv. Nipponbare), two independent transgenic RNAi::*OsPIF14* rice plants (Nipponbare background) generated as described in Chapter 3 (from now on called RNAi#1.2 and RNAi#1.3) and *OsphyB-1* (Takano et al., 2005) (Nipponbare background) mutant plants.

2.2. Rice seedling growth

Non-dehusked seeds were surface sterilized with ethanol (70%, v/v) for 1 min with shaking. Seeds were washed twice with sterile water and then incubated in 2% (v/v) sodium hypochlorite solution for 30 min with agitation. Seeds were then washed 8 times with sterile water and germinated in water at 28ºC, in the dark for 3 days. Germinated seeds were grown in sterile glass tubes containing Yoshida medium supplemented with 0 mg/L (Nipp and *phyB*) or 30 mg/L (RNAi::*OsPIF14* #1.2 and #1.3) Hygromycin, for 4 days at 28ºC, 16h/8h photoperiod (500 μmol m^{-2} s^{-1}) to select mutant plants, and then grown in Yoshida without Hygromycin. For transcriptomic studies, five homogenous 14-day-old seedlings were collected 30 minutes before and after light onset and immediately frozen in liquid nitrogen, except for RNAi::*OsPIF14* line #1.2 to which only two seedlings were available. Time points were collected in triplicate. For *in vitro* assays, eight seedlings were collected 30 minutes before light onset.

2.3. Protein extraction and quantification

Fourteen-day-old seedlings without seed were grinded until fine powder. Total protein was extracted from 100mg of ground material by mixing it with 200μL extraction buffer (25mM Tris.HCl pH 7.5, 10mM NaCl, 10mM $MgCl_2$, 5mM DTT and 1x mix of protease inhibitor cocktail (cOmplete, EDTA-free from Roche (#11873580001) and protease inhibitor from Sigma (I3911) and homogenized with a vortex. Samples were cleared at 17,000 x *g* at 4ºC for 15 min. The supernatant was collected, and total soluble protein was quantified using Amido

Black staining method. Briefly, 10 μL of total protein was precipitated at 17,000 x *g* at 4ºC for 15min using 1mL of Amido Black and supernatant was discarded. After washing the protein pellet with 1mL of destaining solution (10% Acetic Acid and 90% Methanol), this was precipitated at 17,000 x *g* at 4ºC for 15min and supernatant was discarded. The protein pellet was air dried, resuspended in 1mL of 0.2M NaOH, and absorbance measured at 620nm. Total protein concentration was calculated by comparison with BSA calibration curve. The whole procedure was performed under green light conditions.

2.4. In vitro degradation assay

The *in vitro* degradation reaction was performed in 16 μL final volume as previously described (Lourenço et al., 2015) with some modifications specific for light treatments. All the sample manipulation was performed under green light at 28ºC. Briefly, a master mix was done in 200μL tubes using for each reaction 4 μg of total protein extract, 4 μL of 4x degradation assay buffer (100mM Tris.HCl pH 7.5, 40mM $MgCl_2$, 40mM NaCl, 20mM DTT, 20mM ATP, 4x mix of protease inhibitor cocktail (described above)), 2μg of His::OsPIF14 recombinant protein and water to reach a final volume of 16 μL. The reaction time started after the addition of recombinant protein, obtained as described in Chapter 2. In some samples, MG132 (Merck, #474790) was added to reach a final concentration of 100μM (1% of reaction volume) and incubated for 30 minutes under green light before addition of recombinant OsPIF14 protein. The degradation assay was performed by incubating the master mix under continuous red LED light (656nm, 10μmol m^{-2} s^{-1}), continuous far-red LED light (740nm, 10μmol m^{-2} s^{-1}) or dark. Samples of 16 μL were collected from the master mix, and the reaction was stopped by the addition of 4 μL of 5x Laemmli buffer (final concentration 1x) and frozen in liquid nitrogen.

2.5. Western blotting to detect His::OsPIF14

The whole protein sample (20 μL) was boiled at 70ºC for 10 min and loaded into a 10% SDS-PAGE (37.5:1). After transferring the separated proteins

overnight to a PVDF membrane, they were stained with Amido Black dye for 1min and destained for 1min with a destaining solution (10% Acetic Acid and 90% Methanol). The membrane was blocked for 1 hour with skim milk and then incubated with α-His (GE Healthcare, 27-4710-01) (diluted 1:3000) for 1h at RT. Incubation with secondary antibody (α-mouse–HRP; GE Healthcare) diluted 1:20000 in blocking solution was performed for 1h at RT. Detection was carried out with ECL Western Blotting detecting reagent (Amersham) and luminescent light emitted from membrane was record and by CCD camera "Fusion SL Imaging System" by Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany.

2.6. RNA extraction and Microarray data analysis

Whole 14-day-old seedlings, without seed, were ground and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. Total RNA was kept at -80ºC until labeling and hybridization to the Affymetrix Rice (US) Gene1.1 ST Array Strip (ssp. Japonica) at the Affymetrix core facility (Instituto Gulbenkian de Ciência, Oeiras, Portugal). This array contains 816815 probes to query 45207 transcripts. Gene level normalization and signal summarization were performed on Affymetrix Expression Console Software. Chipster software was used for gene filter by statistical significance (Bayes t-test, p value<0.05) and for Benjamini and Hochberg's false discovery rate test (0.05 threshold) of filtered data. To reduce background signals, we considered as differentially expressed genes (DEGs) the ones that showed to have 2.5 or more fold difference. To determine the biological function of the differentially expressed genes, we have performed functional annotation using the Blast2GO (version 4, Sept 2016) software. The characterization was based on BLASTp (version 2.6.0) results against NCBI's non-redundant protein sequences (nr) database (December 2017).

3. Results

3.1. OsPIF14 is degraded in a process dependent on OsphyB

Several attempts to characterize OsPIF14 light regulation *in planta* were made, however without success. The antibody raised again OsPIF14 recognize the recombinant protein, but does not detect the endogenous OsPIF14 in total protein extract. One possible explanation might be related with the low level of OsPIF14. To increase the amount of endogenous OsPIF14, rice *calli* were transformed to overexpress OsPIF14 but the overexpressing lines were not ready to use for this assay. Moreover, to the best of our knowledge, there are no transgenic rice lines overexpressing OsPIF14 available. Thus, as alternative to assess OsPIF14 light stability, an *in vitro* OsPIF14 degradation assay was performed. The recombinant His::OsPIF14 protein was incubated with total protein extract of WT to test OsPIF14 light stability. As shown in Fig. 1 (left panel), under continuous red light exposure, the recombinant His::OsPIF14 is degraded in about 10 minutes after incubation with WT total protein extract. We have previously shown that OsPIF14 interacts weakly with OsphyA and OsphyC and strongly with OsphyB (see Chapter 2). Therefore, we hypothesized that OsphyB could be involved in OsPIF14 degradation. To investigate this, the recombinant OsPIF14 protein was also incubated with total *OsphyB* protein extract and exposed to continuous red light. As shown in Fig.1 (right panel) OsPIF14 degradation is slower in a total protein extract from *OsphyB* mutant plants than in WT. This result was the first indication that OsphyB could be involved in OsPIF14 degradation.

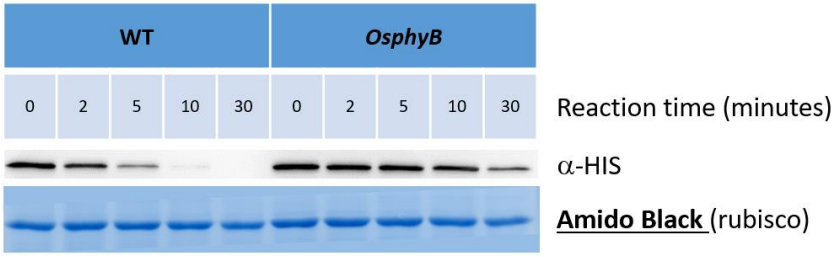


Figure 1. OsPIF14 degradation in WT and *OsphyB* mutant. *In vitro* degradation assay of 2µg of OsPIF14 recombinant protein under continuous red LED light (10µmol m^{-2} s^{-1}), using 4µg of total protein extract from either WT or *OsphyB* mutant over 30 minutes. The recombinant OsPIF14 was detected with α-HIS and rubisco stained with amido black was used as loading control

3.2. OsPIF14 degradation kinetics is unchanged under far-red or dark

In Arabidopsis, AtPIFs (e.g AtPIF3, AtPIF5) degradation rate decreases dramatically under far-red light, however it is not completely stopped (Al-Sady et al., 2006; Shen et al., 2007). AtPIFs are only stable under prolonged dark conditions. To understand if OsPIF14 light-induced protein degradation had the same pattern of AtPIFs, the recombinant OsPIF14 was incubated with WT total protein extract and exposed to far-red light or kept under dark. When incubated with WT total protein extract, OsPIF14 is degraded under both continuous far-red light and dark conditions (Fig.2). The degradation rate is similar to that observed for red light treatment (Fig. 1, left panel), indicating that OsPIF14 is being degraded independently of the light wavelength. This light-independent degradation seems to be caused by some element present in the protein extract since the recombinant OsPIF14 protein is stable without protein extract (Fig.2, No ext). Although we have used two complementary protease inhibitors, we cannot exclude the possibility of protease activity.

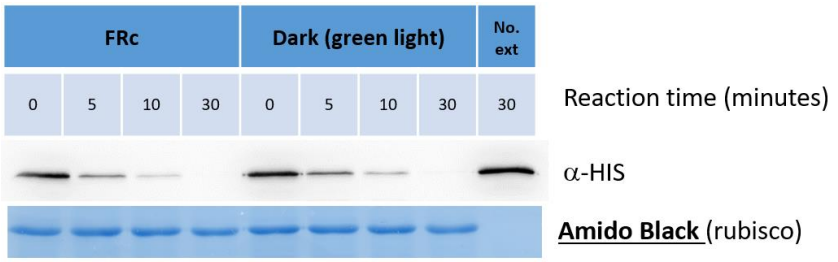


Figure 2. OsPIF14 degradation under continuous far-red (FRc) and dark (simulated by green light). *In vitro* degradation assay of 2µg of OsPIF14 under far-red (10µmol m^{-2} s^{-1}) or green light using 4µg of total protein extract from WT over 30 minutes. Recombinant OsPIF14 protein was incubated for 30 minutes without protein extract (No. ext). Recombinant OsPIF14 was detected with α-HIS and rubisco stained with amido black was used as loading control.

3.3. OsPIF14 degradation is not proteasome-dependent

In Arabidopsis, AtPIFs light-induced degradation is inhibited by MG132, indicating that this degradation is mediated by 26S proteasome (Shen et al., 2005; Al-Sady et al., 2006; Shen et al., 2007). Here, we wanted to investigate whether the proteasome was also involved in OsPIF14 protein degradation. Thus, WT total protein extract was incubated with MG132 for 30 minutes before the addition of recombinant OsPIF14. As shown in Fig.3, the addition of MG132 was not sufficient to prevent OsPIF14 degradation. These results suggest that either 1) the proteasome is not involved in OsPIF14 *in vitro* degradation or 2) a putative protease-dependent degradation activity is faster than proteasome degradation. To answer these questions it is needed to test other proteases inhibitors. Although, it would be better to analyze the OsPIF14 overexpressing lines.

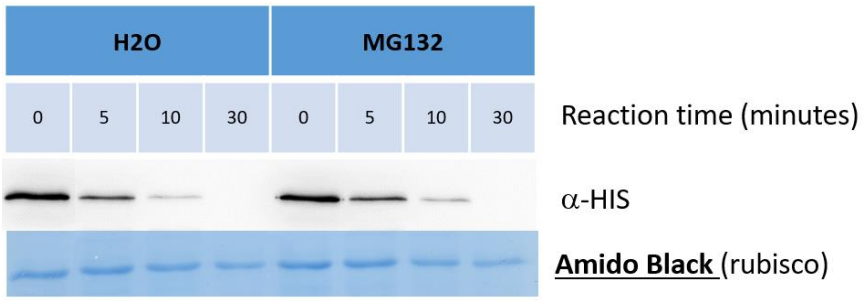


Figure 3. Effect of MG132 on OsPIF14 protein degradation**.** *In vitro* degradation assay of 2µg of OsPIF14 under continuous red LED light (10µmol m^{-2} s^{-1}) using 4µg of total protein extract from WT, pre-incubated during 30 minutes with 100mM of MG132 or with water. Recombinant OsPIF14 was detected with α-HIS and rubisco stained with amido black was used as loading control.

3.4. Dark/light transition has a higher effect on the rice transcriptome than phyB mutation or OsPIF14 silencing

To further study OsPIF14 function on the transition from dark to light and identify new target genes, we analyzed the transcriptome of WT, RNAi::*OsPIF14* silencing lines and *phyB* mutants by microarray. Given that OsPIF14 is more stable in *OsphyB* mutants as compared to WT *in vitro* (Fig. 1), these lines were used based on the possibility that phyB would have increased levels of OsPIF14 after light exposure *in planta*. Rice seedlings were grown under photoperiodic conditions and samples were collected 30 minutes before and 30 minutes after light onset, from now on these samples will be called dark and light samples, respectively.

Genes displaying higher than 2.5 fold change in the mutant lines as compared to WT or between dark and light samples were considered to have a statistically significant difference in gene expression. Thus, the differentially expressed genes (DEGs) were identified and a hierarchical clustering of the transcriptional profiles was performed (Fig. 4A). Two major clusters were observed, one corresponding to dark and the other to light samples. Moreover, in each major cluster, the profile of *phyB* showed to be the most different among all the rice lines analyzed, and the two RNAi::*OsPIF14* lines clustered together. These results confirm that *phyB* knockout and *OsPIF14* silencing induce alteration in gene transcription. However, light continues to be the major factor for gene regulation. As expected, these observations clearly show the hierarchy of light gene regulation. Light is perceived by several photoreceptors, including phyB, which in turn could regulate the function several proteins, including OsPIF14.

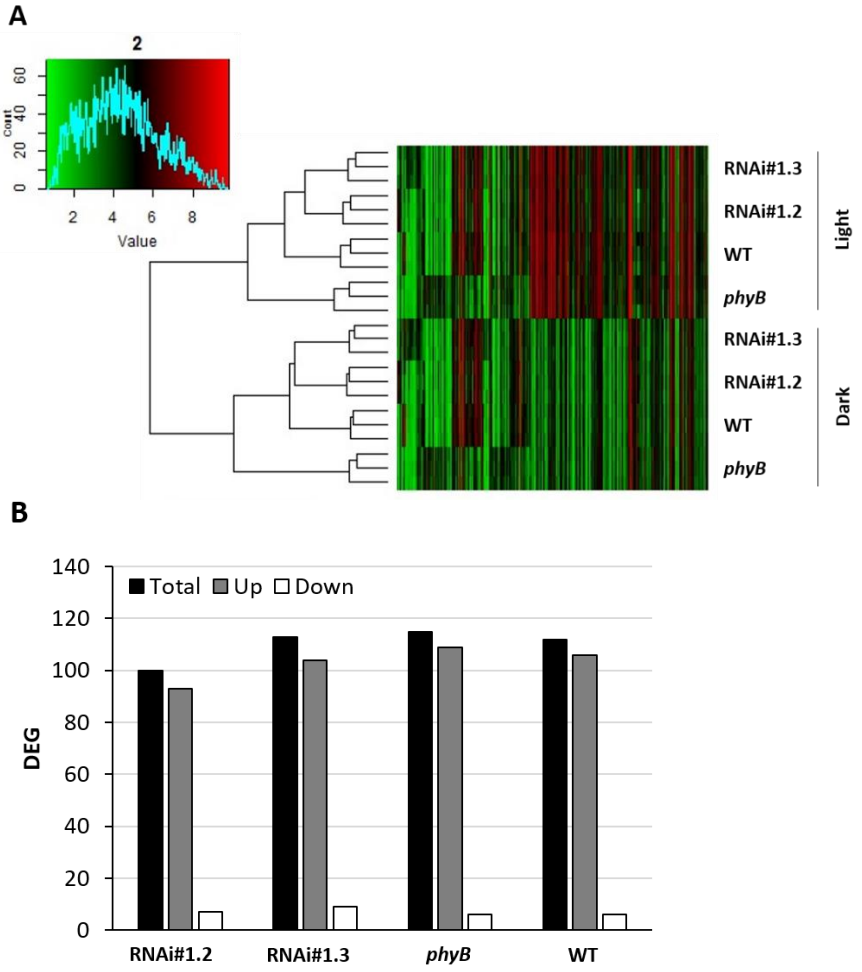


Figure 4. Genes differentially expressed under dark and light conditions. A, Hierarchical clustering of the 260 DEGs found between dark vs. light samples and also between WT vs. transgenic lines. B, DEGs between dark and light samples. Genes were considered to be DEGs if the FDR was ≤0.05 and the fold change was higher than 2.5 fold difference.

3.5. OsPIF14 might be involved in the regulation of a multitude of plant responses in the dark/light transition

In the transition from dark to light, more than 100 DEGs were identified per rice line, among these, more than 92% are induced by light (Fig. 4B). To identify new putative target genes regulated by OsPIF14, we compared the up- or downregulated genes between dark and light samples for all rice lines (RNAi::*OsPIF14*, *phyB,* and WT) and represented it in a Venn diagram.

A total of 66 upregulated genes in response to the dark-light transition were identified in all tested lines (Fig. 5A) meaning that the induction of these genes is independent of the presence of OsPIF14 or phyB. These genes were analyzed for their function using Blast2GO and are listed in Table S1. As expected, we identified light responsive genes, such as, early light-induced genes, high light intensity responsive genes, UVB receptor genes, and genes involved in photosynthesis. We also identified genes coding for heat stress transcription factors, heat shock proteins, chaperones, and others. The induction of such genes could represent a defensive strategy to prevent photoinhibition and photodamage of the photosynthetic apparatus.

Three genes were identified as being more upregulated by light in RNAi lines than in WT or *phyB* (Fig. 5A). These are a two-component response regulator (LOC_Os11g04720), a 1-acyl-sn-glycerol-3-phosphate acyltransferase-like (LOC_Os04g53370) and an AP2 transcription factor (LOC_Os03g09170). Unfortunately, there is not much information about these genes. The two-component response regulator was identified as the *Oryza sativa* response regulator 9 (OsRR9). This protein belongs to the A-type RR gene family, is induced by cytokinin and acts as a repressor of cytokinin signaling (Ito and Kurata, 2006). The acyltransferase-like protein is involved in the metabolism of lipids and phospholipids, namely in the synthesis of phosphatidic acid and triglycerides (Croft et al., 2011). The third identified gene encodes an AP2/ERF transcription factor that belongs to the ERF group Ib and was named OsERF#047 (Nakano et al., 2006). The OsERF#047 does not have an identified function, and the AP2/ERF family has a large range of functions, from plant development to

biotic and abiotic stress responses, thus, is difficult to attribute a pathway or process were OsERF#47 could be involved.

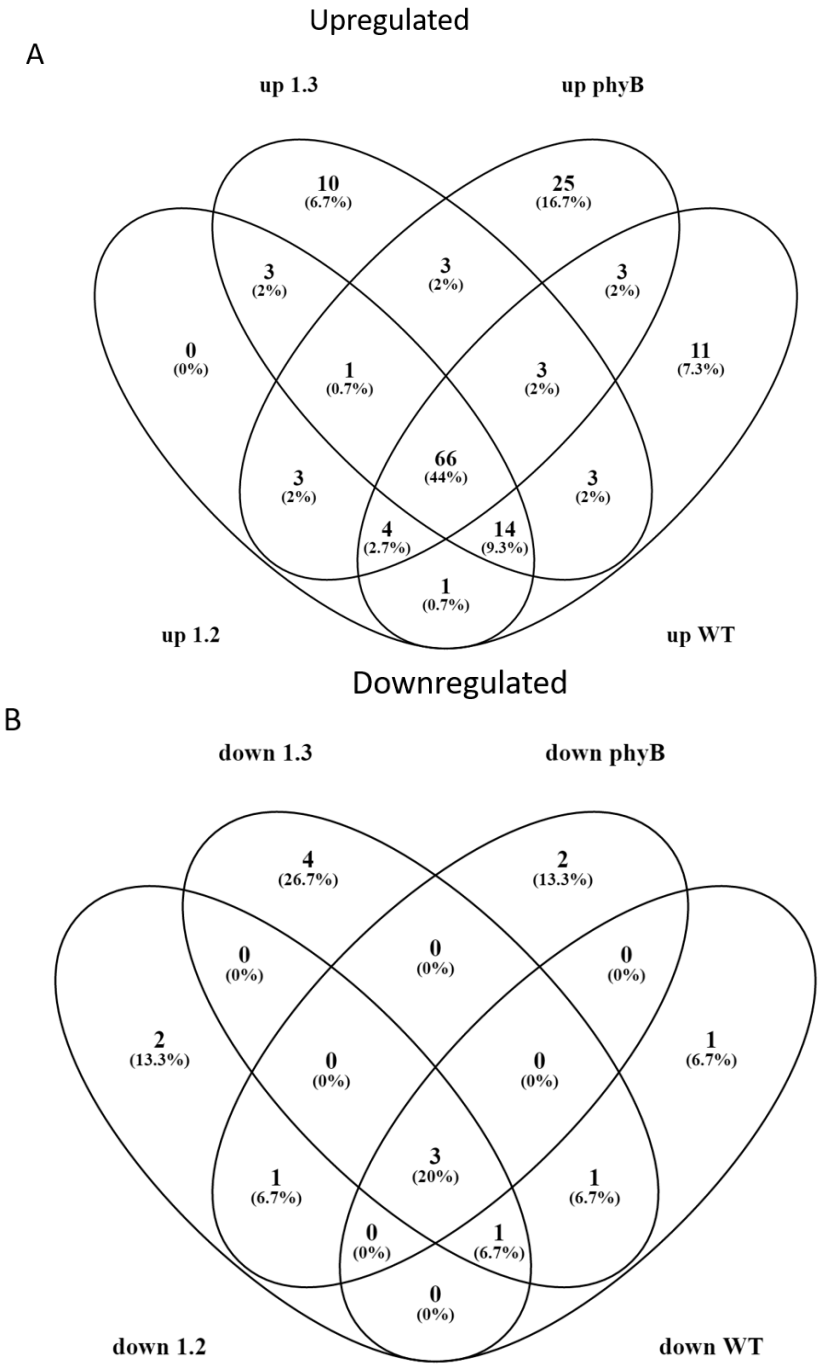
In Chapter 2 and 3, we showed that OsPIF14 can bind to G-box (CACGTG) and extended N-box (CCACGCGG) *cis*-elements. Moreover, it is described that Arabidopsis PIFs can bind to other *cis*-elements, such as the PIF-binding E-box (PBE: CACATG) (Zhang et al., 2013; Kim et al., 2016). Therefore, we analyzed the 2,000 bp upstream of the ATG to identify G-box, extended N-box, and/or PBE *cis*-elements to assess if these genes could be direct targets of OsPIF14. We identified at least one of these *cis*-elements in the promoter of all genes, suggesting that OsPIF14 can bind to their promoter. Moreover, the fact that these genes are upregulated, suggest that OsPIF14 could act as a repressor (as we showed in Chapter 2 and 3), however further studies are needed to confirm that hypothesis. Regarding the detailed analysis of the promoter of these genes, we did not identify any N-box in their promoter. In the promoter of the two-component response regulator four PBE *cis*-elements were identified. In the promoter of the acyltransferase one G-box and one PBE *cis*-element were identified, while in the promoter of AP2 one G-box and three PBE *cis*-elements were identified (supplemental Fig. S1). Overall, these three genes show that OsPIF14 might be involved in the regulation of a multitude of plant responses, such as plant growth and development, lipid metabolism and stress.

Fourteen genes were identified as being upregulated by light in all lines except in *phyB* mutants (Fig. 5A). Seven encode for a chlorophyll a-b binding, and among the other genes, we found a photosystem I subunit, a two-component response regulator-like PRR37, and the transcription factor PIF1-like (OsPIL12) (Table S2). Chlorophyll a-b binding proteins bind to chlorophyll and serve as antenna complex (Jansson, 1994), therefore playing an important role in photosynthesis. The role of Pfr phytochrome conformation was previously shown to be important for the induction of *Chlorophyll a-b binding* gene expression in *Avena sativa* (Lissemore and Quail, 1988) and Arabidopsis (Sun and Tobin, 1990). Moreover, we identified *OsPIL12* among the genes that are induced by light except in *phyB* lines, suggesting that *OsPIL12* gene expression is light-

induced in a phyB dependent manner. Despite the fact that phytochromes regulate PIF protein abundance/activity, it was also shown that phyB can induce *PIL1* expression in Arabidopsis, and that might be an indirect target of the phyB pathway (Tepperman et al., 2004).

Less than ten genes were identified for each line as being downregulated in the transition from dark to light. As shown in the Venn diagram in Fig. 5B, none of these genes was identified to be more repressed in RNAi lines. However, there are three genes that are common to all lines. These genes encode an auxin-repressed protein (LOC_Os09g26620), an auxin-responsive SAUR36-like protein (LOC_Os09g37330), and a probable receptor kinase (LOC_Os01g02710). Auxin biosynthesis and concomitant auxin-regulated genes can be clock regulated (Covington and Harmer, 2007), therefore independent of the phytochromes and/or PIFs, however, the identified SAUR (Small Auxin Up RNA) (previously named as SAUR39) was shown to be downregulated in etiolated OsPIL15-ox plants (Zhou et al., 2014). OsSAUR39 is characterized as a negative regulator of auxin biosynthesis and transport, and rice plants overexpressing OsSAUR39 showed reduced shoot and root growth (Kant et al., 2009). Given that OsSAUR39 is not differentially expressed in RNAi::*OsPIF14* suggest that OsSAUR39 is not regulated by all OsPIFs or it is an indirect target of OsPIF14 which does not respond within 30 minutes of light exposure.

Figure 5. Venn diagram of the DEGs between dark and light samples. A, Comparison of the light-upregulated genes in all rice lines. B, Comparison of the light-downregulated genes in all rice lines. Venn diagram was built using the VENNY tool (http://bioinfogp.cnb.csic.es/tools/venny/)


Upregulated
A
up 1.3
up phyB
10
(6.7%)
25
(16.7%)
3
(2%)
3
(2%)
3
(2%)
0
(0%)
1
(0.7%)
3
(2%)
11
(7.3%)
3
(2%)
66
(44%)
3
(2%)
4
(2.7%)
14
(9.3%)
1
(0.7%)
up 1.2
up WT
Downregulated
B
down 1.3
down phyB
4
(26.7%)
2
(13.3%)
0
(0%)
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(0%)
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(13.3%)
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1
(6.7%)
1
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3
(20%)
1
(6.7%)
0
(0%)
1
(6.7%)
0
(0%)
down 1.2
down WT

3.6. RNAi::OsPIF14 lines showed induced expression of cell wall structure and organization genes

To further characterize RNAi::*OsPIF14* lines and identify genes that are intrinsically regulated by OsPIF14 independently of light transition, the transcriptome of transgenic lines (RNAi lines or *phyB*) was compared to WT in both dark and light samples. Light samples showed less DEGs between transgenic and WT plants as compared to dark samples (Fig. 6). These results might be a consequence of light stimuli due to the high number of light-regulated genes observed in this assay. As expected, *phyB* mutants showed to have the most different transcriptome compared to WT since it shows more DEGs for both dark and light samples (Fig. 6) as compared to RNAi lines.

The two RNAi::*OsPIF14* lines showed some differences, in the total number of DEGs (RNAi#1.2 always showed fewer DEGs as compared to #1.3) and also in percentage of up and down regulated genes (#1.3 showed more induced genes while #1.2 did not) (Fig. 6). To identify putative OsPIF14 target genes, we compared the DEGs of both RNAi lines in dark and light samples and represented in a Venn diagram (Fig. 7A). Five genes were identified as being more upregulated in RNAi lines as compared to WT. A group of four genes is simultaneously induced in dark and light samples, while the other is more induced only in light samples. To understand if these genes are specifically upregulated in RNAi lines, we compared with the upregulated genes of *phyB* dark and light samples, Fig. 7B and 7C, respectively. Indeed, these five genes are more induced in RNAi lines as compared to WT than in *phyB* mutant as compared to WT. The group of four genes encode a retrotransposon unclassified protein (LOC_Os12g24050), a glycine-rich cell wall structural (Os01g0910500), a trichome birefringence-like 14 (LOC_Os12g33194), an expressed protein (LOC_Os03g02470). The other gene encodes a hypothetical protein (LOC_Os01g64949). There are no studies reporting the characterization of these genes. However, it is known that retrotransposons are a sensitive marker of plant stress that probably is a consequence of the rice transformation process. The gene coding for an expressed protein has a domain of unknown function (DUF3778)

and is the gene that shows the highest fold difference (>10 fold as compared to WT). The glycine-rich cell wall structural and the trichome birefringence-like 14 were not yet studied, and no information about their function is available. However, members of these families have been involved in cell wall structure (Mangeon et al., 2010) and secondary wall cellulose synthesis (Bischoff et al., 2010), respectively. No further information was obtained for the gene coding for the hypothetical protein that is more induced only in light samples of RNAi lines.

Seven genes were identified as downregulated in both RNAi lines as compared to WT, five in dark and light samples and two only in dark samples (Fig. 8A). When compared to the DEGs of *phyB,* we observed that these five genes are also downregulated in *phyB* both in dark (Fig. 8B) and light (Fig. 8C) samples, therefore not being specifically regulated by OsPIF14. Among the two genes identified as being downregulated only in dark samples (Fig. 8A), only one is more downregulated in both RNAi::*OsPIF14* lines as compared to WT and *phyB* (Fig. 8B), the LOC_Os01g18970. This gene encodes a class III peroxidase 10 (OsPrx10). Classe III peroxidases are a multifunctional family, which seems to have redundant functions. These genes are involved in several biological processes, such as defense and stress responses, lignification, and auxin catabolism (Cosio and Dunand, 2009). Also, peroxidases are key players during the cell cycle and in particular in cell wall organization (Passardi et al., 2004b). Therefore, further studies are needed to unveil the function of OsPrx10.

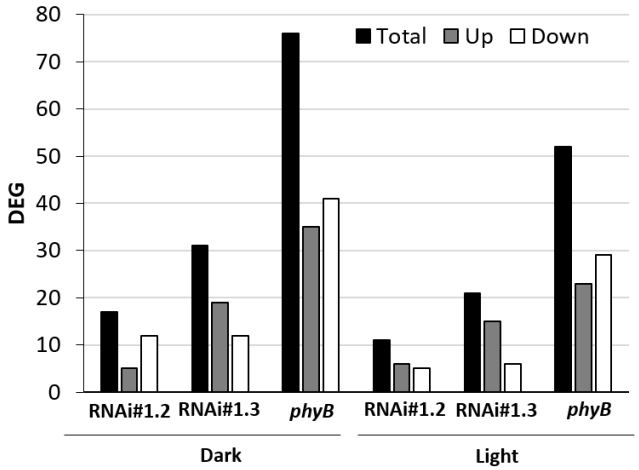

Figure 6. DEGs of dark and light samples of two RNAi::*OsPIF14* silencing lines (RNAi#1.2 and RNAi#1.3) and *phyB* mutant lines compared to WT.

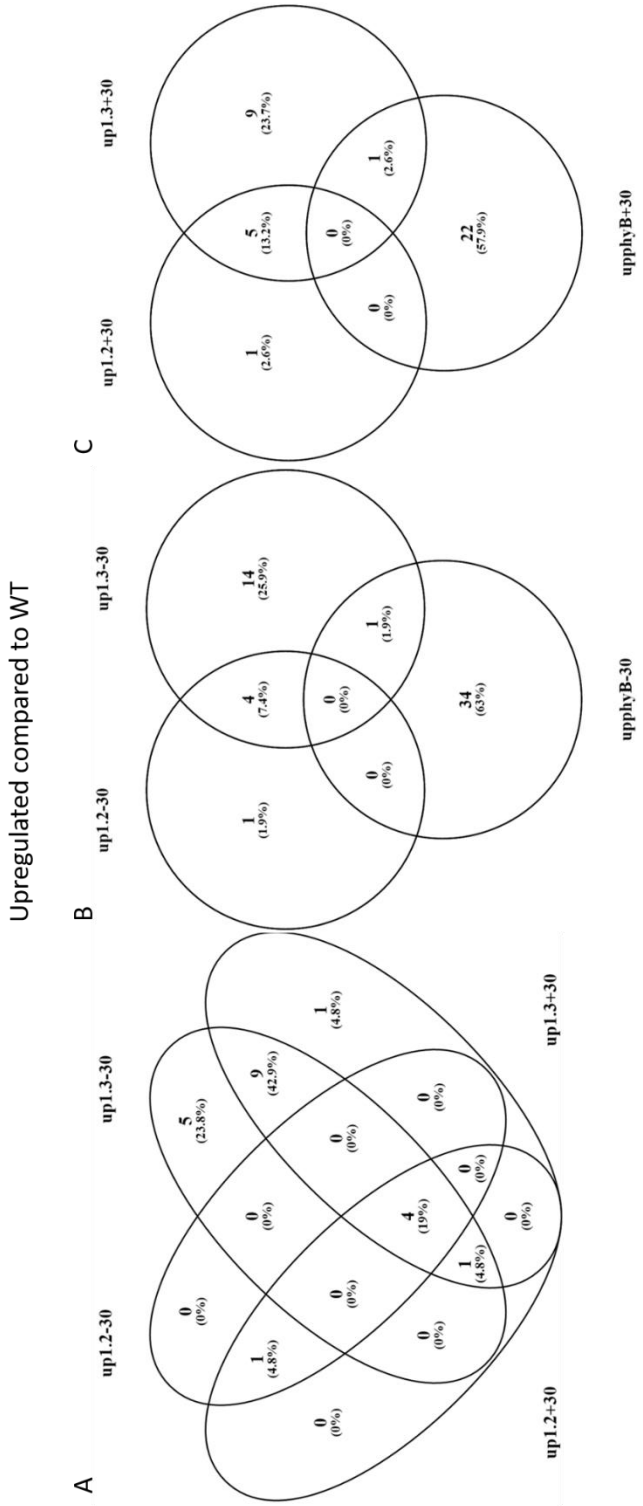

Figure 7. Venn diagram of the upregulated genes between mutant lines and WT. A, Comparison of the upregulated genes under dark (-30) and light (+30) of both RNAi lines #1.2 and #1.3. B, Comparison between dark samples (-30) of RNAi lines and *phyB* mutant. C, Comparison between light samples (+30) of RNAi lines and *phyB* mutant lines. Venn diagram was built using the VENNY tool (http://bioinfogp.cnb.csic.es/tools/venny/)

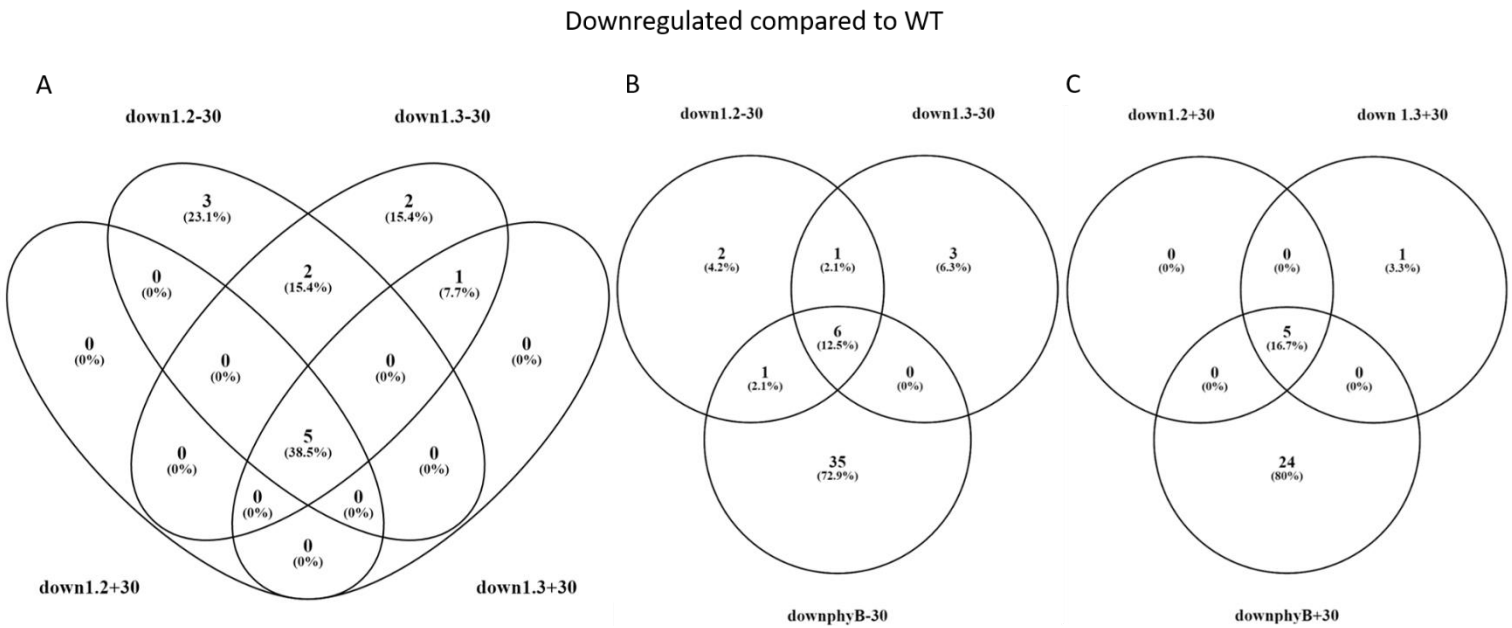

Figure 8. Venn diagram of the downregulated genes between mutant lines and WT. A, Comparison of the downregulated genes under dark (-30) and light (+30) of both RNAi lines #1.2 and #1.3. B, Comparison between dark samples (-30) of RNAi lines and *phyB* mutant. C, Comparison between light samples (+30) of RNAi lines and *phyB* mutant lines. Venn diagram was built using the VENNY tool (http://bioinfogp.cnb.csic.es/tools/venny/)

4. Discussion

PIF protein activity and stability as long been demonstrated to be regulated by light in Arabidopsis (Al-Sady et al., 2006; Shen et al., 2007; Leivar and Quail, 2011). Upon red light, PIFs are phosphorylated and degraded by proteasome due to the interaction with the active phytochromes. Under dark, PIFs are stable and promote plant growth. In Chapter 2, we showed that OsPIF14 preferentially binds to the active form of phytochrome B. However, the light stability of OsPIF14 was not assessed. In the present study, using an *in vitro* degradation assay, we demonstrated that OsPIF14 is faster degraded in WT than in *phyB* mutant, thus showing the involvement of phyB in OsPIF14 degradation.

PIFs are rapidly degraded after red light incubation with an half-life time that can be shorter than 5 min (Shen et al., 2007; Shen et al., 2008), making difficult the full characterization of this degradation process. Therefore, the strategy that has been used to overcome this is to generate plants overexpressing PIFs. In these plants, PIF light stability is characterized in more detail, namely by assessing degradation rate and identification of phosphorylation sites (Al-Sady et al., 2006; Shen et al., 2007; Ni et al., 2013). In rice, there are no OsPIF14 overexpressing plants available, therefore, we generated these plants but they were not ready to use in this assay. Moreover, the antibody that was raised against OsPIF14 did not detect OsPIF14 in total protein extract. We also used plants collected in the end of the dark period to try to accumulate and thus detect OsPIF14 protein using other technics, such as mass spectrometry or size exclusion chromatography followed by Western blotting, but without success. As an alternative to assess OsPIF14 protein degradation, we used an *in vitro* degradation assay (Lourenço et al., 2013) in which the recombinant His::OsPIF14 protein was incubated with rice total protein extract. Using this system, we showed that under red light, the recombinant His::OsPIF14 is degraded in about 10 minutes after incubation with WT total protein extract and that with *phyB* mutant protein extract the degradation is slower. The *in vitro* degradation rate is similar to what was previously observed for other AtPIFs *in planta* (Shen et al., 2007; Shen et al., 2008), however, this needs to be tested in OsPIF14 overexpressing lines. Nevertheless, our results

show, for the first time, that phyB is involved in the degradation of OsPIF14. However, given that OsPIF14 degradation is not abolished in *phyB* mutant plants, we cannot exclude the involvement of the other two rice phytochromes (e.g. OsphyA and OsphyC) in the degradation of OsPIF14. These results are in agreement with what was shown for AtPIF1 (Shen et al., 2008), AtPIF3 (Al-Sady et al., 2006), and AtPIF5 (Shen et al., 2007), which are rapidly degraded after red light incubation and that phytochromes act synergistically to modulate PIFs light-induced degradation.

In Arabidopsis, PIFs accumulate under dark, while under far-red light the degradation rate is slowed down (Al-Sady et al., 2006). Here, using an *in vitro* degradation assay, we observed a similar degradation rate independently of the light condition (red, far-red or dark). In Arabidopsis, under far-red light exposure or a long period of darkness, AtphyB is located in the cytosol, thus inactive and unable to interact with AtPIFs that are located in the nucleus (Klose et al., 2015). The absence of cell compartmentalization in our *in vitro* assay would allow the interaction between rice phytochromes and OsPIF14. We still do not know the reason for this light-independent OsPIF14 degradation, but this interaction could lead to OsPIF14 degradation even in the absence of light activated phytochromes. These observations are not in agreement with literature and our previews results. Huq *et al.*, reported that both photoactivation and nuclear localization are fundamental for AtphyB activity (Huq et al., 2003) and even if the AtphyB Pfr is located in cytosol it is not functionally active. Moreover, in Chapter 2, we showed that OsPIF14 interacts preferentially with phyB Pfr form. Thus, remains unclear how is OsPIF14 degraded under far-red and dark conditions. The recombinant OsPIF14 protein showed to be stable when incubated without protein extract under dark. Therefore, the observed light-independent degradation seems to be caused by some element present in the WT protein extract and despite using two complementary protease inhibitors cocktails, we cannot exclude the possibility of protease activity. To test this hypothesis, the addition of other proteases inhibitors, for instance, iodoacetamide, which inhibits cysteine proteases or even aprotinin,

leupeptin and pepstatin that were used to study AtPIF3 light-induced degradation need to be tested (Al-Sady et al., 2006; Ni et al., 2013).

In Arabidopsis, phosphorylated PIFs are degraded by the proteasome. To test if this mechanism is conserved in rice, we incubated the total protein extract with a protease inhibitor, MG132, before adding the recombinant protein. The results did not show a significant effect of MG132 on OsPIF14 degradation rate, suggesting that other processes are contributing to this *in vitro* OsPIF14 degradation supporting the suspicion of protease degradation. Nevertheless, the results from the *in vitro* degradation assay suggests that the mechanisms underlying the regulation of PIFs in rice may involve phytochromes as it happens in Arabidopsis. To characterize better OsPIF14 light stability the study of the rice plants overexpressing OsPIF14 will be crucial.

In this chapter, our goal was not only to understand how is OsPIF14 regulated by light, but also to identify which genes are regulated by OsPIF14. Since the *in vitro* results show that OsPIF14 is degraded in about 10 minutes in WT, we collected seedlings 30 minutes before and 30 minutes after the light onset and analyzed their transcript profile by microarrays. This is an exploratory study and the results shown here need to be further validated. The RNAi::*OsPIF14* line #1.2 showed 53% and 75%, while the line #1.3 showed 58% and 83%, of *OsPIF14* gene expression under dark and light as compared to WT, respectively (supplemental Fig. S2). The low number of differentially expressed genes (DEGs) in RNAi::OsPIF14, as compared to WT, might be due to the low silencing level of *OsPIF14* in RNAi lines and probably due to the redundant function of PIF family. Therefore, we could not construct a metabolic pathway based on the attributed KEGG pathways. Our transcriptomic data clearly showed two distinct major groups, corresponding to dark and light samples. These results were expected since light gene regulation is a complex process in which all light receptors are involved and not only elements from phytochrome and PIF family are important. To increase the complexity of this light regulation, it was shown that AtPIFs and Osphys family members have a synergistic and cooperative effect in plant development, for instance in hypocotyl growth (Yang et al., 2012) and

flowering time (Takano et al., 2005), respectively, therefore the silencing or knockout of only one gene of the family might not be enough to observe major transcriptional differences.

4.1. DEGs in dark/light transition

4.1.1. Common DEGs in all rice lines

More than 100 DEGs between dark and light collected samples were identified per rice line, and more than 92% of the genes are induced by light in each line. Among the common light induced genes, we identified members of the family of the early light-induced, UVB light receptors, and chlorophyll a-b binding, which have already been shown to be involved in light response (Jung et al., 2008; Fehér et al., 2011). We also identified heat shock genes coding for heat shock proteins (HSP) and two genes responsive to high light intensity. In Arabidopsis, HSPs were shown to be involved in the protection of photosystem II, which may ameliorate the damaging effects of excess light (Downs et al., 1999; Schroda et al., 1999) and protect the plant photosynthesis apparatus. In fact, we grew plants with a total light intensity of 500 μmol m^{-2} s^{-1}, without dawn and dusk periods. Our results suggest that, when light is switched on after the dark period, rice seedlings are under light stress and try to protect their photosystem by inducing the expression of HSPs.

Our transcriptomic data showed that only three genes were downregulated in the transition from dark to light in all rice lines. Two of these are regulated by auxins, which in turn can be regulated by the circadian clock and are involved in plant growth. Only one of theses genes, *SAUR39*, was already characterized. Rice plants overexpressing *SAUR39* are smaller and with less root volume, as compared to WT. These plants also showed increased senescence and lower chlorophyll content (Zhang et al., 2015), showing that SAUR39 levels are toxic for the plant and need to be tightly controlled. Interestingly, it was previousely shown that rice plants overexpressing OsPIL15, which are taller than WT, show reduced expression of a set of genes involved in auxin pathway including the

SAUR39 (Zhou et al., 2014). However, our data indicate that *SAUR39* is not regulated by OsPIF14 30 minutes after light onset.

4.1.2. Three genes more induced by light in RNAi::OsPIF14 lines

We have identified three genes that are more induced by light in RNAi::*OsPIF14* than in *phyB* or WT plants. The OsRR9 belongs to type-A RRs and is induced by cytokinin (Ito and Kurata, 2006). The *OsRR9/OsRR10 Tos17* insertion mutant show a dwarf phenotype, sterility, lesion mimic, and vivipary (Jain et al., 2006), showing that this gene is involved in growth and development in rice. On the other hand, in Arabidopsis, it was shown that AtRR4 interacts with phyB and stabilize its Pfr form (Gallay et al., 2001), in turn the phyB light-degradation is dependent on PIFs (Jang et al., 2010). Taken together, these results suggest that the RR proteins can play a role in the mechanism by which PIFs contribute for light-dependent phyB degradation. Therefore, since the RNAi::*OsPIF14* showed increased levels of *OsRR9*, we speculate that in these lines phyB Pfr would be more stable during the day, which in turn would contribute to the degradation of PIFs, however further studies are needed to confirm this hypothesis.

The AP2/ERF belongs to the ERF group Ib and was previously named ERF#047 (Nakano et al., 2006), however, neither the gene nor the ERF group Ib are characterized, and their function remains unknown. Nevertheless, it is known that ERF family plays a role in many cellular processes in rice, such as hormonal signal and plant defense (Pré et al., 2008), response to abiotic stresses (Dubouzet et al., 2003; Serra et al., 2013). This gene family was also shown to play a role in developmental processes, such as spikelet development in maize indicating that some of these genes regulate hormone-related pathways (Chuck et al., 2002). On the other hand, the acyltransferase-like protein encodes an enzyme involved in the metabolism of lipids and phospholipids, namely in the synthesis of phosphatidic acid and triglycerides (Croft et al., 2011) but no further information is available for this gene. A detailed *in silico* analysis of the promoter of these three genes identified a G-box and/or a PBE *cis*-element in all the promoters (supplemental

Fig. S1), suggesting that these genes could be direct targets of OsPIF14. However further studies are needed to test this hypothesis and to unveil the functions of the three identified genes.

4.2. Intrinsic gene regulation of RNAi::OsPIF14 as compared to WT

To identify new genes regulated by OsPIF14 independently of the transition from dark to light, we analyzed the dark and light samples separately. We compared the transcript profile of the transgenic rice lines with WT and, our data showed that dark samples have more DEGs than light samples. These results may be related to the higher silencing of *OsPIF14* in dark samples as compared to light samples (supplemental Fig. S2). We have identified only a few DEGs common to both RNAi lines, which could be due to the lower silencing level of the transgenic plants (<50%), the weak repressor activity of OsPIF14 (observed in Chapter 2 and 3) and/or to the putative overlapping role of OsPIFs/PILs. Even though, we identified a total of six DEGs common to both RNAi lines. A group of four genes that is upregulated in dark and light samples, one gene that is upregulated only in light samples and one gene that is downregulated only in dark samples. The group of four genes encodes a retrotransposon, a glycine-rich cell wall structural, a trichome birefringence-like, and an expressed protein. The gene upregulated only in light samples encodes a hypothetical protein and, the downregulated gene in dark samples encodes a peroxidase class III (Prx10) protein. Analysis of the promoter of these genes showed that only the upregulated genes showed to have *cis*-elements where OsPIF14 could bind (supplemental Fig. S1). Taken together, our results suggest that OsPIF14 could directly regulate the expression of cell wall structure and organization genes.

RNAi::*OsPIF14* silencing lines have an upregulation of the gene coding for a glycine-rich cell wall structural and a trichome birefringence-like 14 (TBL14), in both dark and light samples, when compared to WT (Fig. 9). Both gene families seems to share a role in cell wall structure and organization, namely in cell elongation (Ringli et al., 2001; Mangeon et al., 2010), and cell wall synthesis (Bischoff et al., 2010). The involvement of OsPIFs/PILs in cell structure was

already observed in rice (Todaka et al., 2012; Zhou et al., 2014). OsPIL13-ox plants are taller than WT and *OsPIL13* was shown to induce the expression of cell wall-related genes responsible for cell elongation (Todaka et al., 2012). Moreover, OsPIL15-ox plants showed shorter above ground portion under dark and *OsPIL15* was shown to repress auxin pathway and cell wall biosynthesis genes at these conditions. However, exposure to red and far-red light promote seedling elongation even with higher levels of OsPIL15 (Zhou et al., 2014). Altogether, these results show that OsPIFs/PILs can control plant growth by regulating cell wall-related genes.

PIFs target genes are not only related to cell wall structure or elongation. PIFs, as bHLH transcription factors bind to conserved DNA *cis*-elements, such as G-box (CACGTG) to regulate the expression of a multitude of growth associated genes (Zhang et al., 2013). In Arabidopsis, PIF4 was shown to be involved in the regulation of growth-regulating hormones such as auxin-associated genes, brassinosteroids, gibberellic acid, and cytokinin (Nomoto et al., 2012). PIF3 was shown to bind to the G-box of the promoter of *PIL1*, *HFR1*, and *XTR7*, and *pif3* mutant plants show shorter hypocotyl (Soy et al., 2012). In addition, the same report shows that PIFs acts synergistically to modulate the expression of these genes and regulate hypocotyl growth. These evidences support our idea that OsPIFs/PILs might have overlapping functions. This, together with the lower silencing level and activity of OsPIF14 may explain why we did not observe any of these genes among the DEGs.

Our transcriptomic data also showed that the *prx10* gene was downregulated in RNAi lines, as compared to WT, only in dark samples (Fig.9). Moreover, the promoter of *prx10* does not have OsPIF14 binding *cis*-elements, suggesting that it might not be a direct target of OsPIF14. Prx10 belongs to Class III peroxidase and is involved in the mechanism of cell elongation, in cell wall construction and differentiation, and in the defense against pathogens (Passardi et al., 2004a). The specific role of prx10 is still unknown, but it is possible that it has a function in cell wall organization, suggesting once more the involvement of OsPIF14 in the regulation of cell wall organization genes.

To the best of our knowledge there are no rice plants overexpressing *OsPIF14*. At the moment, we are generating these plants but they were not ready to use in this assay. As an alternative, we used *phyB* mutants since our results from the *in vitro* degradation assay suggest that after 30 minutes of light onset OsPIF14 is more stable in *phyB* than in WT. In order to unveil genes that are regulated by the pair OsPIF14-phyB, the DEGs of RNAi::*OsPIF14* and *phyB* were compared. Unfortunately, no genes were identified as upregulated in RNAi::*OsPIF14* and downregulated in *phyB* or downregulated in RNAI::*OsPIF14* and upregulated in *phyB* mutants. This might be due to the lower level of silencing of OsPIF14 or, the redundant effect of rice phytochromes in what regards OsPIF14 degradation or even due to the collected time point. Moreover, we cannot discard the possibility that *phyB* mutant lines do not have an accumulation of OsPIF14, since the antibody raised against OsPIF14 is not detecting OsPIF14 in protein extract. The transition from dark to light is a very complex process, which is not only dependent of light but also to the intrinsic gene regulation, also called the circadian clock. To decrease this complexity and identify which genes are indeed regulated by PIFs, other authors have been analyzing the transcriptome of etiolated seedlings. This approach eliminate the circadian clock regulation and unveil the role of PIFs in the regulation of plant growth. In the future, it would be worthwhile to analyze the gene regulation of etiolated rice plants silencing/knockout and overexpressing OsPIF14. The experiments would confirm the results shown here and also identify new putative target genes. Moreover, given that OsPIF14 seems to be involved in the regulation of cell wall-related genes, it would be interesting to analyze the size of the cells of the transgenic plants.

The results shown here, suggest that OsPIF14 is degraded in a process dependent of phyB, however the mechanism is still not clear and the analysis of OsPIF14::HA overexpressing plants would be crucial to validate this regulation further. Our transcriptomic data show that the RNAi::*OsPIF14* lines have several DEGs associated with cell wall structure and organization and also lipid biosynthesis. Thus, we hypothesize that OsPIF14 might be involved in the

regulation of such processes. In the future, we must analyze the cell structure and architecture of *OsPIF14* transgenic lines, which could unveil the role of OsPIF14 in plant growth and development.

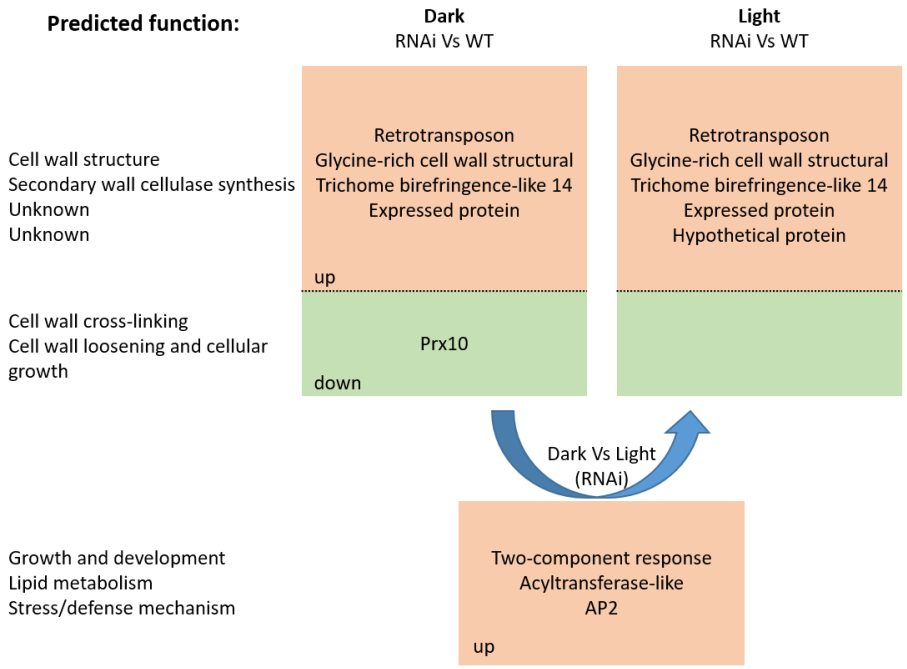

Figure 9. Schematic representation of the identified DEGs in RNAi::*OsPIF14* lines. Representation of the upregulated (up) and downregulated (down) genes common to both RNAi lines as compared to WT (upper panel) or compared between dark and light collected samples in RNAi lines (lower panel). On the left, it is represented the predicted gene function based on BLAST2GO annotation and/or gene family function.

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7. Supplemental data

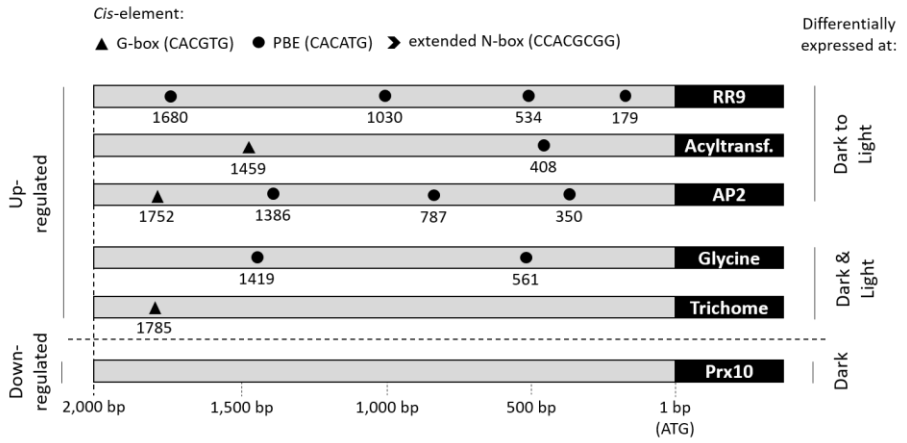

Figure S1. Schematic representation of the promoter of the DEGs specific of RNAi::*OsPIF14* silencing lines and correspondent OsPIF14 binding *cis*-elements position. Grey bar represents the 2,000 bp upstream of the ATG and black bar represents the coding sequence of the identified gene. The top five genes were found to be upregulated in RNAi::*OsPIF14* silencing lines as compared to WT and *phyB* mutants. Among these, the top three we identified in the transition from dark to light and the other two are upregulated both at dark and light conditions. The bottom gene is downregulated and was identified at dark conditions. Triangle represents G-box, circle represents PBE and arrow represents extended N-box. The numbers in the promoter sequence represent the position of each *cis*-element in the promoter.

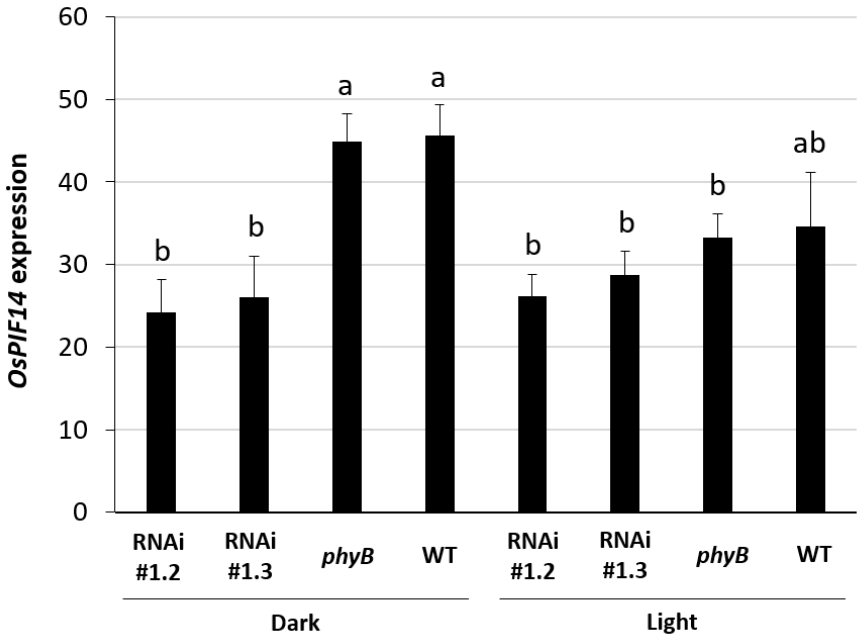

Figure S2. Expression values of the microarray *OsPIF14* probe (16009455). Values are mean +/- standard deviation of microarray data (n=3) of two RNAi::*OsPIF14* silencing lines (#1.2 and #1.3), *phyB* and WT seedlings grown for fourteen days under 16h/8h, 28ºC. Dark samples were collected 30 minutes before light onset and light samples were collected 30 minutes after light onset. Letters represent significant statistical difference ($p<0.05$).

Table S1. Significantly light-induced genes in all tested lines (fold change >2.5, total of 66 genes).

Locus	Putative function
	Light induced genes
LOC_Os07g08150.1	early light-induced
LOC_Os01g14410.1	early light-induced
	Response to high light intensity
LOC_Os03g18200.1	dnaJ ERDJ3A
LOC_Os06g14240.1	kDa heat shock peroxisomal
	Light receptors
LOC_Os01g62810.1	ultraviolet-B receptor UVR8
LOC_Os05g38270.1	ultraviolet-B receptor UVR8
	Photosynthesis
LOC_Os11g13890.1	chlorophyll a-b binding chloroplastic
LOC_Os09g17740.1	chlorophyll a-b binding chloroplastic
LOC_Os04g59440.1	photosystem II 22 kDa chloroplastic
	Heat stress
LOC_Os03g53340.1	heat stress transcription factor A-2c-like
LOC_Os03g06630.1	heat stress transcription factor A-2c-like isoform X1
LOC_Os04g48030.1	heat stress transcription factor B-2a-like
	Heat shock proteins
LOC_Os08g39140.1	heat shock 90
LOC_Os09g35790.1	heat shock factor
LOC_Os10g28340.1	heat shock factor HSF30 isoform X1
LOC_Os05g23140.1	small heat shock chloroplastic-like
Os03g0266900	kDa class I heat shock
LOC_Os06g06490.1	hsp70-Hsp90 organizing 1 isoform X3
LOC_Os01g08860.1	kDa class II heat shock
LOC_Os04g45480.1	hsp70-Hsp90 organizing
LOC_Os03g16860.1	heat shock cognate 70 kDa 2
Os02g0758000	kDa heat shock mitochondrial
LOC_Os02g54140.1	kDa class III heat shock
LOC_Os03g16030.1	kDa class I heat shock
LOC_Os02g04650.1	activator of 90 kDa heat shock ATPase

Table S1. (Continuation)

Locus	Putative function
	Protein folding
LOC_Os02g08490.1	chaperone mitochondrial
LOC_Os04g28420.1	70 kDa peptidyl-prolyl isomerase
LOC_Os05g48810.1	dnaJ homolog subfamily B member 4-like
LOC_Os02g28980.1	70 kDa peptidyl-prolyl isomerase
LOC_Os06g09560.1	chaperone -like
LOC_Os05g44340.1	chaperone 1
	Miscellaneous
LOC_Os02g15930.1	BAG family molecular chaperone regulator 6 isoform X2
LOC_Os02g56900.1	desumoylating isopeptidase 1
LOC_Os08g08850.1	fatty acid desaturase chloroplastic-like
LOC_Os01g36720.1	high affinity nitrate transporter
LOC_Os06g39240.1	multi -bridging factor 1c
LOC_Os06g51260.1	MYB transcription factor
LOC_Os01g61010.1	NUCLEAR FUSION DEFECTIVE 4
LOC_Os03g13450.1	OPI10 homolog
LOC_Os03g49430.1	pre-mRNA-splicing factor 18
LOC_Os03g51920.1	probable zinc metallopeptidase chloroplastic
Os03g0100200	pterin-4-alpha-carbinolamine dehydratase
LOC_Os02g52210.1	RING-H2 finger ATL29 isoform X1
LOC_Os02g40900.1	RNA-binding 42-like
LOC_Os03g15890.1	serine arginine-rich splicing factor SR45a
Os05g0407100	small EDRK-rich factor 2-like
LOC_Os03g60080.1	SNAC1
LOC_Os08g39370.1	tonoplast dicarboxylate transporter-like
LOC_Os03g17570.1	two-component response regulator-like PRR73
LOC_Os09g36220.1	two-component response regulator-like PRR95
LOC_Os08g39150.1	Ubiquitin and WLM domain-containing
LOC_Os07g43740.1	ubiquitin- ligase CIP8
LOC_Os06g30970.1	ubiquitin-conjugating enzyme E2-17 kDa

Table S1. (Continuation)

Locus	Putative function
	unknown
LOC_Os01g17396.1	PREDICTED: uncharacterized protein LOC4326913 isoform X4
LOC_Os03g27019.1	PREDICTED: uncharacterized protein LOC4333004 isoform X1
LOC_Os02g32990.1	PREDICTED: uncharacterized protein LOC4329552
LOC_Os01g31360.2	PREDICTED: uncharacterized protein LOC4324539 isoform X3
LOC_Os02g04510.1	PREDICTED: uncharacterized protein LOC4328240
LOC_Os09g23590.1	PREDICTED: uncharacterized protein LOC4346996
LOC_Os08g40910.1	PREDICTED: uncharacterized protein LOC4346066
LOC_Os06g04210.1	PREDICTED: uncharacterized protein LOC9269221
LOC_Os10g09240.1	PREDICTED: uncharacterized protein LOC4348192
LOC_Os05g38530.1	probable mediator of RNA polymerase II transcription subunit 37c
LOC_Os01g62290.1	probable mediator of RNA polymerase II transcription subunit 37c
LOC_Os09g27670.1	hypothetical protein
LOC_Os07g43950.1	LOC100285628 isoform X1

Table S2. Significantly light-induced genes in all tested lines except in *phyB* mutant lines (fold change >2.5, total of 14 genes).

Locus	Putative function
LOC_Os02g10390.1	chlorophyll a-b binding chloroplastic
LOC_Os07g37240.1	chlorophyll a-b binding chloroplastic
LOC_Os07g38960.1	chlorophyll a-b binding chloroplastic
Os08g0435900	chlorophyll a-b binding chloroplastic
LOC_Os01g41710.1	chlorophyll a-b binding chloroplastic
LOC_Os07g37550.1	chlorophyll a-b binding of LHCII type chloroplastic
LOC_Os06g21590.1	chlorophyll a-b binding 6A
LOC_Os04g33830.1	photosystem I subunit O
LOC_Os03g43810.1	transcription factor PIF1-like
LOC_Os10g41100.1	two-component response regulator-like PRR37
LOC_Os10g05130.1	arf-GAP with SH3 ANK repeat and PH domain-containing 2
LOC_Os03g13870.1	formin 5
LOC_Os09g35880.1	Salt tolerance
LOC_Os03g12700.1	PREDICTED: uncharacterized protein At1g01500

Chapter 5

Final Conclusion and Future Perspectives

Light modulates the behavior of various living organisms affecting many processes throughout their lives. Growth, movement and, reproduction are some examples. Diurnal animals take advantage of their vision to analyze the surrounding environment, for instance to move towards water, chase a prey and, avoid obstacles. Other animals, such as reptiles, dependent on the sun light as energy source to raise their body temperature and become active. Thus, light plays an important role as energy source and as stimulus that modulate the response of diurnal animals to the surrounding environment. Light is also essential for plants, which, as autotrophic organisms, rely on light as energy source to perform photosynthesis. In addition, as sessile organisms, plants cannot move or scape from adverse conditions, therefore, they regulate gene expression by interconnecting light signal with other external cues. In plants, light is perceived by photoreceptors that regulate the activity of transcription factors, such as Phytochrome-Interacting Factors (PIFs), to initiate a light-signaling cascade. Plants use PIFs as central hubs to interconnect light signaling, environmental cues, and internal signals (e.g. hormones) and thus regulate growth and development.

The rice PIF14 (OsPIF14) was identified to bind to *OsDREB1B* promoter, a key regulator of abiotic stress, and interacts preferentially with the active form of phytochrome B (Chapter 2), indicating that OsPIF14 protein is regulated by light and might be the link between light and cold responses. The main goal of this work was to characterize the function of the rice PIF14 (OsPIF14) at different levels. In Chapter 2, we characterized the involvement of OsPIF14 on the transcriptional regulation of *OsDREB1B*. For that, we identified the specific nucleotides where OsPIF14 binds to the *OsDREB1B* promoter and analyzed its function on the regulation of *OsDREB1B* gene expression. To further characterize OsPIF14 function, we produced RNAi::*OsPIF14* silencing lines that were analyzed at phenotypic and molecular level. In Chapter 3, we suggest a possible involvement of OsPIF14 with the jasmonic acid signaling pathway and a possible role in root architecture. In Chapter 4, we used a transcript profile approach to identify new OsPIF14 target genes, and showed that OsPIF14 regulates the expression of genes involved in cell wall structure and/or cell elongation.

The RNAi::*OsPIF14* transgenic lines were very important to unveil OsPIF14 biological functions (Chapter 3 and 4). These lines showed <50% of *OsPIF14* silencing, and we are now producing new tools, namely *OsPIF14* knockout lines by CRISPR/Cas9 and also OsPIF14::HA overexpressing lines to further characterize OsPIF14 functions in rice.

In Arabidopsis, the expression of some *DREB* genes is regulated by light in a PIF dependent manner. In Chapter 2, we show that OsPIF14 interacts preferentially with the active form of phytochrome B suggesting that OsPIF14 is regulated by light. We also show that OsPIF14 binds to two N-box present at *OsDREB1B* promoter and slightly represses the endogenous expression of *OsDREB1B* in rice protoplasts. However, *in planta*, we did not observe an *OsDREB1B* light regulation when rice seedlings are grown at control temperature. Under this condition, *OsDREB1B* expression is very low, which makes it difficult to study the effects of a repressor TF, such as OsPIF14. In Arabidopsis, the light induction observed in *DREB1s* gene expression is dependent on the length of the photoperiod. The expression of *DREB1s* is three- to five- fold higher in short days than in long days. However, these differences disappear in the *pif4pif7* double mutant, showing that PIFs are involved in the regulation of *DREB1s* under long day. Therefore, it would be interesting to analyze the *OsDREB1B* transcript level along the day when rice seedlings were grown in short and long days, under both control and colder temperatures, and test cold tolerance under these conditions. In addition, the analysis of the transgenic rice lines (RNAi::*OsPIF14*, *OsPIF14* knockout plants, and OsPIF14::HA overexpressing) will be crucial to understand whether OsPIF14 has a role in the regulation of *OsDREB1B* and if that is associated with any effect on plant cold tolerance.

In the present work, we suggest an involvement of OsPIF14 with the jasmonic acid (JA) pathway. Even though we still do not fully understand how/if OsPIF14 regulate JA biosynthesis/signaling, the root phenotype of RNAi::*OsPIF14* lines under dark display similarities with JA-induced root curling phenotype. We proved that OsPIF14 binds to the promoter and represses the expression of an important gene in JA biosynthesis, the *allene oxide synthase 1* (*AOS1*).

Surprisingly, RNAi::*OsPIF14* seedlings do not show differences in *AOS1* gene expression as compared to WT. This suggests that *in planta*, other TFs can act together with OsPIF14 or have redundant function regarding the regulation of *OsAOS1* expression (e.g. other bHLHs, including PIFs) or that the level of *OsPIF14* silencing is not enough to see an effect on *OsAOS1* gene expression. It is also possible that the activity of OsPIF14 is blocked by the interaction with other proteins, such as, the HFR1. Using the OsPIF14::HA overexpressing lines, it would be interesting to perform pull-down assays to identify OsPIF14 interactors. Moreover, the ability of other bHLHs, especially PIFs to bind to *AOS1* promoter need to be evaluated. However, we cannot rule out the possibility that OsPIF14 can regulate the expression of other genes from the JA biosynthesis pathway. In the future, it is fundamental to quantify the JA levels (WT vs. RNAi::*OsPIF14*) and analyze the expression of other genes of the JA biosynthesis pathway to understand whether OsPIF14 is involved in the regulation of genes from JA biosynthesis pathway.

Regarding the JA signaling pathway, the RNAi::*OsPIF14* lines showed downregulation of two *jasmonate ZIM-domain* (*JAZ*) genes as compared to WT. JAZs are the constitutive repressors of JA signaling. Due to their interaction with TFs, JAZs inhibit the regulation of genes associated with JA response. After JA stimulus, JAZs are degraded and the expression of JA-responsive genes is resumed. The expression of JAZ genes had already been associated with root curling phenotype, as the RNAi::*HOS1* rice mutant shows lower percentage of root curling and higher expression of these two JAZ genes, as compared to WT. These plants are more tolerant to JA and the JA effect on root curling is only observed after the addition of high concentration of JA. These results suggest that JAZ are indeed involved in root curling and that our RNAi::*OsPIF14* lines might be more sensitive to JA or have a constitutive regulation of JA-responsive genes. To test whether RNAi::*OsPIF14* lines are more sensitive to JA, it would be interesting to analyze the inhibitory effect of JA on root growth rate of OsPIF14 silencing/knockout and overexpressing lines. Moreover, the expression analysis of other JA signaling genes, especially the other members of the JAZ family

would contribute to a better understanding of the role of OsPIF14 in JA signaling pathway. In addition, it would be very interesting to analyze the transcriptome of shoots and roots of etiolated RNAi::*OsPIF14*/*OsPIF14* knockout lines, to gain new insights into the root curling mechanism.

In Arabidopsis, light-dark cycles are important to regulate PIFs stability and concomitantly, plant growth and development. Under light conditions, activated phytochromes regulate PIFs stability (by degradation or sequestration) and consequent downstream targets. In rice, however, nothing is known about OsPIF14 light regulation. Here, we analyzed the light stability of OsPIF14 protein *in vitro*, under different light conditions. Our results showed that the degradation rate of OsPIF14 is decreased in the *phyB* mutant, as compared to WT, suggesting an involvement of phyB in OsPIF14 degradation. Although PIFs are usually degraded via proteasome, the degradation of OsPIF14 did not decrease with the addition of a proteasome inhibitor, suggesting that OsPIF14 is being degraded by other process, such as the action of proteases. Moreover, the observed degradation is light independent, which contrasts with what has been described for this regulation, and reinforce the hypothesis of protease degradation. These results show that this *in vitro* OsPIF14 degradation assay needs to be optimized or the stability of OsPIF14 needs to be assessed by other method. Therefore, we are generating OsPIF14::HA overexpressing lines that would be crucial to better characterize OsPIF14 protein light stability.

To better understand the function of OsPIF14 on the transition from dark to light, we collected a dark sample at 30 minutes before the light onset, and a light sample at 30 minutes after light onset. WT, RNAi::*OsPIF14*, and *phyB* mutant were collected at these two time points, and their transcript profile was analyzed by microarrays. As expected, two major clusters were observed, one for dark and another one for light samples, showing that light is playing the major role in gene regulation. We identified some differentially expressed genes (DEGs) specific for the RNAi::*OsPIF14* rice lines. Only one gene was downregulated in the RNAi lines, as compared to WT and *phyB*, and the analysis of its promoter did not show any putative OsPIF14 *cis*-element binding site, suggesting that it would not be a

direct target of OsPIF14. All the other DEGs specific for RNAi::*OsPIF14* were upregulated and their promoters showed at least one putative OsPIF14 *cis*-element binding site, suggesting that these genes can be direct targets and that OsPIF14 can repress their expression (as previously shown for *OsDREB1B* and *OsAOS1*). The function of these DEGs suggests that OsPIF14 might be involved in the regulation of cell wall structure and/or cell elongation. These results need to be confirmed in *OsPIF14* knockout and overexpressing plants, namely the transcript level of these genes as well as the cell size in these plants as compared to WT. Rice plants overexpressing OsPIL13 showed elongated cells, therefore, if we confirm that OsPIF14 is also involved in cell elongation, both rice PIFs regulate cell structure and elongation.

Most of the current basic knowledge on plant sciences has been acquired from studies using the model plant Arabidopsis. Nevertheless, the translation of that knowledge into plants economically more relevant, such as crops, is crucial to improve their growth, development, and productivity under control and adverse environmental conditions. Despite the growing understanding of the mechanisms by which light regulates crop growth, many of the factors that plants use to integrate several stimuli are still poorly understood.

This work provides new insights into the biological function of OsPIF14 and its role in light-regulated plant growth. We show that OsPIF14 regulate cold, jasmonic acid and light related genes, suggesting that OsPIF14 can be a central hub in the regulation of plant growth and development. Further functional characterization of OsPIF14, and other OsPIFs, is needed to better understand how light and other external stimuli crosstalk to regulate rice plants growth and development