Novel Transcription Factors Regulating the Expression of the Rice Gene *OsDREB1B*

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“Nothing shocks me, I’m a scientist.”

Indiana Jones, Indiana Jones and the Temple of Doom

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I, Duarte Dionísio Figueiredo, declare by my honour to have had active participation in the following research papers:

- **Duarte D. Figueiredo, Pedro M. Barros, André M. Cordeiro, Tânia Serra, Tiago Lourenço, Subhash Chander, M. Margarida Oliveira and Nelson J. M. Saibo.** Seven Zinc Finger transcription factors are novel regulators of the stress responsive gene *OsDREB1B*. Submitted.
Duarte Figueiredo did the research work and wrote the manuscript.

- **Duarte D. Figueiredo, André M. Cordeiro, James Tepperman, Peter H. Quail, M. Margarida Oliveira and Nelson J. M. Saibo**. OsPIF4 is a novel Phytochrome Interacting Factor that unveils a potential convergence of light and cold signalling pathways on the rice gene *OsDREB1B*. Submitted.
Duarte Figueiredo did the research work and wrote the manuscript.

- **Ana Paula Santos, Tânia Serra, Duarte Figueiredo, Pedro Barros, Tiago Lourenço, Subhash Chander, M. Margarida Oliveira, Nelson J. M. Saibo**. Transcription regulation of abiotic stress responses in rice: a combined action of transcription factors and epigenetic mechanisms. Submitted.
Duarte Figueiredo did part of the review work and writing of the manuscript.

List of Abbreviations

3-AT – 3-Amino-1,2,4-Triazole
a.a. – amino-acid
ABA – Abscisic Acid
AP2 – APETALA2
APB – Active Phytochrome Binding domain
bHLH – basic Helix-Loop-Helix
BiFC – Bimolecular Fluorescence Complementation
BLAST – Basic Local Alignment Search Tool
bp – base pair
C2H2 – Cysteine2/Hystidine2
cDNA – complementary DNA
CBF – C-REPEAT BINDING FACTOR
ºC – degrees Celsius
DRE - Dehydration Responsive Element
DREB – DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR
DNA – Deoxyribonucleic Acid
EDTA – Ethylene Diamine Tetraacetic Acid
ERF – Ethylene Response Factor
g – gram
g – gravitational force
h – hours
HD – Homeodomain
HOS – HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE
ICE – INDUCER OF CBF EXPRESSION
kDa – kilo Dalton
L – litre
M – molarity
m - mass

µg – microgram

µL – microlitre

µM – micromolar

min – minutes

mg - miligram

mL – mililitre

mM – milimolar

nm – nanometer

Os – *Oryza sativa*

PCR – Polymerase Chain Reaction

Pfr – Phytochrome active form

Phy – Phytochrome

PIF – Phytochrome Interacting Factor

Pr – Phytochrome inactive form

s - second

TF – Transcription Factor

RNA – ribonucleic acid

rpm – rotations *per* minute

RT – Room Temperature

RT-PCR- Reverse Transcription PCR

RT-qPCR – quantitative/real time RT-PCR

SDS – Sodium Dodecyl Sulfate

v - volume

WT – Wild Type

Y1H – Yeast One-Hybrid

Y2H – Yeast Two-Hybrid

YFP – Yellow Fluorescent Protein

ZF-HD – Zinc Finger Homeodomain

General Abstract

Environmental stresses are responsible for major losses in crop production worldwide. Due to an increasing world population, there is a higher demand for food, feed and fuel, which requires the development of new plant varieties with an increased yield, particularly under adverse environmental conditions. Transcription Factors (TFs) are promising candidates for plant improvement, since a single TF can modulate the expression of several genes. The AP2/ERF family of transcriptional regulators and, namely, the TFs belonging to the DREB1/CBF sub-family were described as major regulators of plant responses to abiotic stresses. Nevertheless, the transcriptional regulation of the genes coding for these TFs is still poorly understood. We focused our research on the rice gene *OsDREB1B*, whose gene expression had been previously described as responsive to abiotic stress, namely cold. Using semi-quantitative RT-PCR, we confirmed the cold-induction of this gene, but also observed that its response is different depending on the severity of the stress. Moreover, we observed that this gene is highly induced in response to drought stress in roots, and also in response to mechanical stress. Using the Yeast One-Hybrid system and the promoter of *OsDREB1B* as bait, we have screened a rice cold-induced cDNA expression library. Thereby we identified eight TFs as binding to *OsDREB1B* promoter: seven Zinc Finger TFs, of which three C2H2-type and four Zn Finger-Homeodomain (ZF-HD) TFs, as well as one bHLH TF, predicted as a putative Phytochrome Interacting Factor (PIF).

Regarding the Zn Finger TFs, we determined their gene expression patterns in response to several abiotic stress conditions and to ABA treatment. We observed that the genes coding for these TFs responded differently to the various stresses imposed. This is illustrative of the cross-talk between stress-signalling pathways. Using a transactivation assay in *Arabidopsis* protoplasts, we observed that all the Zn Finger TFs are

repressors of gene expression when binding to the promoter of *OsDREB1B*. We also tested if the Zn Finger TFs interacted with one-another and verified that the ZF-HD TFs formed several homo- and hetero-dimers, whereas no interactions were observed for the C2H2-type TFs.

Concerning the gene coding for the bHLH TF, that we named OsPIF4, we observed that it was regulated by several abiotic stress conditions at the transcriptional level and also that its transcript was alternatively spliced in response to cold stress. Using a transactivation system we showed that OsPIF4 is a negative regulator of *OsDREB1B* gene expression. This TF had been previously predicted as a putative PIF, since it had an Active Phytochrome Binding domain (APB). We observed that this protein interacted preferentially with the active Pfr form of the rice Phytochrome B (OsphyB). This was the first time that a PIF was shown to interact with phytochromes in rice and was an indication that the activity of this protein could be modulated by light. This was further supported by the finding that OsPIF4 abundance is reduced by light during the day of a diurnal light/dark cycle, compared to plants retained in prolonged darkness during the corresponding subjective day period. Concomitantly, we observed that *OsDREB1B* transcript levels are up-regulated by this daylight treatment, compared to darkness during the subjective day. These findings provide the first indication that OsPIF4 may be a link between light signalling and the OsDREB1 regulon in rice and suggest that light and cold-temperature signalling pathways converge on the regulation of *OsDREB1B*.

This work provides new insights on the transcriptional regulation of *OsDREB1B,* being the first regarding the rice *DREB1/CBF* sub-family. This will allow a better understanding of the abiotic stress signalling pathways, as well as provide new possible targets for plant improvement in the future, particularly in what concerns abiotic stress tolerance.

Resumo Geral

Factores ambientais adversos são responsáveis por avultadas perdas na produção agrícola, por todo o mundo. Devido à crescente população mundial, há cada vez uma maior necessidade de alimento, para pessoas e gado, assim como de combustíveis, o que implica o desenvolvimento de novas variedades de plantas, que possibilitem um maior rendimento, mesmo sob condições ambientais adversas. Factores de Transcrição (FT) são candidatos promissores para o melhoramento de plantas, uma vez que um só FT pode modular a expressão de vários genes. A família de reguladores transcricionais AP2/ERF e, nomeadamente, os FT pertencentes à sub-família DREB1/CBF, foram descritos como importantes reguladores das respostas das plantas a stresses abióticos. No entanto, a regulação da transcrição dos genes que codificam estes FT ainda é pouco compreendida. A nossa investigação focou-se no gene de arroz *OsDREB1B*, previamente descrito como induzido por stresses abióticos, nomeadamente frio. Usando RT-PCR semi-quantitativo, confirmámos a indução deste gene pelo frio, mas também observámos que esta resposta é dependente da intensidade do stress. Também verificámos que este gene é altamente induzido por secura nas raízes, e também por stress mecânico. Utilizando o sistema de Yeast One-Hybrid pesquisámos uma biblioteca de cDNA de arroz, induzida por frio, de modo a identificar FT que se ligassem ao promotor do gene *OsDREB1B*. Identificámos assim oito FT que se ligam a este promotor: sete “Zn Fingers”, três do tipo C2H2 e quatro “Zn Finger HomeoDomain” (ZF-HD), e um FT do tipo “basic Helix Loop Helix” (bHLH), previsto como um “Phytochrome Interacting Factor” (PIF).

Em relação aos FT “Zn Fingers”, determinámos a expressão dos respectivos genes em resposta a vários stresses abióticos assim como a tratamento com ABA. Observámos uma grande variedade de respostas, uma vez que estes genes respondem diferentemente aos diversos stresses aplicados. Isto é ilustrativo da existência de elementos comuns

entre as várias vias de sinalização de stress. Usando um sistema de transactivação em protoplastos de *Arabidopsis*, observármos que todos os FT "Zn Fingers" são repressores da transcrição, quando ligados ao promotor de *OsDREB1B.* Testámos também a interacção entre os vários FT "Zn Fingers": Verificámos que os ZF-HD formavam homo- e hetero-dímeros, mas não detectámos quaisquer interacções para os Zn Fingers C2H2.

Relativamente ao gene que codifica o FT bHLH, a que chamámos OsPIF4, observámos que era regulado por vários stresses abióticos, e também que o seu transcrito sofria "splicing" alternativo em resposta ao frio. Usando um sistema de transactivação mostrámos que o OsPIF é um regulador negativo da expressão do gene *OsDREB1B*. Este TF tinha sido previamente previsto como um PIF putativo, devido à presença de um domínio de ligação a fitocromos (ABP). Confirmámos então que este FT interagia com a forma activa Pfr do Fitocromo B de arroz. Esta foi a primeira vez que foi confirmada em arroz a interacção de um PIF com um fitocromo indicando que a actividade desta proteína poderia ser modulada pela luz. Isto foi corroborado pela observação de que a abundância de OsPIF4 era reduzida pela luz durante o dia de um ciclo luz/escuro, quando comparado com plantas mantidas em escuro constante, durante o respectivo dia subjectivo. Concomitantemente, observámos que o transcrito de *OsDREB1B* era positivamente regulado por este tratamento de luz, comparativamente com a escuridão do dia subjectivo. Estes resultados são a primeira indicação que o OsPIF4 poderá ser um elo de ligação entre a via de sinalização de luz e o regulão OsDREB1. Sugerem também que as vias de sinalização de luz e frio convergem na regulação do gene *OsDREB1B*.

Este trabalho permitiu-nos obter novos conhecimentos acerca da regulação do gene *OsDREB1B,* e foi o primeiro estudo do género em elementos da sub-família *DREB1/CBF* em arroz. Tal permitirá uma melhor

compreensão das vias de sinalização de stress abiótico em arroz, assim como a identificação de novos alvos para o melhoramento de plantas, especialmente no que diz respeito à sua resposta a condições ambientais adversas.

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General Introduction

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A short history of rice

Rice (*Oryza sativa*, L.) belongs to the genus *Oryza* of the Poaceae family and is the staple food for over half of the world population, namely in Asia, where 90% of rice is produced (Khush, 1997; Khush, 2005). There are two species of cultivated rice: the more common *Oryza sativa*, an Asian rice, and *Oryza glaberrima*, an African species, grown on a smaller scale in western Africa. The domestication of wild rice is believed to have started about 9000 to 10000 years ago in Asia and it was probably in China that rice cultivation was refined (Khush, 1997). Some authors claim that the two main sub-species of *Oryza sativa* (Indica and Japonica) have a single origin, having resulted from the dispersal of the species (Schaal *et al.*, 2011), while others argue that these two sub-species were domesticated independently, having arisen from independent genetic pools of a wild rice species, *Oryza rufipogon* (Kovach *et al.*, 2007). Rice was eventually exported from Asia to Europe, probably through the expedition of Alexander the Great to India, and was slowly adopted in the Mediterranean region. At the same time, rice cultures spread throughout Asia, giving birth to different varieties, some tropical and some temperate (Khush, 1997). Portuguese priests are believed to have introduced temperate japonicas from Indonesia in Guinea-Bissau, which eventually led to the spread of this culture in Africa. Moreover, the Portuguese and the Spanish were also responsible for the introduction of rice in South America.

Today, rice is a very diverse crop, being cultivated in very contrasting environments (Khush, 2005). In the past few decades, the green revolution allowed the production of rice to exceed the population growth, but it is now estimated that rice production will have to increase in 40% by 2030 to satisfy the demand (Khush, 2005). Nevertheless, this increase will have to occur using less land, less water and less chemicals. The improvement of

existing rice varieties is therefore of paramount importance in order to increase yield and cope with the increasing population numbers.

Rice in Portugal

The first references to rice cultivation in Portugal date from the XIIIth and XIVth centuries, where it is described as food for the wealthier classes (Silva, 1969). It is likely however, that rice was introduced in the Iberian Peninsula centuries earlier by the Arabs. Nevertheless, it was only some centuries later that rice cultures started to spread, mainly in the basins of the main Portuguese rivers. In the XXth century, with the adoption of improved irrigation techniques, the cultivation and gastronomical use of rice boosted, mainly in the North of the country (Silva, 1969). Several varieties were then adopted and their productivity was improved through the work of the former "Estação Agronómica Nacional". Nowadays, Portugal is the largest rice consumer in Europe, with approximately 15kg of rice *per capita*, *per* year, while the second largest consumer, Spain, consumes 7kg of rice *per capita, per* year (according to the Food and Agriculture Organization of the United Nations (FAO), http://faostat.fao.org). Nevertheless, the production of rice in Portugal is not sufficient for the demand, and around 40 to 50% of rice consumed in Portugal is imported (Negrão, 2008). According to FAO, in the past 50 years, the total production of rice in Portugal has not increased and in several years it was even below the production rates obtained in the beginning of the 1960's.

Rice as a model plant

Even though *Arabidopsis* has long been used as the primary model for plant research, rice has been well established as the second model organism for plant systems (Rensink and Buell, 2004). This is not only due to its social and economical importance, but also to a growing number of

tools available, which have stimulated its study. Moreover, rice represents a different taxonomic group from *Arabidopsis*, the monocots, which comprises many important food crops. Rice has also a relatively small genome (390Mbp), especially when compared to other monocots, and is a diploid species (2n=24) with a relatively short life cycle (3-6 months), for which there are several genetic transformation tools available. Rice was also the second plant species to have its genome fully sequenced (Goff *et al.*, 2002; Yu *et al.*, 2002), which largely increased the amount of resources available for its study.

Plant responses to Abiotic stress

Impact of abiotic stress in plant development and productivity

The negative impact of an abiotic stress condition as well as the response that it triggers in the plant depends on its duration, severity and the frequency by which it is imposed (Bray *et al.*, 2000). Abiotic, as well as biotic stresses, account for a high percentage of crop yield losses every year. So far, the development of crop varieties with increased tolerance to abiotic stress conditions has been slow, since many mechanisms governing plant responses to these conditions are still poorly understood (Khush, 2005).

Water deficit is a common feature of several environmental conditions: Drought conditions limit water availability to the plant, but high salinity and low temperatures can also lead to water deficit in plants (Bray *et al.*, 2000). Under high salt conditions, it is harder for plants to obtain water, due to decreased soil water potential. And, under freezing temperatures, water can also leave the cells and form ice crystals in inter-cellular spaces. Nevertheless, even temperatures that are not low enough for ice to form can cause chilling injury in plants that are normally grown at warm

temperatures, which is the case of many crops (Taiz and Zeiger, 2006). Rice plants are particularly sensitive to low temperature, especially at booting stage, in a process that affects the pollen formation, thus leading to male sterility (Mamun *et al.*, 2006).

Cellular mechanisms of abiotic stress perception and response

In order to respond to adverse environmental conditions, plants have evolved several mechanisms of stress-adaptation, which comprise a series of physiological, biochemical and molecular changes. Even though some of those processes are common between stresses, there are also several response mechanisms that are specific to each kind of stress (Knight and Knight, 2001).

Drought

Although drought stress has been extensively studied, the primary perception of dehydration signals by the plant cells is still unknown. Nevertheless, several reports have highlighted the role of secondary messengers, such as calcium ions and phospholipid-derived compounds in the transduction of such signals (Zhu, 2002). The hormone abscisic acid (ABA) has long been known to play a prominent role in the response to drought stress, and its concentration in leaves can increase up to 50 times under drought conditions (Taiz and Zeiger, 2006). In response to a rapid dehydration, ABA induces stomata closure, which is the earliest physiological response to decrease evaporation of water (Shinozaki and Yamaguchi-Shinozaki, 2006; Taiz and Zeiger, 2006). But, in the case of gradual and prolonged dehydration, other developmental responses occur, like a decrease in leaf area and mobilization of resources to the roots, promoting their growth (Taiz and Zeiger, 2006). At cellular level, however, dehydration induces the accumulation of osmoprotectants. These

compounds, together with other proteins, work to protect the cellular components, namely membranes, from damage as well as to maintain osmotic balance within the cell (Shinozaki and Yamaguchi-Shinozaki, 2006). One major group of proteins that has been described as involved in drought-related responses are the Late Embryogenesis Abundant (LEA) proteins. Although the role of these proteins remains unclear, due to their high hydrophilicity they are believed to help the cells retain water, as well as to prevent the crystallization of other proteins that are important for cellular function (Taiz and Zeiger, 2006).

Salinity

Drought and high salinity share common mechanisms of perception and response, since both these stresses result in the dehydration of the cells and in osmotic imbalance. Salinity stress, however, is composed both of an osmotic stress (physical), as well as of an ionic Na^+ toxicity stress (chemical; Taiz and Zeiger, 2006). This means that some of the perception and response mechanisms are specific to salinity stress, namely in what regards protecting the cells from excessive Na^+ ions.

Although the primary sensor for salinity stress is still unknown, calcium signals have been shown to be major components of the plant response to ionic stress, in a pathway mediated by the SOS (Salt Overly-Sensitive) proteins (Zhu, 2003). This pathway is now known to be conserved in several plant species (Shi *et al.*, 2000; Martinez-Atienza *et al.*, 2007; Olias *et al.*, 2009). SOS3, a calcium-binding protein, is essential for transducing the salt-stress signals in *Arabidopsis* (Liu and Zhu, 1998). This signal transduction is achieved together with SOS2, a protein kinase that regulates the activity of SOS1, a plasma membrane Na^+/H^+ exchanger (Qiu *et al.*, 2002). The SOS1 transporter is active in salt-stressed plants and its greatest activity is detected in root epidermal cells, where it unloads Na^+

ions from the cytosol, preventing its transport to the shoot by the vascular tissues (Zhu, 2003). Under high salt conditions, in addition to Na^+ efflux, cells also work to compartmentalize Na^+ ions in vacuoles and in other organelles, in order to maintain osmotic adjustment in the cytoplasm (Zhu, 2003). Tonoplast ion transporters belonging to the NHX family have been shown in different plant species to play a fundamental role in ion compartmentalization, and consequently in salinity tolerance (Apse *et al.*, 2003; Fukuda *et al.*, 2004; Zorb *et al.*, 2005; Ye *et al.*, 2009). Interestingly, the activity of these proteins in *Arabidopsis* seems to be mainly regulated by ABA (Shi and Zhu, 2002), being at least in part independent of the SOS-pathway.

Low temperature

It is believed that plant cells perceive temperature changes primarily through changes in membrane fluidity (Orvar *et al.*, 2000; Sangwan *et al.*, 2001; Penfield, 2008). In fact, membrane integrity and fluidity are severely affected under extreme temperatures, particularly under freezing stress (Bray *et al.*, 2000). Given the crucial role of membranes under temperature stress, maintaining their stability is a priority for the cell. This is achieved through an increased synthesis of polyunsaturated fatty acids and changes in membrane components, such as sterols, which work to maintain membrane integrity and fluidity (Bray *et al.*, 2000; Wallis and Browse, 2002; Falcone *et al.*, 2004). The cytoskeleton has also been shown to play an important role in the temperature sensing mechanism, since the use of microtubule stabilizers prevents the cold-dependent expression of certain genes (Orvar *et al.*, 2000; Sangwan *et al.*, 2001). The changes in temperature perceived by the membrane are likely transmitted by the cytoskeleton to calcium channels. Oscillations in cytosolic [Ca^{2+}] are believed to mediate temperature signalling, since EGTA, a calcium

chelator, when applied to *Arabidopsis*, reduced the *KIN1* cold-induced gene expression (Knight *et al.*, 1996). Moreover, the use of a calcium channel blocker, was reported to reduce the cold-induced expression of *LTI78* (Henriksson and Trewavas, 2003). Interestingly, the amplitude of calcium waves has even been shown to depend on the temperature shift (Plieth *et al.*, 1999). These waves will most likely lead to the activation of calcium-dependent kinases responsible for signal transduction, and ultimately to changes in gene expression (as reviewed by Chinnusamy *et al*. (2007)).

These mechanisms of perception not only lead to short-term protective responses, but can also result in cold acclimation. Cold acclimation is the process through which plants become more tolerant to freezing after a period of exposure to low non-freezing temperatures (Chinnusamy *et al.*, 2007). Most plants from temperate regions have the ability to cold acclimate. Nevertheless, many important crops, such as rice, tomato and maize do not share this capability. The cold acclimation process is characterized by the reprogramming of metabolism and gene expression and the remodelling of cell and tissue structures (reviewed by Chinnusamy *et al.* (2007)).

Ultimately, the abiotic stress responses result in a massive reprogramming of gene expression. Products of genes induced by stress can be classified in two groups (Yamaguchi-Shinozaki and Shinozaki, 2006): (1) proteins that have a direct role in stress tolerance, such as chaperones, LEA proteins and enzymes involved in osmolyte biosynthesis; and (2) proteins that have a role in signal transduction, such as transcription factors (TFs), kinases and other signalling molecules. Another classification for stress-responsive genes depends on the timing of their induction/repression (Yamaguchi-Shinozaki and Shinozaki, 2006): The transcript level of the first group of genes is rapidly and transiently

regulated and they usually encode TFs and other regulatory proteins, whereas the second group shows a later transcript regulation and encodes LEA proteins and proteins involved in the biosynthesis of protective compounds, but also TFs with a role in late stress-responses.

Regulons in ABA-dependent and -independent signalling

The hormone ABA has long been known to play an important role in plant responses to abiotic stresses, namely drought (Zhu, 2002). For example, it has been reported that *Arabidopsis* plants under water stress modulate their carbon usage and mobilization (Hummel *et al.*, 2010), and ABA was found to have a direct role in that metabolic remodelling (Kempa *et al.*, 2008). Moreover, ABA accumulated during dehydration regulates the accumulation of various amino-acids and sugars, which correlates with the expression of ABA-regulated genes in the respective biosynthetic pathways (Urano *et al.*, 2009). The regulation of stomatal closure under drought conditions has also been shown to be highly dependent on ABA. Mutants deficient in ABA synthesis cannot close the stomata and have a wilty phenotype (Taiz and Zeiger, 2006). Even though ABA seems to play a more prominent role in the response to drought (Lang *et al.*, 1994), the biosynthesis of this hormone is also required for cold acclimatization and thermotolerance (Gilmour and Thomashow, 1991). This is illustrative of the relevance of this hormone in the stress signalling pathways.

Exogenous application of ABA induces stress-related genes, but several cold- and drought-related genes are not induced by this hormone (Yamaguchi-Shinozaki and Shinozaki, 2006). The study of *cis*-acting elements present in the promoter regions of stress-responsive genes allowed a better understanding of the pathways mediated, or not, by ABA. The *Arabidopsis* gene *RD29a* (also known as *COR78* or *LTI78*) was

described as being induced by several abiotic stress conditions and by ABA (Yamaguchi-Shinozaki and Shinozaki, 1993). Nevertheless, the promoter of this gene was found to have both ABA-dependent and -independent *cis*-acting elements, thus revealing the existence of two independent regulatory pathways (Yamaguchi-Shinozaki and Shinozaki, 1993). A summary of the transcriptional pathways involved in abiotic stress signalling can be found in Fig. 1.

ABA-dependent pathway

Many genes that are ABA-responsive have in their promoters a conserved *cis*-acting sequence, known as ABA Responsive Element (ABRE; PyACGTGG/TC). The ABRE was first identified in wheat (Guiltinan *et al.*, 1990) and in rice (Mundy *et al.*, 1990) and it was reported that one copy of this element was not sufficient for ABA-dependent gene expression. Later, it was shown in barley that ABRE, together with the coupling element 3 (CE3), was sufficient to confer ABA-induced gene expression (Shen *et al.*, 1996). These authors also reported the existence of another element (CE1), with similar function, and that can act in synergy with CE3. Coupling elements were further identified in other plant species, such as rice (Hobo *et al.*, 1999a) and maize (Niu *et al.*, 2002), which means that they are a conserved component in ABA-signalling. Interestingly, in *Arabidopsis*, the ABA-independent *cis*-acting element Drought Responsive Element/C-Repeat (DRE/CRT) may function as an ABRE-coupling element for ABA responses (Narusaka *et al.*, 2003). By removing the DRE/CRT core-elements in the promoter of *RD29a*, Narusaka *et al.* (2003) observed that this gene no longer responded to ABA, even though there was an intact ABRE *cis*-element in its promoter.

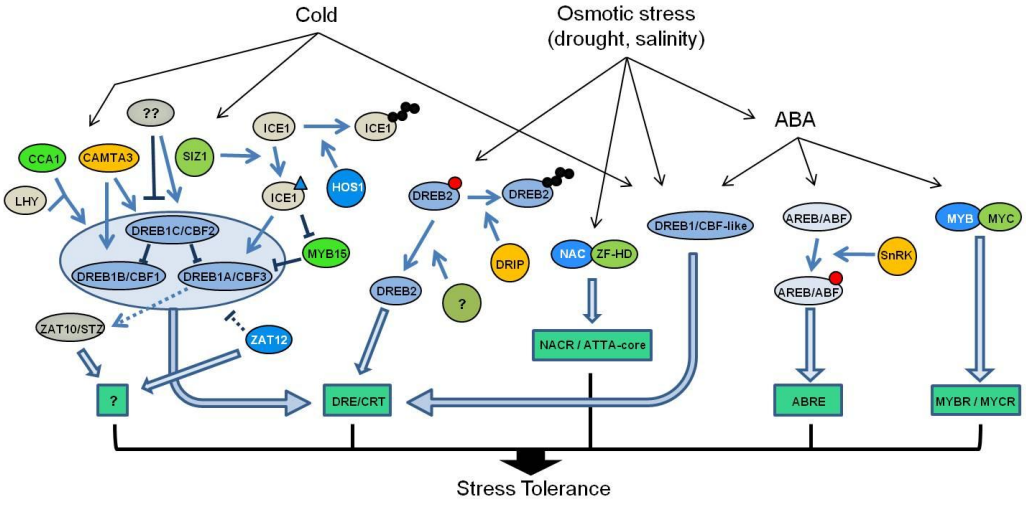

Figure 1. Transcription Factors in ABA-dependent and -independent pathways. TFs are shown in ovals. TF modifying enzymes are shown in circles. Ovals and circles with question marks represent regulatory elements yet to be identified. *cis*-elements for TF binding sites are shown in boxes. Unknown binding elements are indicated with question marks. The red and black circles represent phosphorylation and ubiquitination of target proteins, respectively. The blue triangle represents SUMOylation of ICE1. Dotted lines indicate unknown mechanism of regulation.

Several basic leucine zipper (bZIP) TFs were identified as binding to ABRE *cis*-elements, thereby regulating ABA-dependent gene expression (Hobo *et al.*, 1999b; Choi *et al.*, 2000; Uno *et al.*, 2000). In *Arabidopsis*, these TFs were named ABRE BINDING FACTORS (ABFs) and ABA-RESPONSIVE ELEMENT BINDING PROTEINS (AREBs) by two independent groups (Choi *et al.*, 2000; Uno *et al.*, 2000). Both authors were able to identify AREB/ABFs with the Yeast One-Hybrid system, using the ABRE element as bait. The genes coding for these TFs were described as induced in response to ABA treatments, and also to several abiotic stress conditions, such as dehydration, cold and salinity (Choi *et al.*, 2000; Uno *et al.*, 2000; Fujita *et al.*, 2005). It was also reported that the over-expression of these TFs in *Arabidopsis* resulted in enhanced drought tolerance and

also in hypersensitivity to ABA (Kang *et al.*, 2002; Fujita *et al.*, 2005). In the study by Kang *et al.* (2002), for instance, the authors observed that *Arabidopsis* plants over-expressing *ABF3* and *AREB2/ABF4* had reduced transpiration rates, when compared to the wild type plant, which correlated to smaller opening of the stomata. Moreover, *ABF3* and *AREB2/ABF4* over-expressing plants had increased levels of ABA-responsive genes (Kang *et al.*, 2002). These results provided evidence for the role of AREB/ABFs TFs in the regulation of ABA signalling.

In the work by Fujita *et al.* (2005), the authors reported that the over-expression of *AREB1/ABF2* by itself was not sufficient to induce the expression of ABA-responsive genes. This TF could therefore need to be modified somehow in order to promote the induction of its target genes. This hypothesis was supported by the report that ABI5, which belongs to the AREB/ABF family, was phosphorylated after a treatment with ABA (Lopez-Molina *et al.*, 2001). Moreover, Furihata *et al.* (2006) observed that AREB1/ABF2 is also phosphorylated in an ABA-dependent manner. This post-translational modification was shown to be mediated by the SNF1-Related Protein Kinase 2 (SnRK2) family (Furihata *et al.*, 2006). This mechanism seems to have been maintained throughout evolution, as the TRANSCRIPTION FACTOR RESPONSIBLE FOR ABA REGULATION (TRAB1) (Hobo *et al.*, 1999b), a rice homologue of AREB2/ABF4, was also reported to be phosphorylated in response to ABA (Kagaya *et al.*, 2002). Rice has ten genes coding for SnRK2 kinases, all of which respond to drought and three of them also respond to ABA (Kobayashi *et al.*, 2004). These three ABA-responsive SnRK2 proteins were shown to be responsible for the ABA-dependent phosphorylation of TRAB1 (Kobayashi *et al.*, 2005).

While many data have been gathered on the ABRE-driven ABA-dependent gene expression, it is known that the transcriptional responses

to this hormone are not exclusively dependent on the ABRE *cis*-element. The *Arabidopsis* gene *RD22* has no ABRE motifs in its promoter and, nevertheless, it is ABA-responsive. Its transcriptional regulation was found to be determined by MYC and MYB-type TFs (Abe *et al.*, 2003), which is illustrative of the presence of different signalling pathways regulating ABA-dependent gene expression.

ABA-independent pathway

Even though ABA plays a major role in the plant responses to abiotic stress, namely drought, there are also stress signalling pathways that work independently of this hormone (Shinozaki and Yamaguchi-Shinozaki, 2006). This ABA-independent mechanism was discovered through the observation that the *Arabidopsis* gene *RD29a* is induced by salinity and drought, even in *aba* and *abi* mutants, that are impaired in ABA synthesis and sensitivity, respectively (Yamaguchi-Shinozaki and Shinozaki, 1993). In this section, the regulons involved in ABA-independent signalling will be discussed.

The DREB1/CBF Transcription Factors

The DREB1/CBF regulon is by far the best characterized signalling pathway within the abiotic stress responses in plants. The DREB1/CBF sub-family of TFs is part of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family (Sakuma *et al.*, 2002), since these proteins have a canonical AP2/ERF DNA-binding domain. DREB1/CBF TFs were initially identified in *Arabidopsis* (Gilmour *et al.*, 1998; Liu *et al.*, 1998), but homologues have now been described in many other plant species, which illustrates their prominent role in plant biology (Dubouzet *et al.*, 2003; Zhang *et al.*, 2004; Skinner *et al.*, 2005; Xiao *et al.*, 2006; Qin *et al.*, 2007; Shan *et al.*, 2007; Zhang *et al.*, 2010).

DRE/CRT *cis*-element and *trans*-acting Transcription Factors

The *cis*-regulatory sequence, known as Drought-Responsive Element or C-Repeat (DRE/CRT) was identified in the promoter regions of abiotic stress-responsive genes by two independent groups (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). The nucleotide sequence of the *cis*-element DRE (TACCGACAT) in the promoter of the *Arabidopsis RD29a* gene was shown to be involved in the induction of this gene by cold, salinity and drought, but not by ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). This first report set the ground for this regulatory element as ABA-independent. In parallel, Baker *et al.* (1994) identified a CCGAC core sequence in the DRE motif, named CRT, which was involved in the low-temperature response of the *Arabidopsis COR15a* gene. Using a Yeast One-Hybrid system, a protein containing an AP2/ERF domain, named C-REPEAT BINDING FACTOR 1 (CBF1), was the first TF identified as binding to the CRT/DRE element (Stockinger *et al.*, 1997). Through a similar approach, DRE BINDING PROTEIN 1A (DREB1A), and DRE BINDING PROTEIN 2A (DREB2A) were identified as TFs that bind to the DRE element present in the promoter of *RD29a* (Liu *et al.*, 1998). In this study, DREB1A and DREB2A were also proposed to function in two separate pathways: low temperature and dehydration/osmotic stress, respectively. Medina *et al.* (1999) later identified two other TFs, CBF2 and CBF3, that showed a high similarity to CBF1. It is important to clarify that, although reported by different groups, DREB1 and CBFs are the same proteins. DREB1A corresponds to CBF3, DREB1B to CBF1 and DREB1C to CBF2. These TFs were later on described as binding to the sequence A/GCCGAC, a component of the DRE *cis*-element (Sakuma *et al.*, 2002). Two amino-acids, valine and glutamic acid, located in the AP2/ERF domain of DREBs, were found to be determinant for the binding of these TF proteins to DNA (Sakuma *et al.*, 2002). Recently, it was shown that

mutations in the DREB1 signature sequence PKKPAGR, outside the AP2/ERF domain, abolishes the ability of these proteins to bind DNA (Canella *et al.*, 2010), meaning that regions other than the canonical DNA-binding domain have also a functional role in this process. It is interesting to note that the DREB1A/CBF3 and DREB2A proteins have slightly different DNA-binding specificities (Sakuma *et al.*, 2006). This may help explaining why these TFs have different down-stream regulated genes (Sakuma *et al.*, 2006), which further supports the hypothesis that these two groups of TFs function in different pathways.

The OsDREB1 TFs in rice

In rice, several genes have been identified as homologues of the *Arabidopsis DREB1/CBF*s (Dubouzet *et al.*, 2003; Chen *et al.*, 2008; Wang *et al.*, 2008). *OsDREB1A-D* were initially identified by Dubouzet *et al.* (2003) through sequence homology with the *Arabidopsis* TFs, while *OsDREB1E-G* were later identified by different groups (Chen *et al.*, 2008; Wang *et al.*, 2008). Dubouzet *et al*. (2003) described *OsDREB1A* and *OsDREB1B* as genes highly and quickly induced by cold, and *OsDREB1A* also showed a later induction under high salinity conditions. Drought and ABA did not seem to influence the expression of these genes. More recently, however, the rice gene *OsDREB1B* has been reported to be induced by stress conditions other than cold, such as osmotic stress, high salinity, salicylic acid, dehydration and methyl viologen (which triggers oxidative stress), but not by ABA (Gutha and Reddy, 2008). Regarding other *OsDREB1* genes, *OsDREB1C* was observed to have a constitutive gene expression, not being regulated at transcript level under stress, while the *OsDREB1D* transcript could not be detected under the different conditions tested (Dubouzet *et al.*, 2003). Wang *et al.* (2008) reported that *OsDREB1F* responded to drought, salinity and cold, but also to ABA. There

are no reports on the gene expression of *OsDREB1E* or *OsDREB1G* in response to abiotic stresses.

Similarly to what has been reported for other plants (discussed below), the over-expression of *OsDREB1* genes in rice usually leads to plants with improved tolerance to abiotic stress conditions. Rice plants over-expressing *OsDREB1A* and *OsDREB1B* (Ito *et al.*, 2006) showed a higher tolerance to cold, dehydration and salinity, but also a dwarf phenotype (discussed below). These transgenic plants were reported to accumulate higher amounts of proline and sugars, both in control and stress conditions, when compared to wild-type plants. Moreover, the rice plants over-expressing either *OsDREB1A* or the *Arabidopsis DREB1A* had increased levels of *COR*-regulated genes. In wild type plants, these genes are induced only under cold conditions (Ito *et al.*, 2006). In addition to an improved tolerance to cold, drought and high salinity, transgenic rice plants over-expressing *OsDREB1F* also showed increased transcript levels of ABA-dependent genes (Wang *et al.*, 2008). This, together with the ABA-induction of *OsDREB1F*, means that this gene works in an ABA-dependent fashion. Regarding the other two *OsDREB1* TFs, rice plants over-expressing *OsDREB1G* were reported to have an increased survival rate under drought conditions, but such a phenotype was less evident in the *OsDREB1E* over-expressing lines (Chen *et al.*, 2008). These different phenotypes indicate that OsDREB1 proteins are not redundant and may act differently in abiotic stress signalling.

The study by Gutha and Reddy (2008) unveiled a possible role for *DREB1/CBF*s, and particularly for *OsDREB1B*, in biotic stress signalling. These authors over-expressed *OsDREB1B* in tobacco plants and observed that the plants, not only were more tolerant than the wild type to drought, salinity and cold, but also to oxidative stress and, more interestingly, to infection by tobacco streak virus. Moreover, the transgenic plants also

showed a constitutive expression of some Pathogenesis-Related (PR) genes, which may explain the resistance phenotype (Gutha and Reddy, 2008). So far, this is the only study reporting a direct involvement of *DREB1/CBF*s in biotic stress signalling.

DREB1/CBFs in other plants

As previously mentioned, *DREB1/CBF* genes were initially identified in *Arabidopsis:* three TF encoding genes, *DREB1A/CBF3*, *DREB1B/CBF1* and *DREB1C/CBF2* were described as homologous genes, belonging to the DREB1/CBF sub-family (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). These genes are located in the *Arabidopsis* chromosome IV, in tandem, which means that they probably have a common origin. *DREB1/CBFs* were initially characterized as quickly and transiently induced by low temperatures, but not by drought or ABA (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). However, more recently, it was reported the activation of *DREB1/CBF*s by ABA, through the DRE/CRT element (Knight *et al.*, 2004). Gilmour *et al.* (1998) also reported an induction of *DREB1B/CBF1* and *DREB1C/CBF2*, but not of *DREB1A/CBF3*, by mechanical stress.

Several genes in *Arabidopsis* have been identified as sequence-homologues of *DREB1/CBF*s, but their function in stress signalling was apparently not conserved. These genes are sometimes referred to as *DREB1/CBF*-like genes. CBF4 was described as having extensive sequence similarity to DREB1/CBFs, even outside the AP2/ERF domain (Haake *et al.*, 2002). This homology was even extended to the DREB1/CBF signatures that are specific to these TFs (Canella *et al.*, 2010). Nevertheless, *CBF4* is not induced in response to cold. According to Haake *et al*. (2002), *CBF4* only responds to drought and ABA, and its over-expression results in the up-regulation of *COR* genes. Sakuma *et al.* (2002)

identified three genes that had high nucleotide sequence similarity to *DREB1/CBF*s. They named these genes *DREB1D*, which corresponds to *CBF4*, *DREB1E* and *DREB1F*. Contrarily to Haake *et al*. (2002), these authors reported an induction of *DREB1D/CBF4* by salinity, but not by drought or ABA. In the case of *DREB1E*, its gene expression could not be determined, while *DREB1F* was reported to be induced by salinity, but not by drought, cold or ABA (Sakuma *et al.*, 2002). Haake *et al.* (2002) proposed that *DREB1/CBF*s and their related genes have probably the same evolutionary origin and arose due to gene duplication, followed by promoter evolution. This led to similar genes but with different gene expression patterns, and very likely involved in different signalling pathways.

Since the identification of *DREB1/CBF*s as major components of the abiotic stress responses in *Arabidopsis*, many groups have focused their attention on trying to identify homologues of these genes in other plants. Many homologues have already been described, such as in cotton (Shan *et al.*, 2007), grape (Xiao *et al.*, 2006), soybean (Li *et al.*, 2005), tomato (Zhang *et al.*, 2004) or medicago (Chen *et al.*, 2010), and also in monocots, such as rice (Dubouzet *et al.*, 2003), maize (Qin 2004) and barley (Skinner *et al.*, 2005), among others. In barley, for instance, the *DREB1/CBF* family has been described as composed of at least 20 genes (Skinner *et al.*, 2005). The number of *DREB1/CBF* genes in different barley cultivars was also correlated with the ability of these plants to tolerate cold (Knox *et al.*, 2010). Different numbers of *DREB1/CBF*s are thought to have arisen due to duplication events, and are enough to discriminate between winter and spring barley genotypes (Knox *et al.*, 2010).

Interestingly though, *DREB1/CBF*s seem to have maintained their function along speciation. Over-expression of *DREB1/CBF*s in different species resulted in phenotypes similar to the ones observed in *Arabidopsis*,

like increased tolerance to abiotic stress conditions and growth retardation (Ito *et al.*, 2006; Chen *et al.*, 2010). This was even observed in heterologous expression of *DREB1/CBF*s, meaning that these pathways are at least partly conserved across the plant kingdom. For example, the expression of the *Arabidopsis DREB1A/CBF3* in wheat plants increased their tolerance to drought (Pellegrineschi *et al.*, 2004). Similarly, *Arabidopsis* plants expressing the rice gene *OsDREB1A* showed an increased tolerance to freezing and high salt stresses (Dubouzet *et al.*, 2003). Curiously, the over-expression in tomato plants of *DREB1/CBF*s either from *Arabidopsis* or tomato itself (*LeCBF*s) did not result in an increased freezing tolerance (Zhang *et al.*, 2004). In order to understand this, Zhang *et al.* (2004) analyzed the transcriptome of those plants and observed that very few genes were differentially expressed in response to *DREB1/CBF* over-expression. Therefore, the authors proposed that, even though tomato plants have a functional *DREB1/CBF* gene family, the regulon controlled by these TFs is much smaller in tomato than in other species, which explains the lack of freezing tolerance in these plants.

The DREB1/CBF Regulon

The DREB1/CBFs were confirmed as regulators of *COLD-REGULATED* (*COR*) gene expression and as having an important role in the plant responses to abiotic stresses, through their over-expression in several plant species (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Kasuga *et al.*, 1999; Cook *et al.*, 2004; Gilmour *et al.*, 2004). Transgenic *Arabidopsis* plants over-expressing these genes were shown to have increased transcription of DREB1/CBF down-stream genes, even without cold acclimation, resulting in plants that were more tolerant not only to cold and freezing stresses (Jaglo-Ottosen *et al.*, 1998), but also to dehydration (Liu *et al.*, 1998) and salinity (Kasuga *et al.*, 1999). Nevertheless, in these studies, the authors

also reported that DREB1/CBF over-expression also caused a dwarf phenotype, resulting in plants smaller than the wild type. To better understand the molecular mechanisms triggered by DREB1/CBFs, microarray analysis were performed by several groups, in order to identify their down-stream-regulated genes (Seki *et al.*, 2001; Fowler and Thomashow, 2002; Gilmour *et al.*, 2004; Maruyama *et al.*, 2004; Vogel *et al.*, 2005). Initially, DREB1/CBFs were found to regulate at least 12% of the cold-regulated genes in *Arabidopsis* (Fowler and Thomashow, 2002). Gilmour *et al.* (2004) observed that over-expressing any of the *Arabidopsis DREB1/CBFs* induced the expression of the same down-stream genes. Based on this and also on the fact that the over-expression lines showed similar phenotypes, these authors proposed that the three DREB1/CBFs would have similar functions and, therefore, be redundant. Using two microarray systems, Maruyama *et al.* (2004) were able to identify 38 genes acting down-stream of DREB1A/CBF3. Among these genes, several had already been described as responsive to abiotic stress conditions, such as genes coding for LEA proteins and others such as *RD29a* and *COR15a* (Yamaguchi-Shinozaki and Shinozaki, 2006). Interestingly, genes coding for TFs were also identified as targets of DREB1A/CBF3, such as *STZ/ZAT10* (Maruyama *et al.*, 2004), indicating the presence of sub-regulons down-stream of DREB1/CBFs. Later, Vogel *et al.* (2005), using a full genome array, were able to identify 93 genes as down-stream targets of DREB1C/CBF2. A recent report, however, has described that DREB1C/CBF2 can regulate the expression of as much as 286 genes in mature *Arabidopsis* leaves (Sharabi-Schwager *et al.*, 2010). This growing number of known DREB1/CBF-regulated genes illustrates the relevance of these genes in the responses to abiotic stress.

As discussed above, DREB1/CBFs were initially proposed to be redundant, since plants over-expressing them had similar phenotypes

(Gilmour *et al.*, 2004). Nevertheless, an *Arabidopsis cbf2* mutant was found to have increased tolerance to chilling, when compared to the wild type, and this correlated with increased levels of *DREB1A/CBF3* and *DREB1B/CBF1* (Novillo *et al.*, 2004). The gene expression of these two genes at 4ºC also preceded that of *DREB1C/CBF2* (Novillo *et al.*, 2004; Medina *et al.*, 2011). The authors proposed therefore that this gene plays a different role in the abiotic stress signalling pathway, negatively regulating the expression of *DREB1A/CBF3* and *DREB1B/CBF1*. This was further confirmed by the finding that these two genes have a similar expression pattern during *Arabidopsis* development, that is different from the one observed for *DREB1C/CBF2* (Novillo *et al.*, 2007). Moreover, in this study, RNAi lines for *DREB1A/CBF3* and *DREB1B/CBF1* were impaired in the cold-induced expression of the same DREB1/CBF-target genes, and DREB1C/CBF2 could not compensate for that absence. Novillo *et al.* (2007) suggested that the DREB1/CBF regulon consists of at least two subsets of genes: one regulated in concert by DREB1A/CBF3 and DREB1B/CBF1 and the other one by DREB1C/CBF2.

The changes in gene expression driven by DREB1/CBFs ultimately result in an adaptation to abiotic stress conditions, as discussed above. Plants over-expressing *DREB1/CBF*s showed an accumulation of compatible solutes, such as proline and soluble sugars (Gilmour *et al.*, 2004). In fact, the *Arabidopsis* metabolome was shown to be extensively regulated by DREB1/CBFs (Cook *et al.*, 2004; Maruyama *et al.*, 2009). In the study by Cook *et al*. (2004), non-acclimated *Arabidopsis* plants over-expressing *DREB1A/CBF3* were shown to have a metabolome similar to the one of cold-acclimated plants. These results indicate that stress-tolerant phenotypes of plants over-expressing DREB1/CBF should be due to the accumulation of certain metabolites.

Regulation of *DREB1/CBF*s by hormones and the circadian clock

The phenotypes observed in *DREB1/CBF* over-expressing plants may also reflect the role of these TFs in pathways other than abiotic stress signalling (Fig. 2). As discussed previously, plants over-expressing *DREB1/CBF*s show growth retardation, in addition to their increased tolerance to abiotic stresses. The fact that it was possible to rescue this dwarf phenotype with the application of an exogenous gibberellin (GA), gibberellic acid, without affecting cold tolerance (Hsieh *et al.*, 2002a; Hsieh *et al.*, 2002b), raised the possibility of an interplay between these TFs and hormone signalling. This was further confirmed by the finding that *DREB1B/CBF1* regulates plant growth by reducing GA content (Achard *et al.*, 2008). This TF was observed to promote the expression of genes involved in the degradation of GAs, thus allowing the accumulation of DELLA proteins that, in turn, contribute to arrest plant growth. More recently, DREB1C/CBF2 has also been shown to play a role in suppressing the plant response to the plant hormone ethylene (Sharabi-Schwager *et al.*, 2010). Plants over-expressing *DREB1C/CBF2* had decreased leaf senescence and their life span was extended in two weeks, when compared to wild type plants. These results highlight the role of DREB1/CBFs, not only in abiotic stress signalling, but also in pathways regulating plant development itself. The observation that *Arabidopsis* plants, expressing *GUS* driven by *DREB1/CBF* promoters, show GUS activity during the first stages of seedling development, gradually disappearing as the plants aged, also supports a role for these TFs during plant development (Novillo *et al.*, 2007).

DREB1/CBF genes have also been reported as regulated by the circadian clock (Harmer *et al.*, 2000; Fowler *et al.*, 2005; Bieniawska *et al.*, 2008). The first indications of this were provided by Harmer *et al.* (2000), who studied the transcription of 8000 *Arabidopsis* genes along 48h and

observed that the gene expression of *DREB1A/CBF3* peaked at around midday. *DREB1/CBF* gene expression at 4ºC was also described to be dependent on the circadian clock (Fowler *et al.*, 2005). In fact, the cold-induction of *DREB1/CBF* genes in *Arabidopsis* was found to be gated by the clock, which means that the level of induction depends on the time of the day at which the stress is imposed. *DREB1/CBF* transcripts accumulate the most when stress is imposed 4h after subjective dawn and the least when plants are transferred to cold conditions 16h after subjective dawn (Fowler *et al.*, 2005). Interestingly, in this study, the genes *RD29a* and *COR6.6* that were described as down-stream components of the DREB1/CBF regulon (Jaglo-Ottosen *et al.*, 1998), did not show a circadian cycling, nor was their expression gated by the clock. In addition, cold-responsive [Ca^{2+}] waves have also been shown to be gated by the clock (Dodd *et al.*, 2006). Since these waves are a part of the cold-signal transduction pathway in the cell (as discussed above), this may have an effect on the gating of *DREB1/CBF* cold-dependent gene expression.

The circadian-dependent expression of *DREB1/CBF*s was found to be dependent, at least in part, on the activity of the PHYTOCHROME INTERACTING FACTOR 7 (PIF7; Kidokoro *et al.*, 2009). In the *pif7* mutant, the circadian cycling of *DREB1B/CBF1* and *DREB1C/CBF2* was abolished, when *Arabidopsis* plants were transferred to continuous light, contrarily to what happened with wild type plants, where this cycling continued (Kidokoro *et al.*, 2009). In this study, PIF7 and the circadian clock component TIMING OF CAB EXPRESSION 1 (TOC1) were actually shown to work together to repress the expression of *DREB1C/CBF2*. More recently, the circadian expression of the three *DREB1/CBF* genes in *Arabidopsis* has also been shown to be dependent on the two clock components CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY; Dong *et al*., 2011). In this study the

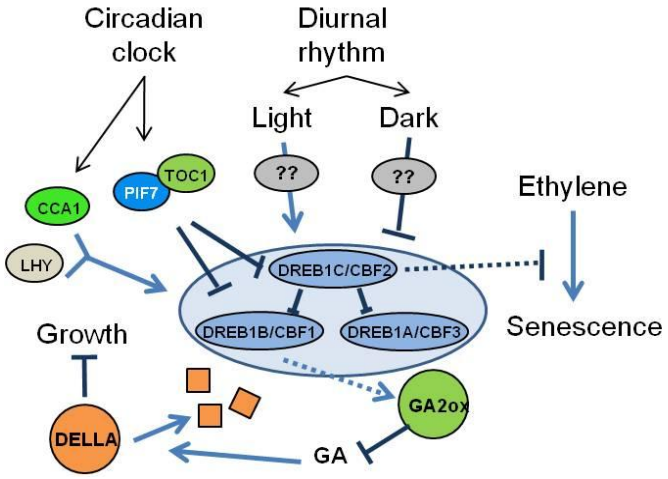

Figure 2. Cross-talk of DREB1/CBFs with other signalling pathways. TFs are shown in ovals. Other proteins are shown in circles. Ovals with question marks represent regulatory elements yet to be identified. Dotted lines indicate unknown mechanisms of regulation. GA – Gibberellin, GA2ox – GA2 oxidase.

authors observed that in the *cca1/lhy* double mutant, the circadian-driven expression of *DREB1/CBF*s was abolished. Whether these two TFs work together or independently to regulate *DREB1/CBF* gene expression, is still unknown.

Phytochrome signalling and its cross-talk with the *DREB1/CBF* regulon

As discussed, the gene expression of *DREB1/CBF*s has been shown to be dependent on circadian rhythms. However, several studies have also observed that the regulation of *DREB1/CBF* genes is dependent on light, whose quality is perceived, in part, by phytochromes (phys) (Kim *et al.*, 2002; Franklin and Whitelam, 2007). Phys are light sensing molecules composed of an apoprotein and of a light absorbing chromophore, which is responsible for the light-induced re-conformation of phys. In response to far-red light (Fr), or in the dark, phys are in their inactive state (Pr), and are localized in the cytosol. Upon a red light (R) pulse, these proteins change

conformation to their active state (Pfr) and migrate into the nucleus of the cell (Kircher *et al.*, 2002). There they interact with a group of bHLH TFs called PHYTOCHROME INTERACTING FACTORS (PIFs) and promote their phosphorylation and ubiquitination, which will ultimately lead to the degradation of PIFs by the proteasome (Shen *et al.*, 2005; Al-Sady *et al.*, 2006; Shen *et al.*, 2007; Lorrain *et al.*, 2008). This regulation of PIF protein levels results in a modulation of gene expression dependent on light quality (Shin *et al.*, 2009).

Phytochrome signalling has been implicated in several biological processes, such as germination, hypocotyl elongation and shade avoidance, among others, as recently reviewed (Franklin and Quail, 2010). There is also evidence on the interplay of phys with hormone signalling (Seo *et al.*, 2006; Nozue *et al.*, 2011), which further illustrates the prominent role of phys in plant development. The involvement of phys in these biological processes has been shown to be dependent, at least in part, on their interacting partners, the PIFs. In *Arabidopsis*, PIF3 was the first member of this group to be identified using the C-terminal non-photoactive domain of phyB as bait for an Yeast Two-Hybrid screening (Ni *et al.*, 1998). This TF has since then been implicated in diverse processes, such as in hypocotyl elongation and cotyledon development (Kim *et al.*, 2003), anthocyanin biosynthesis (Shin *et al.*, 2007), flowering (Oda *et al.*, 2004) and chloroplast development (Monte *et al.*, 2004; Terry *et al.*, 2009). Later on, several other PIFs were identified in *Arabidopsis*, such as PIF1, PIF4, PIF5, PIF6 and PIF7 (Huq and Quail, 2002; Khanna *et al.*, 2004; Leivar *et al.*, 2008a). Several of these proteins, with the exception of PIF7, were described as being targeted to degradation upon interaction with the active form of phyB (Shen *et al.*, 2005; Al-Sady *et al.*, 2006; Shen *et al.*, 2007; Lorrain *et al.*, 2008). PIF7, on the other hand, seems to be light-stable even though it was shown to interact with phyB (Leivar *et al.*, 2008a).

PIFs are therefore negative regulators of phy signalling and several studies have been performed in order to understand their biological functions. Some of these functions seem to be conserved among PIFs, in what regards photomorphogenesis (Leivar *et al.*, 2008b; Shin *et al.*, 2009), but some PIFs have been described as having particular biological roles. PIF6 for instance, was shown to have a role in seed dormancy (Penfield *et al.*, 2010), while PIF4 was described as involved in plant adaptation to high temperature (Koini *et al.*, 2009). PIF3 and PIF4 have also been described as directly involved in GA signalling, mainly through the regulation of their transcriptional activity by DELLA proteins (de Lucas *et al.*, 2008; Feng *et al.*, 2008). Even thought PIFs were shown to play prominent roles in plant biology, these proteins have not been studied in plants other that *Arabidopsis*. In rice, several putative PIFs have already been predicted (Nakamura *et al.*, 2007), but their interaction with rice phys is yet to be tested.

The first report on the regulation of *DREB1/CBF*s by phy signalling was the study by Kim *et al.* (2002), in which R light was observed to positively regulate the DRE/CRT-mediated gene expression. Furthermore, this positive effect was shown to be dependent on phyB, since it was attenuated in *phyb* mutants, but not in *phya*, for example. On the other hand, Franklin and Whitelam (2007) reported a positive effect of a low R/FR ratio on the circadian regulation of *DREB1/CBF*s. These authors also observed that *Arabidopsis* plants grown at a low R/FR ratio showed an increased survival rate after a freezing treatment, when compared to plants grown at a high R/FR ratio. PIFs, being TFs that transduce the phy-mediated light information, are therefore very promising candidates to be the link between phy signalling and the *DREB1/CBF* regulon. Nevertheless, until now, only PIF7 was described as a regulator of *DREB1/CBFs* in *Arabidopsis* (Kidokoro *et al.*, 2009). Moreover, this protein was only shown

to modulate the circadian-regulated expression of *DREB1/CBFs*. The factors responsible for *DREB1/CBF* regulation under light/dark conditions, as well as in response to R and Fr, are yet to be identified.

Up-stream regulators of the DREB1/CBF regulon

When Gilmour *et al.* (1998) identified the *DREB1/CBFs*, they proposed the existence of a TF, which they named INDUCER OF CBF EXPRESSION (ICE), that would be responsible for the regulation of *DREB1/CBFs*. This TF would be present in the cell in normal conditions and activated upon a cold stimulus, to promote the expression of the *DREB1/CBFs*. The same authors later analyzed the promoter of *DREB1C/CBF2* in order to find *cis*-acting sequences that would be responsible for its cold-induced gene expression (Zarka *et al.*, 2003). They identified two sequences, Inducer of CBF Expression region 1 and 2 (ICEr1 and ICEr2) that were sufficient for the cold induction of *DREB1C/CBF2*. But it was another group that, using forward genetics, identified an *ice1 Arabidopsis* mutant impaired in the cold-induction of *DREB1A/CBF3* (Chinnusamy *et al.*, 2003). This allele was shown to code for a MYC-like bHLH TF with transcriptional activation activity and able to bind to MYC-recognition elements in the promoter of *DREB1A/CBF3* (Chinnusamy *et al.*, 2003). These authors also reported that over-expression of *ICE1* in *Arabidopsis* did not induce *DREB1/CBF* expression, unless a low temperature signal was applied, like what had been proposed initially by Gilmour *et al.* (1998): *ICE1* was constitutively expressed, but the encoded protein would need to be post-translationally modified to promote *DREB1/CBF* gene expression in cold conditions. The identification of HIGH EXPRESSION OF OSMITICALLY RESPONSIVE GENE (HOS1) as regulator of ICE1 protein levels shed some light on this subject (Dong *et al.*, 2006a). HOS1 is a RING E3 ligase that had been previously described as a

negative regulator of cold signal transduction (Ishitani *et al.*, 1998), through the repression of *DREB1/CBF*s (Lee *et al.*, 2001). Dong *et al*. (2006a) observed that HOS1 interacted with ICE1, promoting its ubiquitination and targeting to degradation by the proteasome. More recently, however, a SUMO E3 ligase, SIZ1, was also identified as a regulator of ICE1 in *Arabidopsis* (Miura *et al.*, 2007). SIZ1 mediates the SUMOylation of ICE1, which reduces its availability to be ubiquitinated (Miura *et al.*, 2007). The authors proposed that this post-translational modification may help stabilizing or even activate the ICE1 protein, promoting *DREB1/CBF* expression under cold. In fact, SIZ1 was shown to be a positive regulator of *DREB1/CBF* gene expression, and particularly of *DREB1A/CBF3* (Miura *et al.*, 2007).

While ICE1 was identified as binding to a MYC element in the promoter region of *DREB1A/CBF3* (Chinnusamy *et al.*, 2003), another TF, MYB15, was described as binding to MYB elements present in the promoter regions of the *DREB1/CBF* genes (Agarwal *et al.*, 2006). In this study, MYB15 was shown to interact with ICE1 but, in contrast to ICE1, it had a transcriptional repressor activity. The *myb15* loss-of-function mutant had increased levels of *DREB1/CBF* under cold conditions (Agarwal *et al.*, 2006). Interestingly, the over-expression of an ICE-mutated form, impairing its sumoylation, increased the transcript levels of *MYB15*, which means that ICE1 is a negative regulator of this gene (Miura *et al.*, 2007).

Other type of TFs that were described as regulators of *DREB1/CBF*s are the CALMODULIN BINDING TRANSCRIPTIONAL ACTIVATORS (CAMTA). CAMTA3 was shown to bind to a *cis*-acting element in the promoter region of *DREB1C/CBF2* thus inducing its gene expression (Doherty *et al.*, 2009). That *cis*-acting element partially overlaps with the previously described ICEr2 motif (Zarka *et al.*, 2003). This way, CAMTA3 modulates the expression of *DREB1C/CBF2* in response to low

temperature (Doherty *et al.*, 2009). Interestingly, CAMTA3 also showed to be a positive regulator of *DREB1B/CBF1*, even though its binding to the promoter of this gene still needs confirmation.

As discussed above, *DREB1/CBF* gene expression was known to be dependent on both circadian rhythms and light quality (Harmer *et al.*, 2000; Kim *et al.*, 2002; Fowler *et al.*, 2005; Franklin and Whitelam, 2007). The identification of PIF7 as a regulator of *DREB1/CBFs* provided new clues on the mechanisms involved in circadian-dependent *DREB1/CBF* gene expression (Kidokoro *et al.*, 2009). PIF7 was described as binding to the promoters of *DREB1B/CBF1* and *DREB1C/CBF2*, but not so efficiently to the promoter of *DREB1A/CBF3*. Kidokoro *et al.* (2009) reported that the *pif7* mutant did not show a circadian repression of *DREB1B/CBF1* and of *DREB1C/CBF2* under constant light conditions, but it shows repression during dark conditions. Therefore, the TFs responsible for the dark-dependent repression of *DREB1/CBF* are yet to be identified. More recently, Dong *et al.* (2011), observed that the TFs CCA1 and LHY bind to the promoter of the three *Arabidopsis DREB1/CBF* genes, and that both the circadian- and cold-induced expression of these genes is abolished in *cca1/lhy* double mutants.

Even though there are no reports of TFs directly regulating *DREB1/CBFs* in plants other than *Arabidopsis*, the rice TF MYBS3 was shown to be a negative regulator of *OsDREB1* expression (Su *et al.*, 2010). Rice plants over-expressing this TF had a lower cold-induction of the *OsDREB1A-C* genes. Nevertheless, the binding of MYBS3 to the promoter of *OsDREB1s* is yet to be tested.

Regulation of *DREB1/CBF*s by other factors

Besides their regulation at gene expression level, *DREB1/CBF*s were also shown to be regulated by other molecular mechanisms. For example,

a mutation in *TRANSLATION ELONGATION FACTOR 2* was reported to induce the expression of *DREB1/CBF*s under cold conditions (Guo *et al.*, 2002). Interestingly, the induction of these TFs did not correlate with an induction of their target genes. In this mutant, *DREB1/CBF* down-stream genes were actually repressed, as compared to the wild type, and the plants had a lower freezing tolerance. Guo *et al.* (2002) proposed that protein synthesis is impaired in this mutant, especially under cold. If this is the case, then the induction of *DREB1/CBFs* in this mutant does not lead to increased DREB1/CBF protein levels, consequently not promoting the expression of their down-stream genes, and is thus unable to confer cold tolerance.

Another mechanism that was shown to be involved in cold-signalling and specifically in *DREB1/CBF* gene expression was the mRNA export (Gong *et al.*, 2005; Dong *et al.*, 2006b). Gong *et al.* (2005) identified an *Arabidopsis los4-2* mutant that had increased levels of *DREB1C/CBF2* transcripts and, conversely, those plants were more sensitive to chilling than wild type plants. *los4* was shown to code for a DEAD-box RNA Helicase with a role in nuclear export of mRNA (Gong *et al.*, 2005). This effect was further confirmed by the findings by Dong *et al.* (2006b), who reported that a mutation in a Nucleoporin, AtNUP160, had an impact on *DREB1A/CBF3* transcript level. This mutant has a defective mRNA nuclear export mechanism, which was observed to have a role in plant development and particularly in the response to low temperatures (Dong *et al.*, 2006b).

The DREB2 Transcription Factors

In the pioneer work by Liu *et al.* (1998), together with the identification of DREB1 TFs, two DREB2 TFs were also identified as binding to the DRE/CRT element: DREB2A and DREB2B. Later, Sakuma *et al.* (2002)

identified another six genes coding for DREB2-related proteins in the *Arabidopsis* genome (*DREB2C-H*). *DREB2* genes were initially described as highly induced by salinity and drought, but not by cold, in contrast to the *DREB1/CBF*s (Liu *et al.*, 1998). More recently, *DREB2* genes have also been implicated in the response to high temperatures, both in *Arabidopsis* (Sakuma *et al.*, 2006; Lim *et al.*, 2007; Chen *et al.*, 2010) and in maize (Qin *et al.*, 2007).

Unlike what is observed when *DREB1/CBF* genes are over-expressed in different plants, the over-expression of *DREB2* TFs did not result in an up-regulation of their down-stream genes under non-stress conditions (Liu *et al.*, 1998). DREB2 proteins could therefore need some kind of post-translational modification in order to be activated under stress conditions. The finding that PgDREB2, a DREB2-related protein from *Pennisetum glaucum*, is phosphorylated, provided the first piece of evidence to support that hypothesis (Agarwal *et al.*, 2007). The phosphorylation of this protein prevented its binding to the DRE/CRT element in the promoter of the *Arabidopsis RD29a* gene (Agarwal *et al.*, 2007). Still, DREB2 proteins have been shown to be subjected to other types of post-translational modifications. Two proteins, DREB2A-INTERACTING PROTEIN 1 and 2 (DRIP1 and 2) were identified as E3 ubiquitin ligases that promoted the ubiquitination of DREB2A (Qin *et al.*, 2008). In this study, the induction of DREB2-target genes was delayed in *DRIP* over-expressing lines. Qin *et al*. (2008) therefore proposed that DRIPs may function in a way to restrict DREB2 transactivation under non-stress conditions.

It is noteworthy that not only post-translational but also post-transcriptional mechanisms regulate *DREB2*s (Qin *et al.*, 2004). Alternative splicing forms were identified for the maize gene *ZmDREB2A*, which showed different expression patterns in response to stress conditions.

Other regulons involved in abiotic stress signalling

The NAC/ZF-HD regulon

Even though major attention has been given to the DREB regulon, in what concerns ABA-independent signalling, other signalling pathways exist that have a role in abiotic stress tolerance. The NAM, ATAF1,2, CUC2 (NAC) transcriptional regulators are one of the most abundant plant-specific TF families and have a wide functional diversity in plant biology (Olsen *et al.*, 2005). Some of these proteins were described as being involved in abiotic stress signalling, since they bind to the promoter of the *Arabidopsis* gene *EARLY RESPONSIVE TO DEHYDRATION STRESS* (*ERD1*) (Tran *et al.*, 2004). This gene had previously been described as a protein similar to a regulatory ATPase subunit, and its transcription was observed to be induced by drought and high salinity (Nakashima *et al.*, 1997; Simpson *et al.*, 2003). Simpson *et al.* (2003) showed that the drought-induced expression of *ERD1* was dependent on a MYC-like *cis*-acting sequence in its promoter, which was later described as the binding site for NAC TFs (Tran *et al.*, 2004). Interestingly, Tran *et al.* (2004) observed that the over-expression of genes coding for NAC TFs in *Arabidopsis* plants induced the expression of several stress-responsive genes, but not of *ERD1*. The same authors later reported that, when NAC TFs were expressed together with a Zn-Finger Homeodomain TF (ZF-HD1), there was an increased expression of *ERD1* (Tran *et al.*, 2006). ZF-HD1 would bind to another *cis*-element in the promoter of *ERD1*, acting together with NAC TFs to regulate the expression of that gene (Tran *et al.*, 2006).

NAC TFs have also been characterized in other plants, such as rice and wheat (Ohnishi *et al.*, 2005; Hu *et al.*, 2006; Uauy *et al.*, 2006; Nakashima *et al.*, 2007; Hu *et al.*, 2008). The rice TF OsNAC6, for instance, has been described as induced by several abiotic stress conditions, but also by ABA, wounding and jasmonic acid, which indicates a possible role of this TF in

the responses to biotic stress (Ohnishi *et al.*, 2005). This was confirmed by the findings by Nakashima *et al.* (2007), who reported that rice plants over-expressing *OsNAC6* not only had increased tolerance to abiotic stress conditions, but also to fungal infection. In addition, the work by Uauy *et al*. (2006) showed that NAC TFs from wheat have a role in senescence, and also in nutrient mobilization from leaves to developing grains, namely zinc and iron. These results highlight different functions for NAC TFs in different plants. It is yet to be determined whether these functions are conserved within the plant kingdom or if they are species-specific.

The C2H2-type Zn Finger regulons

Among the C2H2-type Zn Finger TFs identified in plants (Meissner and Michael, 1997), several have been described as regulators of abiotic stress responses (Sakamoto *et al.*, 2004; Vogel *et al.*, 2005; Mittler *et al.*, 2006).

Sakamoto *et al.* (2004) described several *Arabidopsis* genes coding for C2H2-type Zn Finger TFs as responsive to abiotic stress conditions. Moreover, these authors observed that all those TFs were transcriptional repressors, through the binding to specific *cis*-acting sequences in the promoters of target genes. One of the genes coding for these TFs is *STZ/ZAT10*, which is strongly induced in response to several abiotic stress conditions, such as cold, drought and salinity (Sakamoto *et al.*, 2004). More interestingly though, both the over-expression and silencing of this gene in *Arabidopsis* resulted in plants more tolerant to abiotic stress conditions (Mittler *et al.*, 2006). The authors of that study suggested that this TF may have a dual function in abiotic stress signalling and might be involved in different stress-responsive signalling pathways. In another report, *Arabidopsis* plants over-expressing *DREB1A/CBF3* were found to have altered levels of *STZ/ZAT10* (Maruyama *et al.*, 2004). This cross-talk between regulons is further supported by the finding that ZAT12, a

transcriptional repressor also described as a regulator of several abiotic stress-responsive genes (Davletova *et al.*, 2005), has a regulon partly overlapping that of *DREB1C/CBF2* (Vogel *et al.*, 2005). Moreover, ZAT12 was also described as a negative regulator of *DREB1A/CBF3* and *DREB1B/CBF1* (Vogel *et al.*, 2005). These results are illustrative of the interplay between different regulons.

The C2H2-type Zn Finger TFs have also been reported as regulators of abiotic stress responses in plants other than *Arabidopsis*. For instance, SCOF-1 is a C2H2-type Zn Finger TF from soybean and its gene expression was described as highly induced in response to cold and ABA, but not by salinity or drought (Kim *et al.*, 2001). SCOF-1 was shown to regulate *COR* gene expression through the ABRE *cis*-acting element. Therefore, Kim *et al.* (2001) proposed that SCOF-1 could be acting through and ABA-dependent pathway to modulate cold-regulated gene expression in soybean. In rice, the over-expression of another C2H2-type Zn Finger, *ZFO252*, was also observed to increase the tolerance of plants to drought and salinity (Xu *et al.*, 2008). These plants showed an accumulation of stress-responsive transcripts, such as *OsDREB1A*, and of several compounds related to osmoregulation, such as proline and soluble sugars. Altogether, these results are illustrative of the important role this type of TFs have in stress signalling and also of the cross-talk between regulons, namely with the *DREB1/CBF*s.

Approaches to improve plant tolerance to adverse abiotic stress conditions

Given that a single TF can modulate the expression of many genes, these proteins have been proposed by several authors as promising targets for plant improvement. For instance, the over-expression of the TF *AP37* in

rice increased tolerance to several abiotic stress conditions, such as low temperature, high salinity and drought (Oh *et al.*, 2009). More importantly, these transgenic plants had an increased grain yield of up to 57% under drought stress, as compared to wild type plants. The over-expression of *SNAC1* and *SNAC2* in rice was also reported to improve abiotic stress tolerance (Hu *et al.*, 2006; Hu *et al.*, 2008). The over-expression of *SNAC1* in particular was observed to increase the seed setting of rice plants subjected to drought conditions in the field (Hu *et al.*, 2006). In both cases, *AP37* and *SNAC1* over-expression, the transgenic plants did not show major phenotypic differences, when compared to the wild type (Hu *et al.*, 2006; Hu *et al.*, 2008; Oh *et al.*, 2009). This is noteworthy, since the over-expression of some TFs results in plants with dwarf phenotypes or other undesired alterations (Ito *et al.*, 2006; Nakashima *et al.*, 2007). These phenotypes are usually due to the use of strong constitutive promoters driving the expression of the *TF* gene of interest. Nevertheless, the use of stress-inducible promoters has proven successful to overcome such negative effects (Nakashima *et al.*, 2007). These reports are illustrative of the importance of TFs as targets for plant improvement. The identification and functional characterization of these regulatory proteins is therefore crucial to improve crop yield, particularly when plants have to grow under adverse environmental conditions.

Thesis outline and research objectives

With this work we aimed to identify and characterize novel components up-stream of the *OsDREB1* genes in rice, namely of *OsDREB1B*, whose regulation is still poorly understood. Our main goal was to provide novel clues for the abiotic stress signalling mechanisms in plants and hopefully identify genes that may play prominent roles in stress tolerance, and that may be interesting targets for crop plant improvement in the future.

To meet this goal, we proposed to screen a cDNA expression library for TFs that would bind to the promoter of *OsDREB1B*. Since this gene was initially described as strongly induced by cold, we prepared this library from cold-treated rice seedlings, hence enriched in cold-responsive transcripts. In order to screen the library for TFs that bind to the promoter of *OsDREB1B*, we used the Yeast One-Hybrid (Y1H) system. For that, we divided the promoter of *OsDREB1B* into four overlapping fragments that were used as baits in the Y1H screening.

Having identified novel TFs that bind to the promoter of *OsDREB1B*, we proposed to perform their functional characterization, using molecular and biochemical tools. This characterization involved analysis of gene expression patterns under different abiotic stress conditions, transactivation activities of the TFs, protein-protein and protein-DNA interaction studies, among others. In order to identify and characterize cross-talks between different signalling pathways, we also proposed to investigate the possible involvement of the novel TFs identified in signalling pathways other than abiotic stress responses.

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Chapter 2.

Identification of Novel Transcription Factors Binding to the Promoter of the Rice Gene *OsDREB1B*

Chapter 2. Table of Contents

Abstract

Plants have evolved several mechanisms in order to cope with adverse environmental conditions. Under abiotic stress, plants undergo dramatic changes in gene expression, which are modulated by different Transcription Factors (TFs). The TF proteins belonging to the DREB1/CBF sub-family have been described as major regulators of the plant responses to several abiotic stress conditions. Our study focused on the rice gene *OsDREB1B*, initially described as highly and specifically induced by cold. Nevertheless, we showed that *OsDREB1B* is not only induced by low temperatures, but also by drought and mechanical stress. In spite of its known relevance in abiotic stress signalling, the transcriptional regulation of *OsDREB1B* is still poorly understood. Therefore, in order to identify TFs that bind to is promoter, we have used a Yeast One-Hybrid system to screen a cold-induced cDNA expression library. Thereby, we have identified eight novel TFs that bind to the promoter of *OsDREB1B*. Among them, there were seven Zn Finger TFs, four Homeodomain (ZF-HD) and three C2H2-type, and one basic Helix-Loop-Helix (bHLH) TF, predicted as a Phytochrome Interacting Factor (PIF). This work provided the first insight into the transcriptional regulation of a *DREB1* gene in rice.

Introduction

Given their sessile nature, plants have adapted to cope with environmental stresses, such as extreme temperatures, drought and salinity. Many of these adaptations take place at the molecular level and are modulated by transcription factors (TFs), which mediate the stress responses. The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of TFs plays a prominent role in the response to abiotic stress conditions, particularly through the action of the DROUGHT RESPONSE ELEMENT BINDING 1/C-REPEAT BINDING FACTOR (DREB1/CBF) sub-family (Gilmour *et al.*, 1998a; Liu *et al.*, 1998; Medina *et al.*, 1999). Elements of this sub-family were first identified in *Arabidopsis*, but are now known to be present in many other plant species, such as rice (Dubouzet *et al.*, 2003), maize (Qin *et al.*, 2004), grape (Xiao *et al.*, 2006) and cotton (Shan *et al.*, 2007), which illustrates their relevance in plant stress signalling. Even though several genes have been identified as targets of DREB1/CBFs (Seki *et al.*, 2001; Fowler and Thomashow, 2002; Maruyama *et al.*, 2004), there is still much to learn about their up-stream regulators. ICE1 in *Arabidopsis* was the first protein identified as a regulator of *DREB1/CBF* gene expression, through the binding to a specific *cis*-motif present in the promoter region of *DREB1A* (Chinnusamy *et al.*, 2003). The TF MYB15 was also described as interacting with ICE1 and binding to the promoters of *Arabidopsis DREB1/CBFs*, working to repress their transcription (Agarwal *et al.*, 2006). Moreover, CAMTA3 was shown to be a positive regulator of *Arabidopsis DREB1C/CBF2*, through its binding to the CM2 *cis*-motif, present in the promoter of that gene (Doherty *et al.*, 2009), while PIF7, a negative regulator of phytochrome signalling (Leivar *et al.*, 2008), was established as a possible link between the light and cold signalling pathways, since it binds to the promoter of *DREB1B* and *DREB1C* (Kidokoro *et al.*, 2009). More recently, CCA1 and LHY have also

been shown to bind to the promoters of *DREB1/CBFs*, and to be involved in their circadian regulation and also cold-induction (Dong *et al* 2011). Nevertheless, other than in *Arabidopsis*, very little is known about the regulation of *DREB1/CBFs*. The rice TF MYBS3 was described as a negative regulator of *OsDREB1A* and *OsDREB1B*, but confirmation of its binding to the promoters of these genes is still lacking (Su *et al.*, 2010).

This work focuses on the rice gene *OsDREB1B*, which was initially described as highly and specifically induced by low temperatures (Dubouzet *et al.*, 2003), but later, it was also found to respond to high salinity, osmotic stress and salicylic acid (Gutha and Reddy, 2008). Our gene expression studies indicate that the cold-induction of *OsDREB1B* depends on the severity of the stress. Moreover, we have observed that this gene is highly induced in response to drought, only in the roots, and also by mechanical damage. In order to better understand the transcriptional regulation of *OsDREB1B,* we screened a rice cold-induced cDNA expression library to identify TFs binding to its promoter. We describe here the identification of seven Zn Finger and one bHLH proteins, which are the first TFs reported as direct regulators of a *DREB1/CBF* gene in plants other than *Arabidopsis*.

Materials and methods

Plant materials and construction of the cold-induced cDNA expression library

Eight-days-old rice seedlings (*Oryza sativa* L. cv. Nipponbare) grown at 28ºC and 12h/12h photoperiod, were subjected to an 8ºC treatment. Whole seedlings were sampled after 2h, 5h, and 24h of cold treatment. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Total RNA samples from the three time points were pooled in equal amounts for mRNA

purification using the PolyATtract mRNA Isolation System IV (Promega). An unidirectional cDNA expression library was prepared in λACTII using the HybriZAP-2.1 XR cDNA Synthesis Kit and the HybriZAP-2.1 XR Library Construction Kit (Stratagene), following the manufacturer's instructions. Three µg mRNA were used to perform the synthesis of first strand cDNA. *In vivo* mass excision of pACTII' phagemid from λACTII was performed as described (Ouwerkerk and Meijer, 2001).

Yeast One-Hybrid Screening

The promoter of *OsDREB1B* was divided into four overlapping fragments to be used as baits for the Y1H screening. Fragment length and primer pairs used can be found in Supplemental Table 1. The fragments were cloned in the pHIS3/pINT1 vector system (Ouwerkerk and Meijer, 2001) and integrated into the Y187 yeast strain (Clontech). For each promoter fragment, over one million yeast colonies were screened in CM-His- medium supplemented with 5mM 3-amino-1,2,4-triazole (3-AT), 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 reporter *HIS* gene.

Abiotic stress treatments

Rice seedlings were grown hydroponically in rice growth 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 dawn. At the same time, control seedlings were transferred to fresh growth medium (mock control). Cold treatments were

performed by transferring the seedlings to growth chambers at either 5ºC or 10ºC in pre-cooled medium. For salt and ABA treatments, seedlings were transferred to growth media supplemented with 200mM NaCl or 100µM ABA, respectively. Drought treatment was performed by maintaining the seedlings over dry absorbent paper. The mechanical stress assay was carried out by damaging the seedlings before they were transferred to new growth medium (the leaves and stems were bent and broken). All other conditions were maintained throughout the assays. Ten plants were sampled for each time point, and roots and shoots were harvested separately.

Semi-quantitative RT-PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). First strand cDNA was synthesized from 1µg total RNA, using an oligo-dT primer (Invitrogen) and the SuperscriptII reverse transcriptase (Invitrogen), following the manufacturer's instructions. The cDNA was then amplified by PCR using gene-specific primers (Supplementary Table 2). *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* (*eELF1-α*; Os03g08060) and *UBIQUITIN-CONJUGATING ENZYME E2* (*UBC2;* Os02g42314) were used, respectively (Jain *et al.*, 2006). The results shown are representative of at least two biological replicates.

Results

OsDREB1B transcript level is differentially regulated by several abiotic stress conditions

We have tested *OsDREB1B* (Os09g35010) gene expression in response to several abiotic stress conditions, using semi-quantitative RT-PCR. For this, two-week-old rice seedlings were subjected to cold (5º and 10ºC), salt (200mM NaCl), drought and ABA treatments (100µM), for up to 24h. Our results confirmed that *OsDREB1B* is highly regulated by cold, as previously described (Dubouzet *et al.*, 2003a). In addition, we observed that this regulation is temperature-dependent and shows a similar pattern in both shoots and roots (Fig. 1a). When rice seedlings were subjected to 10ºC, the *OsDREB1B* transcript level was rapidly induced (10min), reached a peak (1h/2h) and then started to decrease, returning to basal levels afterwards. At 5ºC, however, the induction of *OsDREB1B* only started after 40min of cold and remained high until the end of the assay. Rice seedlings treated with ABA or subjected to high salinity showed a similar gene expression pattern for *OsDREB1B* in both shoots and roots. The transcript level of *OsDREB1B* was rapidly (10min) up-regulated after the onset of stress and followed by a down-regulation after 20 to 40min. This pattern was also observed in shoots under drought stress, whereas in roots the transcript level of *OsDREB1B* was kept high at least during 24h after the start of the drought treatment. This suggests that OsDREB1B may play an important role in the plant response to drought, particularly at root level. In the case of NaCl and ABA treatments, there was also a transient up-regulation of *OsDREB1B* after 5-10h of NaCl treatment in shoots and 1-2h of ABA treatment in roots. Nevertheless, these increases also appear in the mock control, which may indicate that they are not specific to the NaCl and ABA treatments.

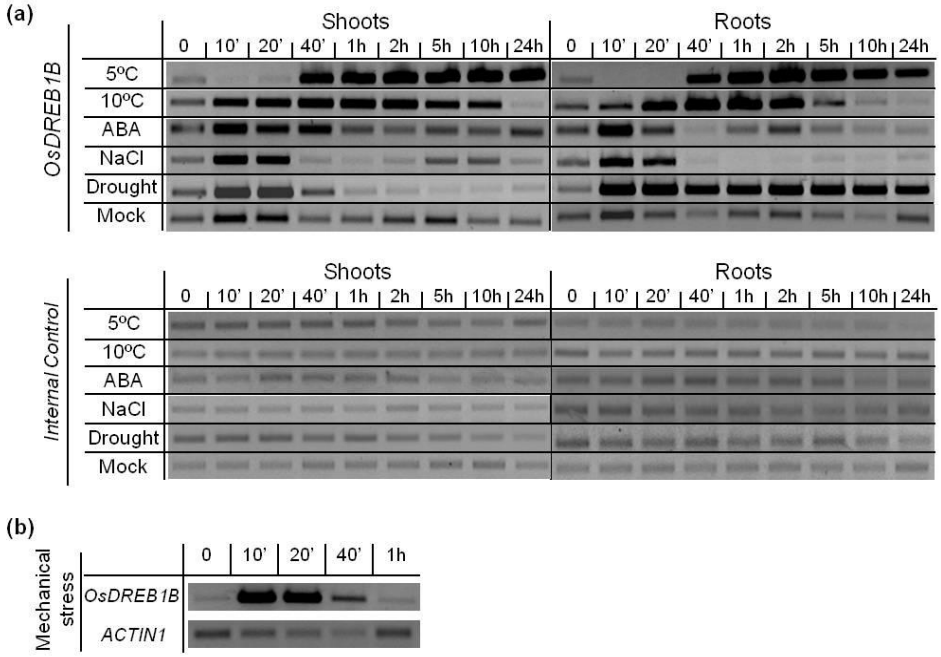


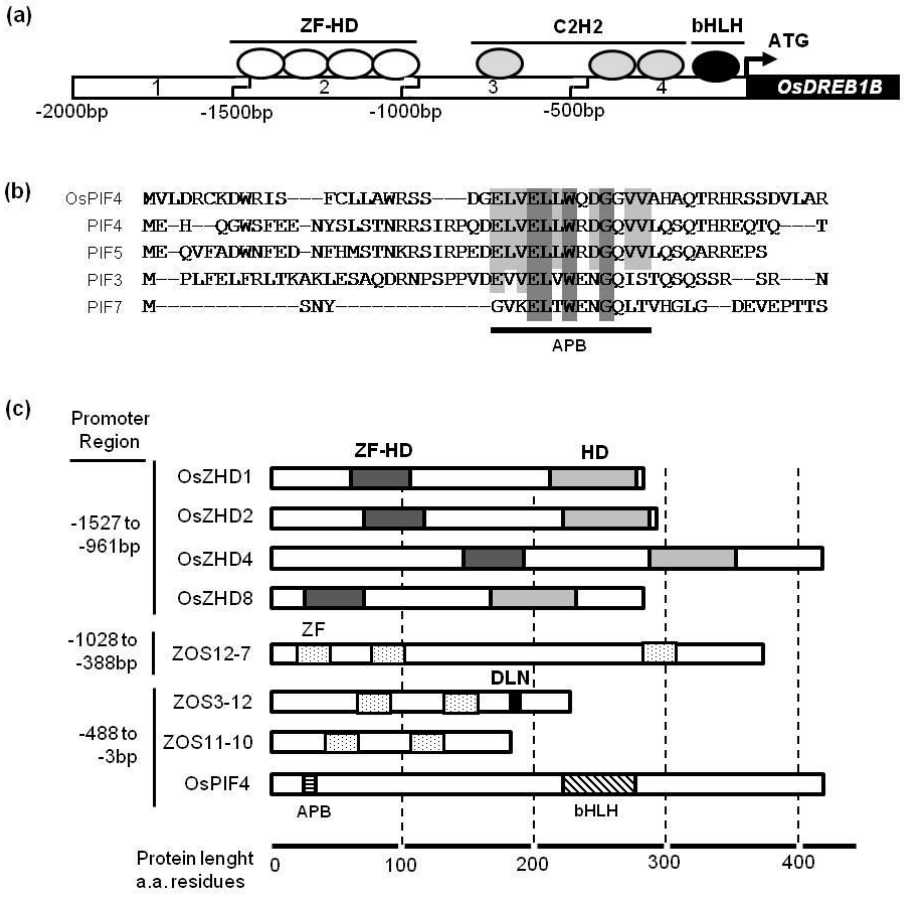
Figure 1. Analysis of *OsDREB1B* gene expression in rice seedlings subjected to different stress treatments.
(a) *OsDREB1B* gene expression was analyzed by semi-quantitative RT-PCR in plants subjected to cold (5º and 10ºC), ABA (100µM), NaCl (200mM), drought and mock control. All treatments started 4h after dawn (time 0). Roots and shoots were analyzed separately. Internal control for each assay is described in the Materials and Methods section.
(b) *OsDREB1B* gene expression was analyzed by semi-quantitative RT-PCR in plants subjected to mechanical stress. Whole plants were assayed.

Under mock treatment, a circadian regulation of *OsDREB1B* could be observed, with the transcript level reaching a peak 2-5h after the start of the assay (6-9h after dawn), decreasing afterwards. Interestingly, *OsDREB1B* also showed a transient increase of gene expression at 10 and 20min. Since, in this case, the only change in conditions was the transfer of the plants to new growth medium, we hypothesised if this up-regulation could be due to a response to mechanical stress. Therefore, another assay was

performed, in the same conditions as above, but in which the plants were damaged and transferred to new nutritive medium. We observed that the *OsDREB1B* transcript level was rapidly and highly induced 10min after mechanical stress, stayed high for at least 20min and returned to basal levels afterwards (Fig. 1b). This may also explain the transient up-regulation that we observed in the salt, drought and ABA treatments around 10 and 20min after stress initiation (Fig. 1a), although in the salt and drought treatments the transient induction observed seems to be more significant than what is seen in the mock treatment.

Eight TFs identified that bind to the promoter of *OsDREB1B*

In order to identify TFs that bind to the promoter of *OsDREB1B*, we have performed an Y1H screening, using that promoter as bait. We considered the 2000bp up-stream of the *OsDREB1B* start codon (ATG), and divided that sequence into four overlapping fragments of around 500bp, numbered from 1 to 4 (Supplemental Table 1). Number 1 being the fragment further away from the start codon and number 4 the one closest to it (Fig. 2a). The four yeast bait strains (each fragment corresponds to one bait strain) were used to screen a rice cold-induced cDNA expression library, and at least one million yeast colonies were screened for each fragment. This allowed us to identify seven Zn Finger and one bHLH TFs as binding to the *OsDREB1B* promoter. Fig. 2a shows the relative position of these TFs along the promoter of *OsDREB1B*. Four Zn Finger Homeodomain (ZF-HD) TFs were found as interacting with the DNA sequence between -1527 to -961bp up-stream of the *OsDREB1B* start codon; one C2H2-type Zn Finger TF was found between -1028 to -388bp, and two others between -488bp and -3bp. The bHLH TF was also identified in the promoter region spanning from -488bp to -3bp. No TFs were found interacting with the promoter fragment ranging from -1945 to -1447bp up-stream of the start codon. In



or

Figure 2. Transcription factors binding to the *OsDREB1B* promoter.
(a) Schematic representation of the *OsDREB1B* promoter, divided in four fragments used as bait in the Y1H screening. The oval shapes represent the TFs identified as binding to each of the promoter fragments: White – Zn Finger-HD; Grey – C2H2-type Zn Finger; Black – bHLH. Positioning of the TFs along each promoter fragment is not indicative of the position of their binding sites.
(b) Alignment of the N-terminal a.a. sequence of OsPIF4 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 and light shaded boxes show residues conserved between OsPIF4 and some of the Arabidopsis PIFs within the APB domain.
(c) Schematic representation of the protein domains of each TF. Dark grey boxes – Zn Finger Homedomain (ZF-HD); light grey boxes – Homeodomain (HD); dotted boxes – Zinc Fingers (ZF); black – DLN-box/EAR-motif; horizontal stripes – putative APB domain; oblique stripes – bHLH domain.

der to confirm the protein-DNA interactions, the yeast bait strains were then re-transformed with the plasmids containing the TF coding sequences.

Table 1 shows the names, gene locus and number of times each TF was identified in the screening. The seven Zn Finger TFs identified were named according to previous studies (Agarwal *et al.*, 2007; Hu *et al.*, 2008). Regarding the bHLH, predicted as a PIF, it had been previously named PHYTOCHROME INTERACTING FACTOR 3-LIKE 14 (OsPIL14) by Nakamura *et al.* (2007). When comparing its predicted APB domain with that of some of the *Arabidopsis* PIFs (Fig. 2b), several amino acid residues are found conserved between all the proteins, but the rice PIF seems to have the highest degree of similarity to PIF4 and PIF5. This gene had been previously clustered with both these *Arabidopsis* PIFs, together with another putative rice PIF (Nakamura *et al.*, 2007; Carretero-Paulet *et al.*, 2010). We therefore named this novel TF as rice PHYTOCHROME INTRACTING FACTOR 4 (OsPIF4).

Fig. 2c shows the protein domains identified in each of the TFs under study. The ZF-HD TFs had a protein sequence with the Zn Finger Homeodomain up-stream of the C-terminal DNA-binding Homeodomain (HD), as described (Windhovel *et al.*, 2001). OsZHD1 and 2 had very similar domain structures with the HD close to the C-terminus, whereas in OsZHD4 both domains were more central in the protein. In the case of OsZHD8, the HD domain was localized similarly to the one on OsZHD4, but the ZF-HD domain was closer to the N-terminal of the protein, when compared to the other ZF-HD TFs. As for the C2H2-type TFs, ZOS12-7 was found to have three Zn Fingers, while the other two, ZOS3-12 and ZOS11-10, only had two. ZOS3-12 showed an additional feature at its C-terminus: a DLN-box/EAR-motif, which was described as a transcriptional repressor domain (Ohta *et al.*, 2001). No such motifs were identified in the other Zn Finger TFs under study.

Table 1 – TFs identified as binding to the promoter of *OsDREB1B*

Promoter Fragment	RGAP[1] Gene locus	Conserved Domains	TF Name	Ref.	Number of times identified in screening
-1527 to -961bp	Os09g29130	ZF-HD	OsZHD1	Hu et al. (2008)	1
	Os08g37400	ZF-HD	OsZHD2		2
	Os11g13930	ZF-HD	OsZHD4		10
	Os04g35500	ZF-HD	OsZHD8		2
-1028 to -388bp	Os12g38960	C2H2	ZOS12-7	Agarwal et al. (2007)	2
-488 to -3bp	Os03g32230	C2H2	ZOS3-12		1
	Os11g47630	C2H2	ZOS11-10		1
	Os07g05010	bHLH APB	OsPIF4	Nakamura et al. (2007)	1

[1]Rice Genome Annotation Project

The coding sequence of OsPIF4 was predicted differently in two public databases (Genebank reference Os07g0143200 and Rice Genome Annotation Project reference Os07g05010). We identified a 1245bp coding sequence encoding a 414 amino acid-long protein (JN400276), with a molecular weight of 44.49kDa and a pI of 4.714. This protein has a conserved bHLH DNA binding domain, extending from a.a. 222 to 275, and

a putative phytochrome B interacting region (APB) at the N-terminal of the protein (Figure 2c).

Discussion

The rice gene *OsDREB1B* was initially reported as specifically and strongly induced by cold (Dubouzet *et al.*, 2003) and shown to be also regulated, to some extent, by salt, osmotic and oxidative stresses and salicylic acid (Gutha and Reddy, 2008). We observed that *OsDREB1B* gene expression is indeed highly induced by low temperature and, interestingly, the pattern of induction is dependent on how low the temperature is (Fig. 1a). Moreover, we have also observed that *OsDREB1B* is very responsive to drought conditions, but only in the roots. This highlights the importance of analyzing different tissues separately, in order not to mask differences in gene expression between them. Gutha and Reddy (2008) reported an up-regulation of *OsDREB1B* under salt stress, which was not observed in our gene expression studies. The fact that these authors used a different rice variety may be an explanation for this difference. The experimental design may also account for some of the gene expression differences observed. However, these authors do not describe the conditions they used for the abiotic stress treatments. Additionally, we observed an early induction of *OsDREB1B* in the mock treated plants, which turned out to be a response to mechanical stress (Fig. 1b). The fact that this gene responded to the moving of the plants from one flask to another, *i. e.*, responded to touch, indicates that *OsDREB1B* is highly responsive to mechanosensing. This is in agreement with the findings by Gupta and Reddy (2008), who observed that *OsDREB1B* transcription level is induced in response to salicylic acid and that tobacco plants over-expressing this gene have an enhanced resistance to viral infection.

Moreover, Gilmour et al. (1998) also reported an activation of the *Arabidopsis DREB1B/CBF1* in response to mechanical stress. Therefore, the involvement of this gene in mechanosensing and biotic stress responses seems to be conserved in different species.

In spite of the fact that DREB1/CBF regulon has been the subject of many studies, in several plants, little is still known about the TFs that control the expression of the *DREB1/CBF* genes. In *Arabidopsis*, at least six TFs were reported to bind to the promoters of *DREB1/CBF*s and regulating their transcription: ICE1, MYB15, PIF7, CAMTA3, CCA1 and LHY (Chinnusamy *et al.*, 2003; Agarwal *et al.*, 2006; Doherty *et al.*, 2009; Kidokoro *et al.*, 2009; Dong *et al.*, 2011). In rice however, no TFs had been identified as direct regulators of *OsDREBs*.

In this work we have identified eight TFs as binding to the promoter of *OsDREB1B*: seven Zn Finger and one bHLH TFs. The abundance of Zn Finger TFs, both Homeodomain and C2H2-type, is noteworthy. Zn Finger TFs had already been shown to interplay with *DREB*s in the response to abiotic stresses, both up- and down-stream of the DREB1/CBF regulon (Maruyama *et al.*, 2004; Vogel *et al.*, 2005). However, to the best of our knowledge, there are no reports on the direct regulation of *DREB1*/*CBF*s by Zn Finger TFs. Additionally, the identification of a putative Phytochrome Interacting Factor (OsPIF4; belonging to the bHLH family) as a regulator of *OsDREB1B,* suggests a possible regulation of *DREB1/CBFs* by light signalling in rice. This type of regulation has already been described in *Arabidopsis* (Kim *et al.*, 2002; Franklin and Whitelam, 2007; Kidokoro *et al.*, 2009). In addition, Kidokoro *et al.*, (2009) showed that PIF7 is a negative regulator of *DREB1C* and *DREB1B* circadian rhythm in *Arabidopsis*. Nevertheless, among the aligned APB motif sequences, OsPIF4 showed the highest similarity to PIF4 and PIF5, whereas PIF7 showed the least degree of similarity (Figure 2b). In rice, however, there are no reports on a

possible light regulation of *OsDREBs*, nor on the possible interaction of PIFs with rice phytochromes.

This work allowed us to identify TFs that directly regulate an *OsDREB1* gene in rice. In the following chapters, the molecular and functional characterization of these TFs will be addressed.

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Supplemental Materials

Supplemental Table 1. Primers used to isolate the promoter fragments of *OsDREB1B*

Fragment	Primer Sequence[1] 5'-3'	Fragment Size (bp)	Promoter Region
1	GCTCTAGATGAATTTGGCTTGAAGGATG	498	-1945 to -1447 bp
	GGACTAGTTCCGTCATTAACAAATGTGAGTT		
2	GCTCTAGATCACGAGATGAATCTTTTGAGC	566	-1527 to -961bp
	GGACTAGTGCACCGTCAATTCTTCGATA		
3	ATGCGGCCGCCAAAGTTTTGGCTTCTAATTGGT	640[2]	-1028 to -388bp
	GGACTAGTTGACTCTCTCTGGTTCACTTCG		
4	ATGCGGCCGCTCGGAGTAACACTCGTGCAG	485	-488 to -3bp
	GGACTAGTTGACTCTCTCTGGTTCACTTCG		

[1]Adaptors with restriction enzyme sites are underlined.

[2]Fragment obtained by enzymatic digestion with *Xba*I of a 1025bp fragment obtained with the primers indicated.

Supplemental Table 2. Primers used to for semi-quantitative RT-PCR

Gene	Primer Sequence 5'-3'
OsDREB1B	CGCGAGGGGGGTCAGGGA
	TAGTAGCTCCAGAGCGGCAT
ACTIN1	GTCGCACTTCATGATGGAGTTG
	CATGCTATCCCTCATCTCGAC
eEF1α	ACCCTCCTCTTGGTCGTTTT
	AAATACCCGCATTCCACAAC
UBC2	CAAAATTTTCCACCCGAATG
	ATCACATGAATCAGCCATGC

Chapter 3.

Seven Zinc Finger transcription factors are novel regulators of the rice gene *OsDREB1B*

Chapter 3. Table of Contents

Abstract

Abiotic stress conditions are major constraints for crop development and productivity. Transcription Factors (TFs) belonging to the AP2/ERF family have been shown to mediate many abiotic stress responses, therefore playing a major role in plant responses to these conditions. The *DREB1/CBF* sub-family, in particular, has been described as a major component of the abiotic stress signalling pathways. In Chapter 2 we have reported the identification of eight TFs that bind to the promoter of the *OsDREB1B* rice gene: seven Zn Finger TFs and one bHLH TF. Here we will present and discuss the molecular and functional characterization of the Zn Finger TFs identified. Gene expression studies showed that these TFs are differentially regulated at transcriptional level by different abiotic stress conditions, which is illustrative of the cross-talk between stress signalling pathways. Using a transactivation assay in *Arabidopsis* protoplasts, we have observed that all the TFs identified repressed the expression of the reporter gene, driven by the promoter of *OsDREB1B*. Protein-protein interaction studies revealed the formation of homo- and hetero-dimers among the ZF-HD TFs identified, but not for the C2H2-type. Our results suggest a prominent role of Zn Finger TFs in the regulation of *OsDREB1B*.

Introduction

Plants, being sessile organisms, have evolved several molecular mechanisms to respond to adverse environmental conditions and many transcription Factors (TFs) have been described as major regulators of abiotic stress responses in plants. Namely, the TFs belonging to the *DREB1/CBF* sub-family are known to play a prominent role in the stress signalling pathways in several species (Jaglo-Ottosen *et al.*, 1998; Dubouzet *et al.*, 2003; Ito *et al.*, 2006; Shan *et al.*, 2007; Zhang *et al.*, 2010; Medina *et al.*, 2011). Nevertheless, and despite the fact that many genes down-stream of DREB1/CBFs have been reported, the TFs that regulate *DREB1/CBFs* gene expression are mostly unknown. In *Arabidopsis* ICE1, MYB15, CAMTA3, PIF7, CCA1 and LHY were described as TFs regulating the expression of the *DREB1/CBFs* through binding to their promoters (Chinnusamy *et al.*, 2003; Agarwal *et al.*, 2006; Doherty *et al.*, 2009; Kidokoro *et al.*, 2009; Dong *et al.*, 2011), but no such regulators have been identified in other plants. In rice, only MYBS3 was proposed as a regulator of *OsDREBs* (Su *et al.*, 2010), but it is unknown whether this TF actually binds to the their promoter regions.

There is much interplay in the stress signalling pathways, namely between different families of TFs. One group of TFs that has been described as interplaying with DREB1/CBFs, and having a major role in abiotic stress signalling, is the Zn Finger group. Among the different families of Zn Finger TFs, the C2H2-type were described as involved in several stress signalling pathways (Sakamoto *et al.*, 2004; Mittler *et al.*, 2006; Huang *et al.*, 2007; Xu *et al.*, 2008). These TFs, also referred to as TFIIIA-type finger, are characterized by two Cys and two His residues that bind to a zinc ion (Pabo *et al.*, 2001). Among the members of this class, ZAT12 was described, together with DREB1C/CBF2, as a negative regulator of the DREB1/CBF regulon in *Arabidopsis* (Vogel *et al.*, 2005), while the gene expression of

STZ/ZAT10 was shown to be dependent on DREB1A/CBF3 (Maruyama *et al.*, 2004). C2H2-type TFs are therefore signalling components that can be located either up- or down-stream of the *DREB1/CBF* genes. Another family of Zn Finger TFs that has been implicated in abiotic stress signalling is the Zn Finger Homeodomain (ZF-HD) proteins. These TFs are characterized by the presence of Zn Finger-like motifs up-stream of a Homeodomain (Windhovel *et al.*, 2001) and have been described as activators of *EARLY RESPONSIVE TO DEHYDRATION* (*ERD*) gene expression, when over-expressed together with NAC transcription factors in *Arabidopsis* (Tran *et al.*, 2006). Nevertheless, there are no reports on the role of these TFs in the responses to abiotic stress in rice.

In Chapter 2, we used the Yeast One-Hybrid (Y1H) system to identify TFs that bind to the promoter of the rice gene *OsDREB1B*. This gene had been previously described as highly induced by different stress conditions and as playing a role in both biotic and abiotic stress signalling in rice (Dubouzet *et al.*, 2003a; Ito *et al.*, 2006; Gutha and Reddy, 2008). Our Y1H strategy allowed the identification of seven Zn Finger TFs, three C2H2-type and four ZF-HD, and of a bHLH TFs, as binding to the promoter of *OsDREB1B*. In this chapter, the functional characterization of the Zn Finger TFs will be discussed. We observed that the genes coding for these TFs are regulated by different abiotic stress conditions. Moreover, all these TFs function as transcription repressors and only the ZF-HD can form homo- and/or hetero-dimers. We report for the first time a direct regulation of a *DREB1/CBF* gene by Zn Finger TFs.

Materials and methods

Plant materials and treatments

Rice seedlings (*Oryza sativa* L. cv. Nipponbare) were grown hydroponically in rice growth medium (Yoshida et al. 1976) at 28ºC, 700µmol fotons.m^{-2}.s^{-1}, 70% humidity and 12h/12h photoperiod for 14 days. Treatments were performed as described in Chapter 2. Ten plants were sampled per each time point, and roots and shoots were harvested separately.

Semi-quantitative RT-PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). 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 (Supplemental Table 1). *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* (*eELF1α*; Os03g08060) and *UBIQUITIN-CONJUGATING ENZYME E2* (*UBC2*; Os02g42314) were used, respectively. The results shown are representative of at least two biological replicates.

Transactivation assay

The reporter plasmids were built using the pCAMBIA1391z promoter-cloning vector backbone, where the kanamycin plant resistance gene, down-stream of the full CaMV35S promoter, was removed using *Xho*I and replaced by the *Luciferase* gene, excised from plasmid pGL3-basic (Promega) with *Xho*I and *Sal*I. The minimal CaMV35S promoter (-90 to +8bp) was cloned up-stream of the *GUS* gene as a *Sma*I-*Eco*RI fragment. This plasmid was confirmed by restriction analysis and sequencing, and

named pLUCm35GUS. The *OsDREB1B* promoter fragments used in the Y1H screening in Chapter 2 were cloned in the pLUCm35GUS reporter vector, using the restriction sites *Sal*I and *Pst*I. Effector plasmids were constructed by cloning the coding region of the TFs in pDONR221 (Invitrogen), according to the manufacturer's instructions, to obtain the vector pENTR-TF. The sequences were then recombined into plasmid pH7WG2 (VIB, Ghent), to be under the control of the full CaMV35S promoter.

Arabidopsis protoplasts were prepared as previously 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 450*g* for 1min in a swing-out rotor. Cell lysis was performed by ressuspension in 150µL 100mM K_2PO_4 (from a 1M pH7.8 stock solution), 1mM EDTA (from a 0.5M pH8 stock solution), 7mM 2-mercaptoethanol, 1% Triton X-100 and 10% glycerol, followed by two freeze-thaw cycles. The lysate was then cleared by centrifugation 2min at 17000*g*. For the GUS quantification assay, 0.5µL 50mM MUG (4-methylumbelliferyl-β-D-glucuronide) were added to 20µL of the lysate (in triplicate). Reactions were carried at 37ºC in the dark for 1h and stopped with 180µL 200mM Na_2CO_3. Fluorescence was detected using a spectrofluorimeter (Fluoromax-4 with Micromax plate reader, Horiba) with excitation at 365nm, emission at 455nm and a slit of 1.5nm. Readings were performed in triplicate. Luciferase levels were determined by adding 150µL LUC reagent (20mM Tricine pH7.8, 5mM MgCl2, 0.1mM EDTA, 3.3mM DTT and 2mM ATP) to 20µL of the cell lysate. Seventy five microliters of 1.5mM luciferin were added to each sample and light intensity was read for 10s in a luminometer (Modulus Microplate, Turner Biosystems). Readings

were performed in triplicate. Activation of gene expression was calculated as a GUS/LUC ratio.

Yeast Two-Hybrid

The full coding sequences of the TFs under study were cloned into vector pAD-WT (Stratagene), by replacement of the coding region of the wild-type lambda cI (fragment C) down-stream of the GAL4 activation domain, using the enzymes *Eco*RI and *Pst*I. Cloning of the TFs to be in fusion with the GAL4 binding domain was performed by recombining their coding sequences from vector pENTR-TF (see above) into vector pBD-GW. This vector was obtained by cloning a Gateway (GW) cassette into plasmid pBD-GAL4 Cam (Stratagene).

The pAD and pBD plasmids were transformed into yeast strain YRG2 (Stratagene), as described (Ouwerkerk and Meijer, 2001). Yeast colonies were plated on CM-Leu-Trp-His media and growing colonies were confirmed by PCR. The vectors pAD-WT and pBD-WT (Stratagene) were used as positive control.

Bimolecular Fluorescence Complementation

The regions coding for the TFs in analysis were recombined from plasmid pENTR-TF (see above) into vectors YFP^C43 and YFP^N43, to be in fusion with the N- and C-terminal portions of the Yellow Fluorescent Protein (YFP), respectively. Cloning was done according to Gateway technology (Invitrogen). To use as negative control in the interaction assays with the TFs, the Arabidopsis SNF1 KINASE HOMOLOG 10 (Akin10) was tested in fusion with the N-terminal portion of the YFP.

Transformation of Arabidopsis protoplasts was performed as described for the transactivation assay. For each transformation 3µg of each plasmid

were used. The protoplasts were incubated overnight in the dark at 22ºC and observed under a fluorescence microscope (Leica DMRA2).

Results

The genes encoding the Zn Finger TFs identified are differentially regulated under abiotic stress

In Chapter 2 we reported the identification of seven Zn Finger TFs, as binding to the promoter of *OsDREB1B*. Among them, there are four ZF-HD TFs and three C2H2-type TFs. To understand whether the seven Zn Fingers identified have a role in the plant response to abiotic stress conditions, we have analyzed their gene expression in rice seedlings subjected to cold (5ºC and 10ºC), high salinity (200mM NaCl), ABA (100µM) and drought. We observed that all genes under analysis showed a constitutive basal expression in both roots and shoots, and the mock control revealed that some genes undergo a circadian rhythm regulation (Fig. 1). This is most striking for genes *OsZHD2* and *ZOS3-12* in roots and *ZOS11-10* in roots and shoots.

Regarding the four *ZF-HD* genes, their expression in shoots was not significantly or consistently altered throughout most treatments. There were however some exceptions, such as the induction of both *OsZHD4* at 5ºC and *OsZHD8* by high salinity and drought. We also observed a repression of *OsZHD2* and *OsZHD4* 2h after onset of drought and of *OsZHD8* 10min after the start of the ABA treatment. Nevertheless, for most stress assays, the changes in *ZF-HD* gene expression in shoots were not significant. The same does not apply however to root transcription profiles. The transcript level of *OsZHD1* in roots seemed to be transiently induced by cold (only at 5ºC) and by drought. The expression of gene *OsZHD2*, on the contrary, was induced by all stress conditions, even if only for a short period,

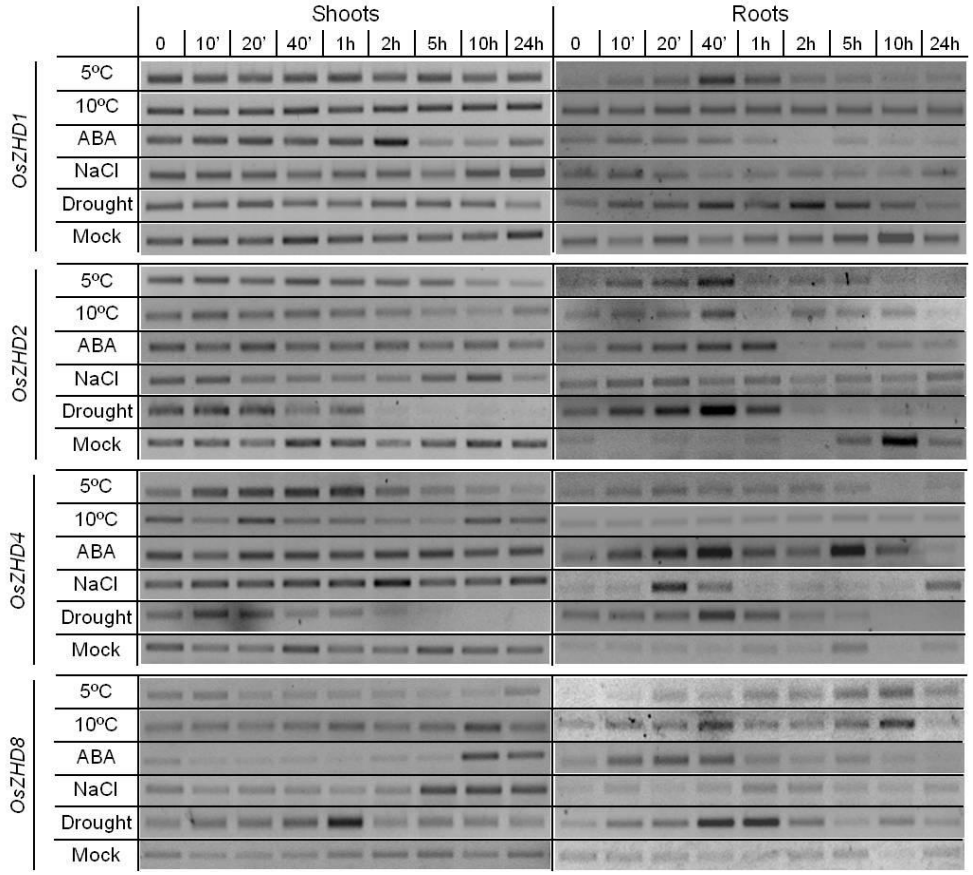

Figure 1. Transcriptional profile of the TFs identified as binding to the promoter of *OsDREB1B*.
The transcriptional profile was obtained by semi-quantitative RT-PCR for plants subjected to cold (5º and 10ºC), ABA (100µM), NaCl (200mM) and drought, and mock control. All treatments started 4h after dawn (time 0). Genes used as internal controls for each assay are described in the Material and Methods section. (continued on the next page).

followed by a return to basal levels. *OsZHD4* was mainly induced by ABA, but also by NaCl and drought to a lesser extent. *OsZHD8* showed an increase in expression under several stress conditions – cold, ABA and drought, with a return to basal levels at the latter stages of the treatments.

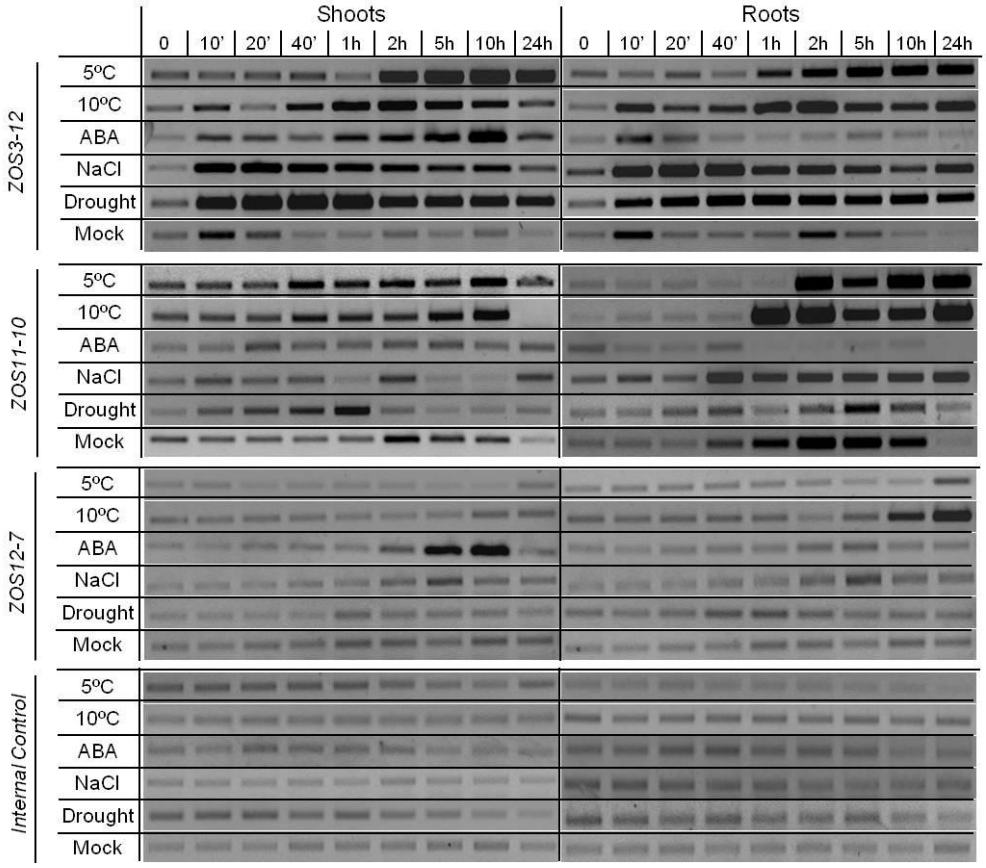

Figure 1. (continued)

Concerning the C2H2-type Zn Finger TFs, the three genes showed very distinct expression patterns. The *ZOS3-12* transcript level was rapidly and highly up-regulated in response to all the stress conditions tested, both in roots and in shoots (except for ABA in roots). The transient induction of this transcript after 10min in the mock control suggests a response to mechanical stress, as observed for *OsDREB1B* (see Chapter 2). Therefore, the rapid induction (10-20min) upon 10ºC and ABA might be biased. Interestingly, and similarly to *OsDREB1B*, the *ZOS3-12* transcript level is more rapidly induced at 10ºC than at 5ºC. The *ZOS11-10* gene expression

seemed to be particularly induced under cold in both shoots and roots. The induction occurs later in the roots, but with higher transcript accumulation. *ZOS11-10* was slightly induced under salt (roots) and drought (roots and shoots), but this regulation might be biased by the circadian rhythm observed in the mock control. In roots, this gene was down-regulated in response to ABA treatment. Regarding *ZOS12-7*, it was late (5-10h) and transiently induced in shoots by ABA and in roots it was induced at 10ºC (after 10h of treatment).

The Zn Finger TFs binding to the *OsDREB1B* promoter have repressor activity

In order to determine whether the TFs identified as binding to the promoter of *OsDREB1B* were repressors or activators of gene expression, we have performed transactivation assays in *Arabidopsis* protoplasts. The constructs used for this assay can be found in Fig. 2a. We have used different reporter vectors in which the *GUS* gene is under the control of the minimal CaMV35S promoter plus the respective *OsDREB1B* promoter fragment that was used in the Y1H screening described in Chapter 2. Using this strategy, this assay also allowed us to validate the interactions of the TFs with the promoter fragments of *OsDREB1B*.

Regarding the C2H2-type TFs, ZOS3-12 had a predicted transcriptional repressor DLN-box/EAR-motif domain (Ohta *et al.*, 2001), but for ZOS11-10 or ZOS12-7 no trans-acting domains could be predicted (see Chapter 2). Nevertheless, in *Arabidopsis*, C2H2-type TFs were shown to repress transcription through a A(G/C)T-X_{3-4}-A(G/C)T *cis*-element (Sakamoto *et al.*, 2004), which is present in both *OsDREB1B* promoter fragments used in the Y1H screening to identify ZOS11-10 or ZOS12-7. As for the ZF-HD TFs, no predicted repressor or activator domains, that we could identify, were present in any of these proteins. Our results indicated that, in the conditions

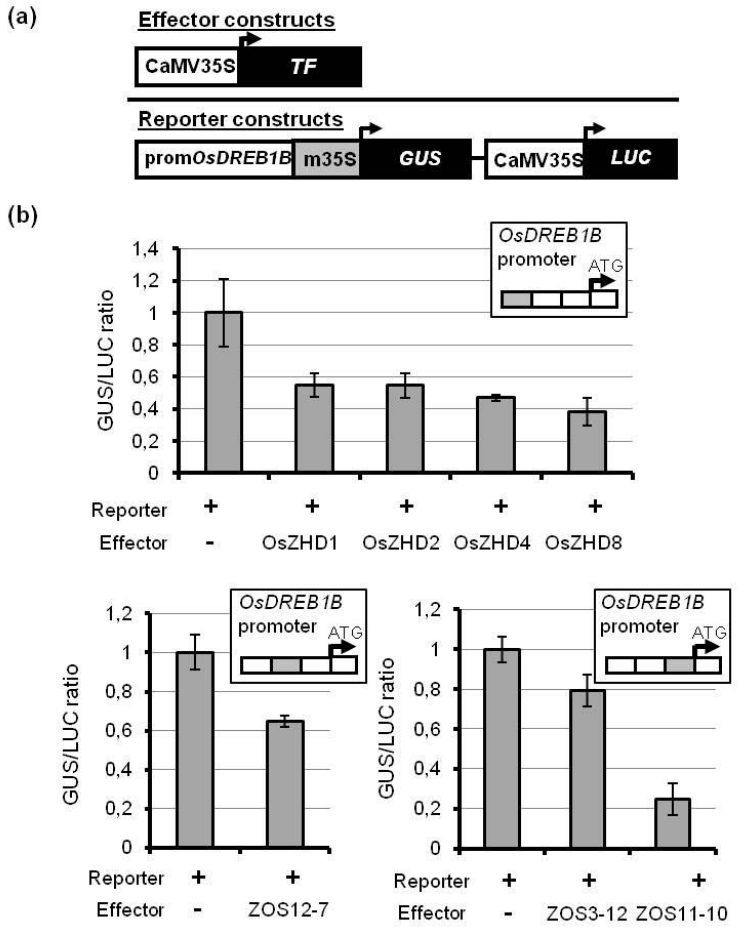

Figure 2. Transactivation activity of the TFs identified as binding to the *OsDREB1B* promoter.

(a) Constructs used for *Arabidopsis* protoplast transformation. Effector constructs used correspond to the *TF* coding region under the control of the full CaMV35S promoter. Reporter constructs contain the *GUS* gene driven by the minimal CaMV35S promoter (m35S) plus the fragment of the *OsDREB1B* promoter (promOsDREB1B) used as bait in the Y1H screening. The *LUC* gene under the control of the full 35S promoter was used to normalize GUS expression levels.

(**b)** Transactivation or repression analysis as a GUS/LUC activity ratio. Each plot refers to a specific *OsDREB1B* promoter fragment, as used for the Y1H screening in Chapter 2, which is highlighted in the schemes on top right corners. Values shown are multiples of the GUS/LUC ratio obtained with the reporter vector without effector. Data represents mean +/- SD (n=3). Differences are statistically significant (t-test, $p<0,05$).

tested, all the seven TFs are repressors of gene expression (Fig. 2b). The repressor activity, measured as a GUS/LUC ratio, ranges from 20% for ZOS3-12 to almost 80% for ZOS11-10, which was the strongest repressor. All the other TFs had intermediate activities, in the range of 40-60% of the initial GUS/LUC ratio. Curiously, the only Zn finger having a canonical repressor domain (ZOS3-12) showed the lowest repressor activity.

The Zn Finger-HD, but not the C2H2-type, TFs form homo- and hetero-dimers

To test whether the TFs under study would form homo- or hetero-dimers, and also to verify if they would interact with one another, we have performed a direct Yeast Two-Hybrid (Y2H) assay. Each TF was tested for interactions with itself and with each of the other six TFs under study. As shown in Fig. 3, we could only detect interactions for the ZF-HD TFs, and not for any of the C2H2-type TFs under study. The only TFs that were shown to homo-dimerize in this assay were OsZHD1 and OsZHD4. These two proteins also interacted in yeast with all the other ZF-HD TFs under study, whereas OsZHD2 and OsZHD8 only interacted with OsZHD1 and with OsZHD4, but not with each other.

In order to confirm these interactions, we performed a BiFC assay with the TFs that yielded positive results for the Y2H (Fig. 4). Using this system we were able to validate all the interactions previously observed. In all cases, the fluorescence signal was clearly localized, most likely in the nucleus of the cell. For some of the interactions several spots of fluorescence could be seen, in what could be nuclear bodies.

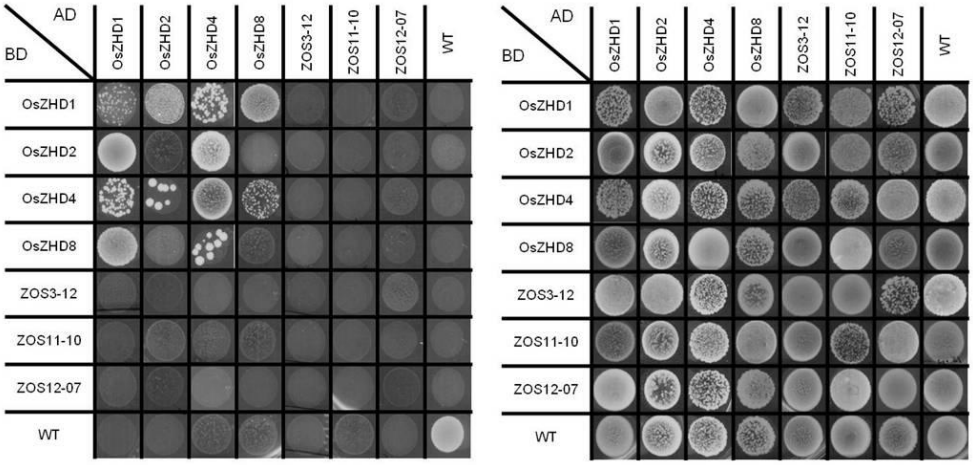

Figure 3. Direct Yeast Two-Hybrid assay to test interactions between the Zn Finger TFs.
TFs were cloned in fusion with both the GAL4-AD and GAL4-BD and all the plasmid combinations were transformed into the yeast strain YRG2. Left panel: yeast growth on -His-Leu-Trp selection medium. Right panel: yeast growth on +His-Leu-Trp selection medium. Positive control used was the interaction of pAD-WT and pBD-WT. Selection medium for the interactions of pAD-ZOS3-12 and pAD-ZOS11-10 was supplemented with 5mM 3-AT, whereas for pAD-OsZHD2 10mM 3-AT was used.

Discussion

In Chapter 2 we reported the identification of novel TFs binding to the promoter of *OsDREB1B*. This gene had been previously described as playing an important role in abiotic stress responses in rice (Dubouzet *et al.*, 2003; Ito *et al.*, 2006; Gutha and Reddy, 2008), but there were no reports on the TFs regulating its expression. We identified eight TFs as novel regulators of *OsDREB1B* and in this chapter we reported the molecular characterization of seven of them: three C2H2-type Zn Finger TFs and four ZF-HD TFs. In Chapter 4 the characterization of the other TF identified, a bHLH TF, will be addressed.

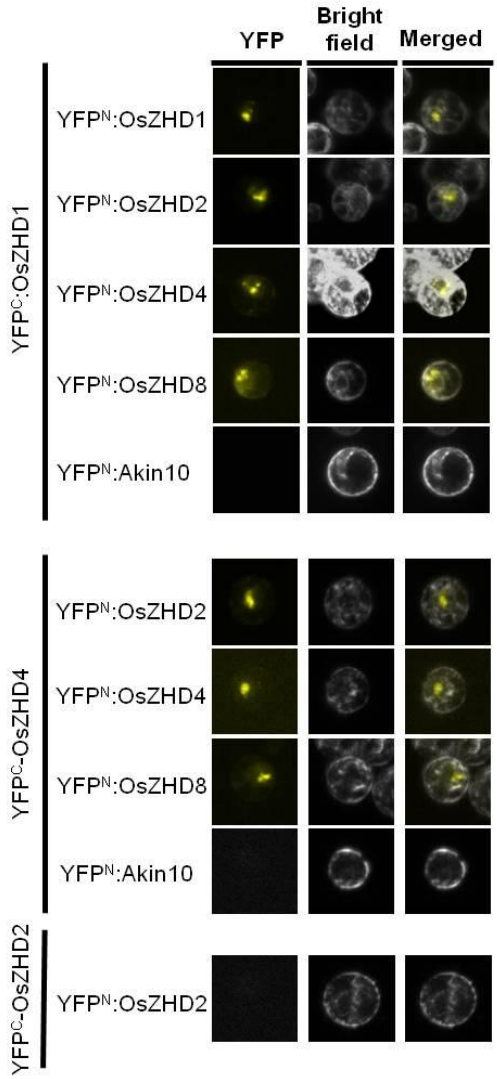

Figure 4. BiFC assay to confirm interactions between the Zn Finger-HD TFs.
On the top panel is the interaction of OsZHD1 with itself and with the other three ZF-HD TFs that bind to the *OsDREB1B* promoter. On the middle panel is the interaction of OsZHD4 with itself and other two Zn Finger-HD TFs. Negative control used was the interaction of the TFs of interest with the *Arabidopsis* Akin10, in fusion with N-terminal portion of YFP as well as the absence of homodimerization between OsZHD2 (bottom panel).

The gene expression of ZF-HD and C2H2-type TFs has been already described as regulated by abiotic stress conditions in *Arabidopsis* (Sakamoto *et al.*, 2004; Tan and Irish, 2006) and in rice (Agarwal *et al.*, 2007; Jain *et al.*, 2008). In our gene expression assays (Fig. 1), we could identify genes whose expression was highly altered by all stresses applied (such as *ZOS3-12*), others that did not show significant variations (such as

OsZHD1), and others that responded specifically to one particular type of stress (such as *ZOS12-7*, both in shoots and roots). Again, similarly to what we described in Chapter 2 for *OsDREB1B*, for some of the genes analyzed here we observed major differences between their expression patterns in roots and shoots, indicating that stress responses are tissue-specific. In *Arabidopsis* it has been observed that several genes are differentially regulated in shoots and roots in response to several abiotic stress conditions (Kreps *et al.*, 2002; Kilian *et al.*, 2007). The fact that the expression of genes encoding regulators of *OsDREB1B* is modulated by these many environmental conditions is indicative of the cross-talk between different stress signalling pathways, where OsDREB1B seems to play an important role.

Interestingly, the faster induction of *OsDREB1B* gene expression at 10ºC, as compared to 5ºC, which we described in Chapter 2, was also observed for *ZOS3-12* and *ZOS11-10* (Fig, 1). This may be due to the fact that 5ºC is a very severe stress for rice plants. When subjected to such severe conditions, rice plants are most likely biochemically impaired, resulting in a delay in molecular responses. In our lab, this response has also been observed for other rice genes (Serra *et al*., unpublished results).

Microarray data was already available on the expression profiles of rice ZF-HD TFs under abiotic stress conditions (Jain *et al.*, 2008). These authors observed an up-regulation of *OsZHD1* in drought and of *OsZHD4* in cold, which correlates with our data. Regarding the C2H2-type TFs, our gene expression data for *ZOS3-12*, indicating an induction by several types of stress, also correlates with previous microarray data that report its up-regulation by cold, salt and drought (Agarwal *et al.*, 2007). In that study, the authors have also observed an induction of *ZOS11-10* by cold, similarly to what we have observed both in roots and shoots. The fact that a time course up to 24h was analyzed here, instead of single time-point, as

performed by Jain et al. (2008) and Agarwal et al. (2007), may explain why some gene expression responses described here were not observed in those studies.

In our transactivation assay in Arabidopsis protoplasts, the TFs identified as binding to the promoter of *OsDREB1B* all functioned as transcription repressors. Among previously described ZF-HD TFs, the *Arabidopsis* ZHD11/ATHB29/ZFHD1 was described as having transcriptional activation activity, while all the other ZF-HD TFs in this plant did not show such activity (Tan and Irish, 2006; Tran *et al.*, 2006). It was not determined however if those TFs would work to repress transcription. Interestingly, the members of this family have been proposed to be redundant in *Arabidopsis* (Tan and Irish, 2006), which may explain the fact that the ZF-HD we identified in rice as regulators of *OsDREB1B* all act in the same way, to repress its transcription. Regarding the C2H2-type TFs, different members of this family had already been described as transcriptional repressors under several abiotic stress conditions, including cold, in *Arabidopsis* (Sakamoto *et al.*, 2004). Moreover, a *cis*-element present in the *OsDREB1B* promoter fragments used in the Y1H screening (see Chapter 2) was described as a binding site for C2H2 TFs and as a negative regulator of transcription (Sakamoto *et al.*, 2004), which correlates with our findings. Nevertheless, other C2H2-type TFs involved in abiotic stress conditions have also been described as transcriptional activators (Huang *et al.*, 2009; Sun *et al.*, 2010).

The identification of seven transcriptional repressors binding to the promoter of *OsDREB1B*¸ a gene known to be highly induced by cold (Dubouzet *et al.*, 2003), lead to the question of why no TFs that promote transcription were identified. Our hypothesis is the following: when rice seedlings are subjected to 10ºC, the *OsDREB1B* gene expression is quickly induced, reaches a peak at 2h and then starts to decrease (see

Chapter 2). In addition, the cold-induced cDNA expression library used for the Y1H screening was prepared from plants subjected to 8ºC and collected 2h, 5h and 24h after the start of the stress (see Materials and Methods in Chapter 2). Thus, if at 8ºC the regulation of *OsDREB1B* gene expression is similar to what happens at 10ºC, it is expected that after 2h at 8ºC, the cDNA library will be enriched in *OsDREB1B* repressors rather than activators.

We also tested whether the Zn Finger TFs we have identified interact with themselves and one-another. We have only observed interactions between the ZF-HD TFs. The C2H2-type TFs did not interact with one-another, nor with the ZF-HD TFs, in yeast. Homo- and hetero-dimers had already been described for ZF-HD TFs in *Arabidopsis* (Tan and Irish, 2006) and in *Flaveria* (Windhovel *et al.*, 2001), meaning that this feature is conserved in several species for this type of TFs. It is possible that hetero-dimerization plays a role in regulating the binding of the TFs to DNA and also in their transactivation activity. The heterodimerization of two Homeodomain proteins from the mushroom *Coprinus cinereus* was shown to be necessary for their function as transcriptional regulators, namely in what concerns their targeting to the cell nucleus (Spit *et al.*, 1998). Moreover, homo- and hetero-dimers of two homeodomain proteins, Alx4 and Gcs, were described as having different DNA-binding specificities, as well as transactivation activities (Tucker and Wisdom, 1999). Nevertheless, the functional significance of ZF-HD homo- and hetero-dimers is yet to be analyzed.

With this work we described seven novel players in the abiotic stress signalling pathway. These novel TFs bind to the promoter and repress the expression of *OsDREB1B* and are regulated by several abiotic stresses. Together with previous reports, our data suggests that Zn Finger TFs are a pivotal component in the regulation of *DREB1/CBF* genes in plants.

Acknowledgements

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Supplemental Materials

Supplemental Table 1 – Primers used for semi-quantitative RT-PCR

Gene	Primer Sequence 5'-3'
OsZHD1	CAGCAGTTCTGTGACGAGGT
	TAGAGCCGAGAACGAATGCT
OsZHD2	GGATGCACAACAACAAGCAC
	AGAACCCAACCACAATGGAA
OsZHD4	AGCCATTGTTGCTGTGTCTG
	CCCATTGTTACCCACATGCT
OsZHD8	CTGAGCCTGGTGCCCTAC
	ACGGACGACGGGAGGAAG
ZOS3-12	AGGAGGAGGAGGTGCAGAGT
	TTGAGCAGATTGAGTACATTAATTTTT
ZOS11-10	GGTGAGTCGTAATGGCACAA
	TGAACATTACAAAATCAAAGACGAA
ZOS12-7	GTGGTGTGTCACGAGCAATC
	GCATGCAACATGGAAATCAG
ACTIN1	GTCGCACTTCATGATGGAGTTG
	CATGCTATCCCTCATCTCGAC
eEF1α	ACCCTCCTCTTGGTCGTTTT
	AAATACCCGCATTCCACAAC
UBC2	CAAAATTTTCCACCCGAATG
	ATCACATGAATCAGCCATGC

Chapter 4.

OsPIF4 is a novel Phytochrome Interacting Factor that unveils a potential convergence of light and cold signalling pathways on the rice gene *OsDREB1B*

Chapter 4. Table of Contents

Abstract

Plants have evolved several molecular mechanisms in order to cope with adverse environmental conditions. *DREB1*/*CBF* genes, which encode members of the AP2/EREBP transcription factor (TF) family, are rapidly and transiently induced by low temperatures and have been described as major regulators of cold-stress responses. In Chapter 2, we identified OsPIF4*,* a putative Phytochrome Interacting bHLH Factor, as a TF that binds to the *OsDREB1B* promoter. Here we present the molecular characterization of this TF, regarding its involvement in abiotic stress, but also in light signalling. The *OsPIF4* transcript is repressed by different treatments, such as drought, salt, cold and ABA. Under cold stress, the *OsPIF4* transcript also undergoes alternative splicing, leading to the formation of a premature stop codon. Using a transactivation assay, we showed that OsPIF4 is a repressor of *OsDREB1B* gene expression. The activity of OsPIF4 may be negatively modulated by light, as it preferably binds to the Pfr active form of rice phytochrome B. Additionally, its abundance is reduced by light during the day of a diurnal light/dark cycle, compared to plants retained in prolonged darkness during the corresponding subjective day period. Concomitantly, *OsDREB1B* transcript levels are up-regulated by this daylight treatment, compared to darkness, during the subjective day. The data suggest that OsphyB may target the OsPIF4 protein for degradation or sequestration during the daylight period. These findings provide the first indication that OsPIF4 may be a link between light signalling and the *OsDREB1* regulon in rice and suggest that light and cold-temperature signalling pathways converge on the rice gene *OsDREB1B*.

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, like the production of osmoprotectants and regulatory proteins involved in signalling pathways (Saibo *et al.*, 2009; Hirayama and Shinozaki, 2010). Among these proteins, TFs play a very important role in the 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 DREB1/CBF TFs belong to the AP2/ERF family and have been described as being quickly and transiently induced by low temperatures (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999; Dubouzet *et al.*, 2003). When present in the cell nucleus, DREB1/CBF 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). Our work focuses on the gene *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 osmotic and oxidative stress as well as to ABA and salicylic acid (Gutha and Reddy, 2008). Gutha and Reddy (2008) also observed that this TF plays an important role in the response to biotic stress.

Temperature and light signalling have been previously described to cross-talk (Franklin, 2009), and phytochrome signalling 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 (R) absorbing Pr and the active far-red light (FR) absorbing Pfr (Franklin and Quail, 2010). In *Arabidopsis* there are five genes that code for phytochromes (A-E), whereas in rice there are three members (A-C), which function as the only photoreceptors to perceive R and FR light (Takano *et al.*, 2009). Upon activation by R, the Pfr active form of phytochromes migrates into the nucleus, where it interacts with a series of TFs, of the basic helix-loop-helix family (bHLH), referred to as Phytochrome Interacting Factors (PIFs; as reviewed by Franklin and Quail, 2010). This interaction usually results in a proteasome-dependent degradation of the PIFs and is a way to modulate the expression of genes regulated by these TFs. 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 in the case of the more recently identified PIF7, even though it co-localizes with phyB in nuclear speckles after a R pulse, this protein appears to be light-stable (Leivar *et al.*, 2008). A set of putative PIFs has 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 expression of *DREB1/CBFs* in *Arabidopsis* was described as being dependent on both the circadian clock and light signalling (Kim *et al.*, 2002; Fowler *et al.*, 2005; Franklin and Whitelam, 2007; Kidokoro *et al.*, 2009; Dong *et al.*, 2011). Phytochrome B was initially reported as a positive regulator of cold-stress signalling in response to light, particularly regarding the expression of *DREB1/CBFs* during cold stress (Kim *et al.*, 2002). These results were further supported by the finding that PIF7, a negative regulator of phytochrome-mediated signalling (Leivar *et al.*, 2008), is a transcriptional repressor of *DREB1/CBF* under circadian control (Kidokoro *et al.*, 2009).

On the other hand, Franklin and Whitelam (2007) reported that a low red to far-red ratio (R/FR) increased *DREB1/CBF* gene expression in a circadian clock-dependent manner.

In Chapter 2 we reported the identification of a bHLH protein, predicted as a putative PIF, as binding to the promoter of *OsDREB1B*. We named this protein OsPIF4. In this chapter, we report that *OsPIF4* transcript level is regulated by different abiotic stresses and that it is alternatively spliced under cold stress conditions. Moreover, we demonstrate that OsPIF4 can act as a repressor of *OsDREB1B* transcription. OsPIF4 is also shown to interact with the active form of rice phyB, suggesting a possible involvement in the light-dependent expression of *OsDREB1B* that we also observe.

Materials and methods

Plant materials treatments

Rice seedlings (*Oryza sativa*, L. cv Nipponbare) 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. Treatments were performed as described in Chapter 2. For semi-quantitative RT-PCR analysis, time points consisting of ten plants were sampled (roots and shoots separately). For quantitative RT-PCR analysis, seedlings were grown in the conditions described above and two different assays were performed both at 28ºC and 5ºC. The first assay (light/dark) was performed using the above-mentioned conditions, while in the second (continuous dark), the lights were maintained switched off during the 24h of treatment. A time-point common to all assays was taken at the transition from dark to light. For the 28ºC assays, plants were transferred to pots with new nutritive medium 4h after the start of the light period (light/dark assay)

or four hours after start of subjective dawn (continuous dark assay). The same was done for the 5ºC assays but, in this case, the plants were transferred to pre-cooled nutritive medium. For all treatments an average of 10 whole plants were harvested at each time-point.

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-days-old seedlings were transferred to 5ºC 4h after the start of the light period, and kept there for 1h or 4h. Ten whole seedlings were collected at each time point.

Semi-quantitative RT-PCR and RT-qPCR

Total RNA from both rice and *Arabidopsis* was extracted using the RNeasy Plant Mini Kit (Qiagen). For semi-quantitative RT-PCR analysis, first strand cDNA was synthesised from 1µg total RNA, using an oligo-dT primer and the SuperscriptII reverse transcriptatse (Invitrogen) following the manufacturer's instructions. The cDNA was then amplified by PCR using gene-specific primers (Supplemental Table 1). 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* (*eELF1α*; Os03g08060) and *UBIQUITIN-CONJUGATING ENZYME E2* (*UBC2*; Os02g42314) were used, respectively. The results shown are representative of at least two biological replicates.

For quantitative PCR analysis, RNA extraction was conducted as described above. First strand cDNA synthesis was performed using 2µg total RNA with an oligo-dT primer using Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to the manufacturer's instructions. *UBC2* was used as internal control. Real-time PCR was done in a Lightcycler

480II (Roche), using Lightcycler 480 SYBR Green I Master mix (Roche). Besides a biological replicate, measurements were performed in triplicate for each time point and efficiency curves were prepared in duplicate. The sequences for the primers used for RT-qPCR can be found in Supplemental Table 2.

Transactivation assay

The *OsPIF4* full coding sequence was cloned in vector pDONR221 (Invitrogen), according to the manufacturer's instructions. The effector plasmid was built by recombining *OsPIF4* coding sequence from pDONR221 into vector pH7WG2 (VIB, Ghent), to be under the control of the full CaMV35S promoter. The reporter vector used was pLUCm35GUS, described in Chapter 3. The *OsDREB1B* promoter fragment used in the Yeast One-Hybrid screening to identify OsPIF4 (see Chapter 2) was cloned up-stream of the minimal CaMV35S promoter, as a *Sal*I fragment.

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, luciferase and GUS determinations were performed as described in Chapter 3. Readings were performed in triplicate. Activation of gene expression was calculated as a GUS/LUC ratio.

Yeast-two hybrid assay

The full coding sequence of the *OsPIF4* gene was cloned into vector pAD-WT (Stratagene), by replacement of the coding region of the wild-type lambda cI, fragment C, down-stream of the GAL4 activation domain, using the enzymes *Eco*RI and *Pst*I. 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, 654-1172 for OsPHYB and 620-1138 for OsPHYC. These sequences were then recombined into vector pBD-GW (see Chapter 3). 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.

Bimolecular Fluorescence Complementation

The *OsPIF4* coding region was recombined from pDONR221 into the vector $YFP^{C}43$, 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 $YFP^{N}43$, to be in fusion with the C-terminal portion of the YFP. Cloning in $YFN^{C}43$ and $YFC^{N}43$ vectors was done according to Gateway technology (Invitrogen). To use as negative control in the interaction assays with OsPIF4, 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 LBA4004. 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).

In vitro co-immunoprecipitation assay

The vectors for this assay were prepared as following: the OsPIF4:GAD was constructed by removing the Arabidopsis *PIF7* coding region from plasmid PIF7:GAD (Leivar *et al.*, 2008) and replacing it with the *OsPIF4* 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 R (660nm) or 4min of R followed by 4min of FR (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).

OsPIF4 antibody production and antibody inhibition assay

The non-conserved peptide H2N-TPTPRAAARSDDVSSR-CONH2, spanning a.a. 112 to 127 of OsPIF4, was used as an antigen to raise a rabbit polyclonal antibody (Eurogentec). The synthetic peptide was coupled to keyhole limpet hemocyanin (KLH). The competition assay was performed by pre-incubation of anti-OsPIF4 antibody with the immunizing peptide (10µg peptide per µL of antibody) overnight at 4ºC in blocking solution.

Protein extraction and Western blotting analysis

Plant tissue samples (100mg) were ground in liquid nitrogen and mixed with 200µL protein extraction buffer (50mM TrisHCl pH8, 150mM NaCl, 2mM EDTA pH8, 0.4% Triton X-100, 2xComplete protease inhibitor (Roche)). Samples were cleared at 17000*g* at 4ºC for 15min and quantified using Bradford's method (Bio-Rad). Total protein extracts (100µg) were separated on a 12% SDS-PAGE gel and transferred to PVDF membranes (Amersham). After blocking for 1h in PBS-T 5% skim milk, the membrane was incubated with anti-OsPIF4 (1:2000) in blocking solution overnight at 4ºC. Incubation with secondary antibody (anti-rabbit-HRP; Abm) diluted 1:20000 in blocking solution was performed for 1h at RT. Detection was carried out with ECL Western Blot Analysis System and Hyperfilm ECL (Amersham). Loading control was performed with Ponceau S staining.

Results

OsPIF4 transcript is regulated by alternative splicing under cold stress

In Chapter 2 we identified OsPIF4 as a TF that binds to the promoter of *OsDREB1B*. To understand how *OsPIF4* influences the plant responses to cold, and whether it is also involved in the responses to other stresses, we

have investigated *OsPIF4* gene expression under different abiotic stress conditions. Two week-old rice seedlings were subjected to cold (5ºC and 10ºC), salt (200mM NaCl), drought and ABA (100µM) treatments over a period of 24h (Fig. 1).

The gene expression studies showed that *OsPIF4* transcript level slightly increases in shoots under control conditions during late afternoon and night period (5h and 10h in Fig. 1a, respectively), indicating a light/dark responsive behavior, as previously described (Nakamura *et al.*, 2007). In rice seedlings subjected to different treatments, the *OsPIF4* gene expression appeared to be repressed after 20min under salt and drought and 1h after ABA treatment. The transcript was then up-regulated some hours afterwards, except in shoots under drought and in ABA-treated roots.

The transcriptional regulation of *OsPIF4* under cold was unique in this set of stress assays, since an upper band appeared after 20 to 40min of cold, both in shoots and roots and at 5ºC and 10ºC. After cloning and sequencing, we found that the second band was an alternative splice form of the transcript (Fig. 1b). Under cold conditions, the alternative splicing of the *OsPIF4* transcript leads to the retention of the first intron, and consequent formation of a premature stop codon. We named this splice form *OsPIF4β*, whereas the splice form under control conditions will be referred to as *OsPIF4α*. Interestingly, the gene expression pattern of both splice forms seems to be slightly different when rice seedlings were subjected to different temperatures (Fig. 1a). At 5ºC, transcripts of the *β*-form started to be detected 40min after stress onset, increasing along the treatment, whereas the *α*-form started to decline after 1-2h. 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

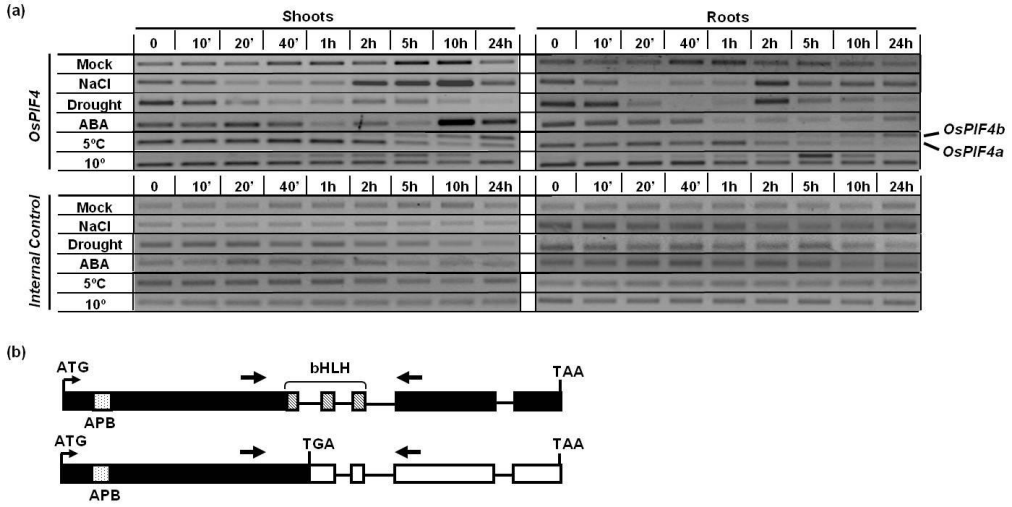

Figure 1. Transcriptional profile of *OsPIF4.*
(a) Analysis of *OsPIF4* gene expression under different treatments as determined by semi-quantitative RT-PCR. Treatments started 4h after the beginning of the light period. All time-points correspond to the light period, except the 10h, which corresponds to 2h after the start of the dark period.
(b) Schematic representation of the *OsPIF4* transcript present under control conditions (top) and the alternative splice form that appears under cold stress (bottom). APB - Active Phytochrome Binding motif (dotted box); bHLH - basic Helix Loop Helix domain (oblique stripes). Thick arrows represent RT-PCR primer locations. Black boxes represent translated exons, white boxes represent non-translated exons and lines represent introns.

treatment. The expression pattern of both forms was similar in both roots and shoots.

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. 2). We identified alternative splice forms for *PIF3, 6* and *7*. The *PIF6* splice

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. 2b), leading to the formation of premature stop codons, similarly to what happens with *OsPIF4*. Nevertheless, none of these splicing events seems to be triggered by cold, since they are present even at 22ºC.

OsPIF4 is a repressor of OsDREB1B

To investigate whether OsPIF4 was a repressor or activator of transcription, we have performed a transactivation assay in *Arabidopsis* protoplasts. The full length OsPIF4 was used as effector, while the *GUS* gene driven by the *OsDREB1B* promoter fragment, used as bait in the Y1H screening (Chapter 2), was used as reporter (Fig. 3a). Fig. 3b shows that co-transformation of OsPIF4 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 OsPIF4 and consequent repression of transcription. These results also provide further evidence that OsPIF4 indeed binds to the promoter of *OsDREB1B*.

OsPIF4 interacts with the active form of OsphyB

The interaction between rice phytochromes (A, B, and C) and putative PIFs had not yet been shown in rice. Thus, we have analyzed whether OsPIF4 interacted with any of the rice phytochromes. We performed a Yeast Two-Hybrid assay (Y2H), using as prey the coding region of *OsPIF4*, and as baits the C-terminal non-photoactive coding regions of the three rice phytochromes (Fig. 4a). From the yeast growth in His-lacking medium (Fig.

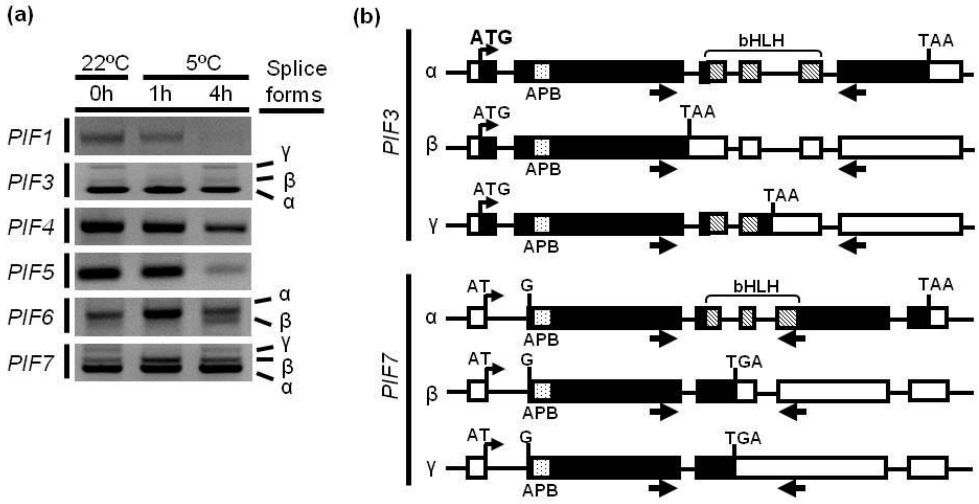

Figure 2. Expression pattern and alternative splice forms of the *PIF* genes in Arabidopsis seedlings subjected to low temperature conditions.
(a) Analysis of the Arabidopsis *PIF* transcript levels by semi-quantitative RT-PCR in cold-treated plants. Indication of bands corresponding to alternative splice 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 (dotted box); bHLH - basic Helix Loop Helix domain (oblique stripes). Thick arrows represent RT-PCR primer locations. Black boxes represent translated exons, white boxes represent non-translated exons and lines represent introns.

4b), our results indicated that OsPIF4 interacts with OsPHYB in a stronger manner than it does with OsPHYA or C. To further confirm these results, a Bimolecular Fluorescence Complementation assay (BiFC) was also performed (Fig. 5). YFP fluorescence signals could be detected in the nucleus of *Nicotiana benthamiana* leaf cells co-infiltrated with the OsPIF4::YFP^N and OsPHYB::YFP^C fusion proteins (Fig. 5b). No fluorescence was detected when leaves were co-infiltrated with OsPIF4::YFP^N plus either OsPHYA::YFP^C or OsPHYC::YFP^C.

The Y2H and BiFC results support the hypothesis that OsPIF4 can bind to the C-terminal of OsPHYB. In order to test whether this binding was

dependent on the active or inactive form of the phytochrome, we have expressed OsPIF4 and the full length OsphyB proteins *in vitro* and analyzed their interaction using a co-immunoprecipitation assay (Fig. 6). As shown in Fig. 6b, OsPIF4 interacts preferably with the active Pfr form of OsphyB. Interestingly, OsphyB also interacts conformer-specifically with *Arabidopsis* PIF3.

The expression of *OsDREB1B* is dependent on light conditions

Given that OsPIF4 binds to the promoter of *OsDREB1B* to repress its transcription and that it binds preferably to the active form of OsphyB, we wanted to test whether the expression of *OsDREB1B*, under control or cold stress conditions, was influenced by light conditions. We therefore analyzed the expression of *OsDREB1B* in plants previously grown in control conditions (photoperiod 12h/12h and 28ºC) and then transferred or not to 5ºC, under control photoperiod or in constant dark (Fig. 7). At 28ºC and in photoperiodic conditions, *OsDREB1B* showed an increased expression during the day time, reaching a peak 8h after the start of the light period, and then decreased during the night period (Fig. 7a). Interestingly, this cycling was attenuated when the plants were transferred to continuous dark, indicating a light regulation of this gene. At 5ºC, *OsDREB1B* expression was induced to a level of more than 100-fold greater than at 28ºC, but no clear difference in expression between the two light treatments (photoperiod vs. continuous dark) was observed. However, after 24h of cold stress, we observed a more pronounced decrease in the expression of *OsDREB1B* in continuous dark conditions, as compared to normal photoperiod. We also analyzed the expression of both splice forms of *OsPIF4* at 5ºC under both light and dark conditions, but no differences were found between them (data not shown).

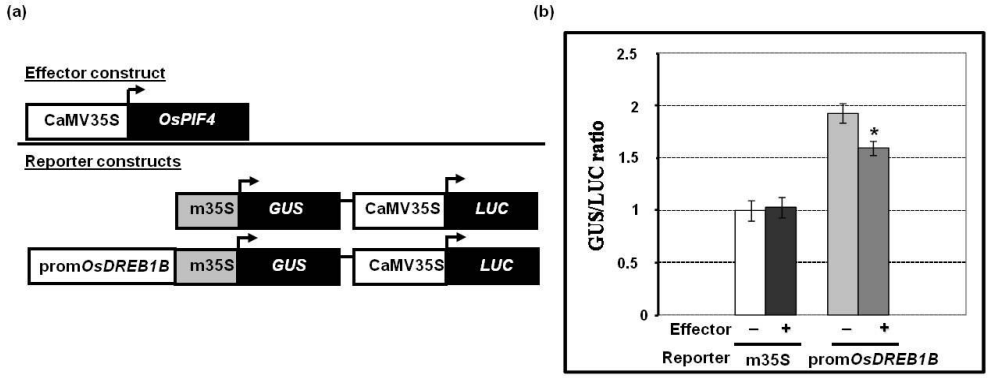

Figure 3. Transactivation activity of OsPIF4 in *Arabidopsis* protoplasts.
(a) Constructs used for protoplast transformation. Effector construct used corresponds to the *OsPIF4* coding region under the control of the full 35S promoter. Reporter constructs contain the *GUS* gene driven by the minimal 35S promoter (control vector) or under the minimal 35S promoter plus the fragment of the *OsDREB1B* promoter used as bait in the Y1H screening (Chapter 2). The *LUC* gene under the control of the full 35S promoter was used to normalize GUS expression levels.
(b) Transactivation analysis of OsPIF4 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). *** -** Differences statistically significant (t-test, p<0,05).

The OsPIF4 protein level is stable under white light and not regulated by cold

In order to test whether the abundance of the OsPIF4 protein is dependent on light conditions, and also if the expression of *OsDREB1B* correlates with OsPIF4 levels, we have raised an antibody against an OsPIF4 specific peptide. The immunoblot analysis detected a protein of a little less than 50kDa, which is the expected size for OsPIF4 (Fig. 7c). An antibody depletion assay was carried out to test the specificity of the antibody to OsPIF4, using the OsPIF4-specific peptide as competitor. In this case, the expected band could no longer be detected, indicating that it indeed corresponds to OsPIF4 (Fig. 7c).

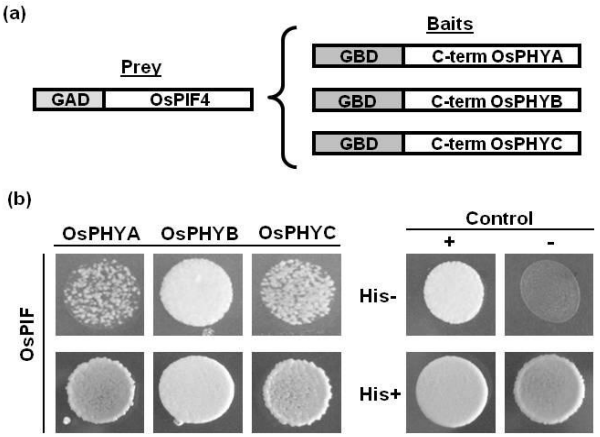

Figure 4. Analysis of the interaction between OsPIF4 and the three rice phytochromes, using a Yeast Two-Hybrid assay.
(a) Protein constructs used in the Y2H assay. OsPIF4 was used as prey, in a translational fusion with GAD. The C-terminal non-photoactive regions of rice PHYA, B and C were fused with GBD to be used as baits.
(b) Analysis of protein-protein interactions in yeast growing in His-lacking medium. Positive control used was the interaction between pAD-WT and pBD-WT, and negative control was the absence of interaction between GAD:OsPIF4 and pBD-WT. Bottom panel shows yeast growth in His-supplemented medium.

The OsPIF4 protein levels were analyzed for the same time points and using the same assay design previously described for the RT-qPCR. Fig. 7d shows that no major differences were detected between the 28ºC and 5ºC assays. In addition, under the 12h/12h photoperiod, OsPIF4 levels did not show a significant fluctuation when the plants were exposed to white light (Fig. 7d, top panels). This indicates that OsPIF4 protein levels remain relatively stable during the light period of the diurnal day/night cycle. By contrast, however, there was an apparent accumulation of the protein during the subjective day of the prolonged darkness treatment (bottom panels), suggesting that OsPIF4 protein level is repressed by light during the day under normal diurnal conditions. Interestingly, after 24h of

continuous dark, at both 28ºC and 5ºC, the protein could no longer be detected.

Given that *OsPIF4* transcript is alternatively spliced in response to cold, it could be expected that the *OsPIF4β* form of the transcript would yield a truncated protein. This form would have a predicted molecular weight of 29.59kDa. Nevertheless, in our immunoblot conditions, no band was detected in that size range for the samples subjected to the 5ºC assays.

Discussion

The DREB1 /CBF regulon has long been shown to play an important role in the cold stress response in plants. Furthermore, it had already been reported that, in *Arabidopsis, DREB1/CBF* genes are light regulated, their expression being dependent on light quality (Kim *et al.*, 2002; Franklin and Whitelam, 2007). PIF7 was recently described as a possible link between light and cold signalling 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 Chapter 2, using the Y1H system, we have identified OsPIF4 as a TF binding to the promoter of *OsDREB1B* and in this chapter we aimed to test its role in the regulation of *OsDREB1B* and possible involvement in the link between light and cold signalling in rice. Even though PIF7 was described as a regulator of *DREB1C* and *DREB1B* in *Arabidopsis* (Kidokoro *et al.*, 2009), among the aligned APB motif sequences, OsPIF4 showed the highest similarity to PIF4 and PIF5, whereas PIF7 showed the least degree of similarity (see Chapter 2).

OsPIF4 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,

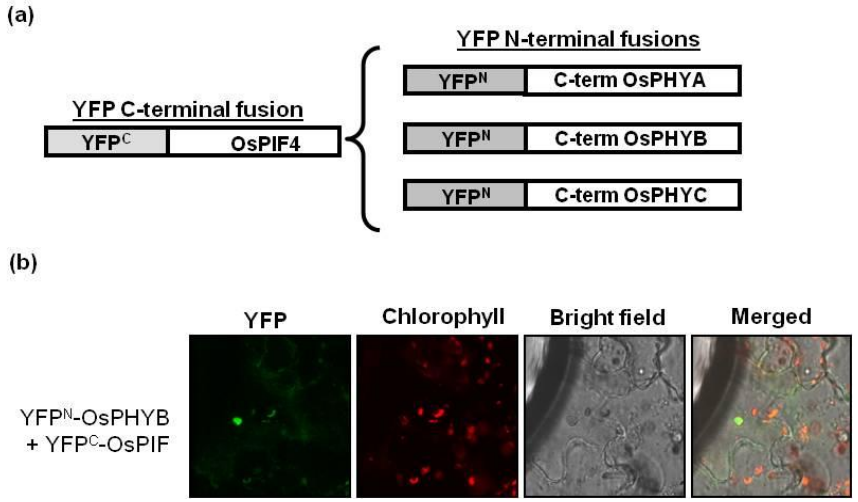

Figure 5. Analysis of the interaction between OsPIF4 and different rice phytochromes, using a BiFC system in detached tobacco leaf epidermis.
(a) Protein constructs used for the assay. The C-terminal region of YFP was expressed in fusion with OsPIF4 and the N-terminal region of YFP was expressed in fusion with the C-terminal non-photoactive domains of the rice phytochromes.
(b) YFP fluorescence obtained under 520nm emission for the interaction of OsPIF4 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 OsPIF4 and rice OsPHYA or C or for the negative control, in which a fusion of YFPN with *Arabidopsis* Akin10 was used.

in most cases, the levels are recovered after a few hours (Fig. 1a). This suggests that *OsPIF4* could have a potential role in rice responses to different abiotic stresses, through the regulation of *OsDREB1B* and/or possibly other down-stream genes. Phytochrome signalling 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*

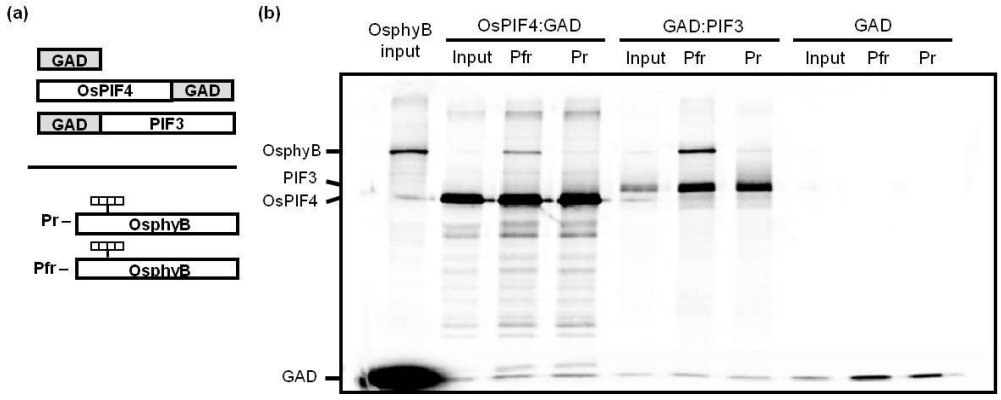

Figure 6. Analysis of the interaction between OsPIF4 and the Pr and Pfr forms of OsphyB, using a co-immunoprecipitation assay.
(a) Schematic representation of the protein constructs used for the assay. OsPIF4 was used as bait, in a translational fusion with GAD. GAD alone was used as a negative control 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.

al., 1994). Nevertheless, so far, in *Arabidopsis* and rice, there are no reports regarding a possible cross-talk between these pathways.

In contrast to the above-mentioned treatments, the effect of phytochrome signalling 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). In our work, we observed that low temperatures induced the formation of an alternative splice form of the *OsPIF4* transcript (Fig. 1b). Interestingly, the expression patterns of both splice forms, *α* and *β*, was different when rice seedlings were subjected to 5ºC or 10ºC (Fig. 1a). In response to a more severe stress (5ºC), the constitutive *α*–form seems to be replaced by the *β*–form along the treatment. When rice seedlings were subjected to a mild stress (10ºC), the

expression of the *α*–form is not significantly altered, whereas the *β*–form is only transiently expressed. These results indicate a differential response of *OsPIF4* to low temperatures, depending on how severe the stress is. We also demonstrated that the *Arabidopsis PIF3, 6* and *7* also show alternative splice forms (Fig. 2), similarly to what happens with *OsPIF4*. *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, but also to regulate phytochrome signalling itself. The *Arabidopsis* PIF3 has been previously described to control 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). 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.*, 2008). This was further confirmed by the recent finding that PIF3, 4 and 5 modulate phyB levels by enhancing its *in vitro* polyubiquitination by COP1 (Jang *et al.*, 2010). A question thus arises on whether the *OsPIF4β* transcript, that has a longer 3'UTR when compared to the *α*-form, is eliminated by nonsense-mediated mRNA decay (Kertesz *et al.*, 2006) or results in a shorter, truncated protein. If the *OsPIF4β* 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 signalling pathway,

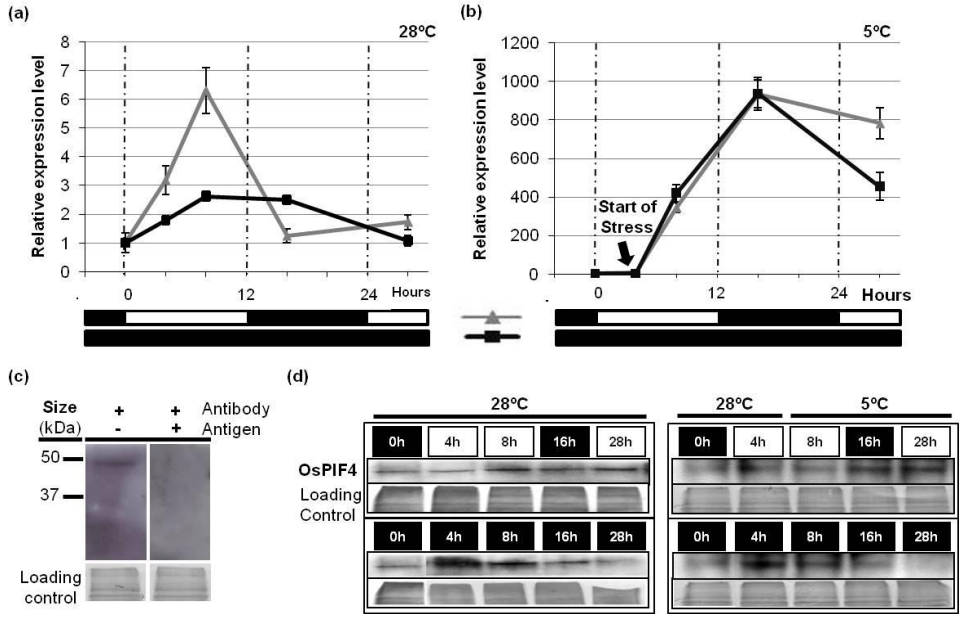

Figure 7. Expression of *OsDREB1B* and OsPIF4 protein levels in rice seedlings subjected to control or cold treatments in different light regimes.

(a) *OsDREB1B* gene expression pattern as determined by RT-qPCR in 14-day-old rice seedlings grown at 28ºC, 12h/12h photoperiod and maintained in the same temperature conditions during the assay. The seedlings were exposed or not to a continuous dark period (black and grey lines, respectively). 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).

(b) Same as above but with plants subjected to a 5ºC treatment.

(c) Depletion assay for the antibody raised against a OsPIF4 peptide. The left panel shows immunoblot performed with anti-OsPIF4 serum, while the panel on the right shows immunoblot performed with anti-OsPIF4 serum previously incubated with the antigen. Loading control is the total protein extract dyed with Ponceau S.

(d) Immunoblot assay to analyze OsPIF4 protein levels in the same samples described above for qRT-PCR. The 28ºC assay is shown on the left and the 5ºC assay on the right. White boxes represent samples taken during the day time, and black boxes represent samples taken in dark conditions.

for example under cold conditions. In our immunoblot assays, using an antibody against OsPIF4, we could only detect a band corresponding to the *OsPIF4α* transcript form, even in the 5ºC assays (Fig. 7d). This may indicate that either *OsPIF4β* transcript is not translated into a protein or its protein level could not be detected in our assay.

Since the *OsPIF4* transcript level was down-regulated during cold, this raised the question of whether the OsPIF4 protein was an activator or repressor of *OsDREB1B* transcription. Our transactivation studies in *Arabidopsis* protoplasts showed an activation of the reporter gene even without the effector (35S::OsPIF4). This induction is most probably due to the fact that *OsDREB1B* promoter is stress-inducible, and the process of *Arabidopsis* protoplast preparation surely triggers a stress response. Nevertheless, OsPIF4 represses the expression of the reporter gene driven by the *OsDREB1B* promoter, reducing its induction. These results are in agreement with what was described for the *Arabidopsis* PIF7, which acts as a repressor of *DREB1B/CBF1* and *DREB1C*/*CBF2* (Kidokoro *et al.*, 2009). On the other hand, it is interesting to note that several *Arabidopsis* PIFs, including PIF7, have been described as activators of transcription (Huq *et al.*, 2004; Al-Sady *et al.*, 2008; Leivar *et al.*, 2008). This may indicate that transcriptional activity of these proteins depends on promoter context, as recently suggested (Leivar and Quail, 2010).

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.*, 2008). However, to the best of our knowledge, no reports have yet been published on the interaction between PIFs and phytochromes in rice. Our co-immunoprecipitation results showed that OsPIF4 preferably binds to the active form of OsphyB (Fig. 6). 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 (Fig. 4b). Similar results had already been published for PIF3 in *Arabidopsis* (Ni *et al.*, 1998; Zhu *et al.*, 2000). Interestingly, the Y2H assays also showed that OsPIF4 could bind weakly to the C-terminal domains of the rice phytochromes A and C (Fig. 4b). 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 OsPIF4 does (Fig. 6b).

Given that OsPIF4 can act as a repressor of *OsDREB1B* expression, and interacts preferably with the active form of OsphyB, we hypothesized that light could influence the expression of *OsDREB1B*. We found that this is indeed the case: the up-regulation of *OsDREB1B* expression that was observed in response to light during the normal diurnal cycle at 28ºC was attenuated in response to prolonged darkness during the corresponding subjective day period (Fig. 7a). OsPIF4 protein levels rose concomitantly in darkness during this subjective day, but not in the light of the diurnal cycle, a correlation consistent with the possible action of OsPIF4 in repressing *OsDREB1B* expression in the dark. This repression of OsPIF4 accumulation in the light might result from light-activated-phyB-induced degradation of OsPIF4, a suggestion supported by the Pfr specific binding of OsphyB to OsPIF4 demonstrated here. It may be that the increase in OsPIF4 protein levels during subjective day is somehow circadian-driven, being suppressed, under light conditions, by degradation of OsPIF4 through interaction with photoactivated OsphyB. Alternatively, this interaction might result in the sequestration of OsPIF4 instead of targeting it for degradation. In *Arabidopsis*, PIF7 has also been described as light stable even though it interacts with the active form of phyB (Leivar *et al.*, 2008). It is possible, therefore, that OsPIF4 is functionally more similar to PIF7, even though its a.a. sequence is more similar to PIF4 and 5 (see

Chapter 2). Although the above suggestion that the light-dependent changes in *OsDREB1B* expression at 28ºC are mediated by phyB-regulated OsPIF4 abundance is consistent with the data, this remains to be rigorously tested. The analysis of a rice *OsPIF4* knock-out or silencing line will be crucial to test this hypothesis.

When rice seedlings are subjected to low temperature (5ºC), the light effect on *OsDREB1B* gene expression is not as evident as it is in control conditions and is not correlated with OsPIF4 levels. In addition, the absolute level of induction is more than 100-fold greater than observed for light under diurnal conditions at 28ºC. This suggests that the mechanism of cold induction of this gene is different from that of light regulation. However, the decline in *OsDREB1B* transcript levels, after about 12h of cold stress, is somewhat faster for plants that were maintained in continuous dark. This indicates that light also plays a role controlling *OsDREB1B* gene expression under prolonged cold stress. Given that we could not detect the OsPIF4 protein after 24h of cold in continuous dark (Fig. 7d), the faster decrease (as compared to photoperiodic conditions) in *OsDREB1B* expression is likely to be due to other TFs.

Our results, together with the results by Kidokoro *et al.* (2009), suggest that *DREB1/CBF* genes may be negatively regulated by PIFs both in rice and in *Arabidopsis* under normal diurnal conditions. These results correlate with what was shown by Kim *et al.* (2002), who reported a red light activation of *DREB1/CBF* gene expression through the C/DRE element in *Arabidopsis,* dependent on phyB. However, results by Franklin and Whitelam (2007) showed that at 16ºC, but not at 22ºC, phytochrome signalling negatively regulates expression of *DREB1/CBF*, as well as of their down-stream targets (*COR* genes). These findings put together suggest that the phyB-PIF-DREB1/CBF signalling pathway may be differentially regulated at different temperatures. In our work we have

shown that the *OsPIF4* transcript is regulated by low temperature and, in addition, it has been reported that phyB protein levels are dependent on PIFs in *Arabidopsis* (Khanna *et al.*, 2007; Leivar *et al.*, 2008; Jang *et al.*, 2010). Thus, it may be that the regulation of PIFs by temperature has an effect in phytochrome levels, modulating their down-stream signalling at different temperatures.

Overall our data provide evidence that both light and cold-temperature signalling pathways converge on the *OsDREB1B* regulon of rice, likely via different mechanisms. Fig. 8 shows a scheme of the proposed model for the regulatory network identified in our work. The action of OsPIF4 may be regulated through its preferential interaction with the red light active form of OsphyB, which in turn can target OsPIF4 for degradation or, alternatively, sequester it and abrogate its function (as repressor of *OsDREB1B* gene expression). It will be important to determine whether OsPIF4-OsphyB interaction prevents OsPIF4 from binding *OsDREB1B* promoter. The OsPIF4 activity regulating *OsDREB1B* may be also modulated through alternative splicing of *OsPIF4* 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.

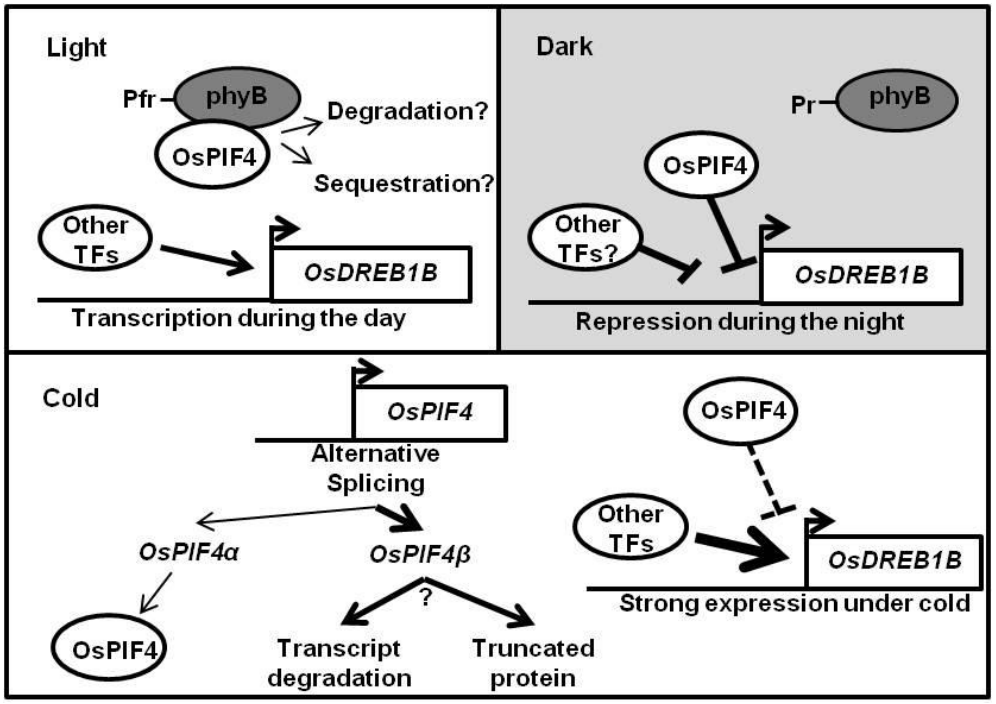

Figure 8. Proposed model for the regulation of *OsDREB1B* by OsPIF4 under light/dark and cold conditions.

Top panels represent regulation under different light conditions at optimal growth temperature. During the day OsPIF4 interacts with the Pfr form of OsphyB, leading to its degradation or sequestration. Therefore, the expression of *OsDREB1B* is not repressed by OsPIF4 and other TFs activate its expression. During the night time, OsPIF4 does not interact with the Pr-form of OsphyB, and is available to repress the transcription of *OsDREB1B*. Bottom panel represents the regulation of *OsDREB1B* expression under low temperature conditions. In these conditions the *OsPIF4* transcript is regulated by alternative splicing yielding *OsPIF4α* and *β*. The *OsPIF4β* transcript may be degraded or result in a truncated protein that does not have the bHLH domain, hence not being able to bind DNA. In this case, the transcription of *OsDREB1B* is highly induced through the action of other TF (activators) that are induced by low temperature. Thickness of the arrows reflects response intensity.

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Supplemental Materials

Supplemental Table 1 – Primers used for semi-quantitative RT-PCR

Gene	Primer Sequence 5'-3'
OsPIF4	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

Supplemental Table 2 – Primers used for quantitative RT-PCR

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

Chapter 5.

Final Conclusions and Future Perspectives

In today's world, with a fast-growing population, the demand for food, feed and fuel will substantially increase in the next few decades. Challenging these increasing needs, abiotic stress conditions account for massive losses in crop production worldwide, what results in reduced yield and higher prices. The development of novel crop varieties that are more tolerant to environmental stresses is therefore of paramount importance in the coming years. However, unlike improving resistance to biotic stress conditions, which is often dependent on a single gene, the tolerance to abiotic stresses is a multigenic trait. This makes plant improvement much more complex and time-consuming. Transcription Factors (TFs) have emerged as promising candidates for plant breeding, given that one TF alone can regulate the expression of many target genes. Nevertheless, our understanding of the transcriptional regulatory pathways is still scarce, especially in plants that are economically relevant. The main goal of the work presented here was to characterize the regulation of the rice gene coding for OsDREB1B, a protein belonging to the DREB1/CBF sub-family of TFs, described as key regulators of abiotic stress responses in plants. In order to identify TFs that bind to the promoter of *OsDREB1B,* we have used a Yeast One-Hybrid (Y1H) system to screen a cold-induced cDNA expression library. Thereby, eight TFs were identified as binding to that promoter (Chapter 2). Among these TFs there were seven Zn Finger proteins and one bHLH TF, which was predicted as a putative rice Phytochrome Interacting Factor (OsPIF4). In chapters 3 and 4, the characterization of the TFs identified was addressed.

Even though Zn Finger TFs had already been described as interplaying with DREB1/CBFs, our work is the first report on Zn Finger TFs as direct regulators of a *DREB1/CBF* gene. The identification of seven Zn Finger TFs as binding to the promoter of *OsDREB1B* suggests a prominent role of this type of TFs in the DREB1/CBF regulon in rice. It is also noteworthy that all these TFs identified as binding to the promoter of *OsDREB1B* are

repressors of gene expression. This might be due to the fact that the cDNA expression library, used for the Y1H screening, was prepared from plants stressed at 8ºC. As described in Chapter 2, we observed that at 10ºC the *OsDREB1B* transcript level reaches a peak at 1-2h after stress, declining after that. Since the cDNA library was prepared from rice seedlings collected after 2, 5 and 24h after the stress initiation, it should be enriched in TFs that are repressors of *OsDREB1B* transcription and not activators. For comparison, it would be interesting to screen a cold-induced cDNA expression library prepared from seedlings collected earlier during the cold stress, or even subjected to a harsher stress, such as 5ºC. This would probably allow the identification of TFs that activate the expression of *OsDREB1B*. Moreover, we observed that *OsDREB1B* is also induced in response to drought in roots, and to mechanical stress. The screening of cDNA libraries prepared from seedlings subjected to those stresses would also be interesting, since the TFs that regulate *OsDREB1B* gene expression may be different in these conditions.

Regarding the Zn Finger TFs that we identified as binding to the promoter of *OsDREB1B*, we observed that each gene coding for these TFs responded differently to different abiotic stress conditions. Moreover, the expression of some of these genes did not show much variation under stress. It is possible that the activity of those TFs may be modulated by other mechanisms, such as post-translational modifications. Interestingly, two of these proteins (ZHD1 and ZOS12-7) have a high probability of being SUMOylated (SUMOplot, Abgent). It will be important to confirm these bioinformatics predictions in an *in vitro* SUMOylation system, which is currently being developed in our group. If these are confirmed, it will also be interesting to understand what role SUMOylation plays in regulating the activity of these TFs. Nevertheless, we cannot rule out that the TFs we identified may be targets for other types of post-translational modifications, like ubiquitination or phosphorylation.

We have also observed the formation of homo- and hetero-dimers for some of the ZF-HD TFs, but not for the C2H2-type. The functional significance of these interactions is still to be determined. Transactivation assays using combinations of these TFs could help understand if the formation of dimers affects the transcription of *OsDREB1B*, or of other down-stream genes. It may be that dimerization influences the DNA-binding specificity of the proteins. It is also possible that it allows the integration of different stimuli, in case there is a differential expression of the genes coding for these TFs, under different environmental or developmental conditions.

In this work, we showed for the first time the interaction of a rice PIF with Phytochromes. OsPIF4 binds preferentially to the active form of the rice phytochrome B, as well as to the promoter of *OsDREB1B*, repressing its expression. This TF is therefore a promising candidate for regulating the light-dependent gene expression that we have observed for *OsDREB1B*. The analysis of a T-DNA insertion line (close to the *OsDREB1B* 5´UTR) is currently underway. Only further studies will allow drawing robust conclusions on the possible link between the light and OsDREB1 regulon in rice.

Our gene expression studies showed an alternative splice form for *OsPIF4* that is triggered by cold. Moreover, we also identified alternative splice forms for *Arabidopsis* PIF encoding genes, which means that this might be a common mechanism for the regulation of *PIF* transcripts in different plant species. The biological significance of these alternative splicing events is mostly unknown and should be addressed in the future. It is interesting to note that we could not detect a protein corresponding to the alternative *OsPIF4β* transcript. Future work should therefore focus on trying to understand whether *PIF* alternative splicing forms code for proteins, functional or not, and if so, test their putative role in the light signalling pathway. If these transcripts do not code for proteins, it will also be

interesting to understand whether they have a functional role or if they are only a way to target *PIF* transcripts for degradation.

Similarly to what was previously observed for *DREB1/CBFs* in *Arabidopsis*, we observed that the rice gene *OsDREB1B* shows a circadian rhythm in light/dark photoperiodic conditions. The biological significance of the light regulation and circadian rhythm observed for genes coding for *DREB1/CBFs* is yet to be fully understood. It may be that an increased expression of *DREB1/CBFs* during the day results in an increased amount of DREB1/CBF-targets during the night, which is when temperatures are lower. Interestingly, it has also been shown that *DREB1/CBFs* are differentially expressed during some developmental stages in *Arabidopsis*. Again, the question remains on whether that increased expression occurs in stages when plants are more sensitive to low temperatures, or if DREB1/CBFs have other roles, such as in plant development. The cross-talk between the DREB1/CBF regulon and gibberellin signalling has already been described in *Arabidopsis*. It may be that these TFs play a role in plant growth even in normal, non-stressful, conditions.

The role of the TFs we have identified as regulators of *OsDREB1B* in abiotic stress signalling is yet to be explored. The analysis of mutant and/or over-expressing rice lines will provide more insight into the biological function of these proteins. Several rice mutant lines for the genes that encode these TFs are currently under study in our group. This will allow a better understanding of the function of these TFs in abiotic stress signalling in rice. Interestingly, preliminary analyses are showing that a mutation in the gene coding for ZOS11-10, which is mainly expressed in roots under cold conditions, has an impact in root development. Therefore, the functional studies of these TFs might also shed some light on the cross-talk between abiotic stress and other signalling pathways, some possibly involved in plant development.

Our work provides clues on the transcriptional regulation of *DREB1/CBFs* by novel TFs. Nevertheless, there is a growing knowledge of other mechanisms that regulate transcription, such as DNA and histone modifications. These epigenetic factors can be responsible for the modulation of gene expression and can therefore be responsible, at least in part, for some of the changes observed in gene transcript levels. In our group there is on-going work focused on the characterization of possible epigenetic factors involved in the regulation of *OsDREB1B* gene expression. To have an integrative view and a comprehensive understanding of transcription regulation, it is essential to combine our knowledge regarding the DNA-binding TFs and the possible involvement of epigenetic mechanisms.

Even though there is a growing understanding of the molecular mechanisms governing plant responses to abiotic stress conditions, many intervening players are yet to be identified and many pathways remain undiscovered. Moreover, most of what is known is in the model species *Arabidopsis*, which does not have social or economical relevance. While fundamental research is certainly required, the study of plant species that are relevant to society, such as food crops, should be intensified.

With this work we were able to gain new insights into the regulation of the rice gene *OsDREB1B*. We have identified and characterized eight novel TFs that bind to its promoter and are involved in the abiotic stress signalling pathway. The further functional characterization of these TFs will prove useful, not only to better understand the stress signalling pathways in plants, but also to provide new possible candidate genes for plant improvement in the future.

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