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ABSTRACT

STUDIES ON COLD RESISTANCE IN PALMS: ANALYSIS OF *CBF*-LIKE GENES

By Li Lu

Cold resistance of plants is an important characteristic that restricts plant distribution, growth and biomass productivity. Cold acclimation is a phenomenon that occurs in some plants as an increase in cold tolerance upon exposure to low, non-freezing temperatures. The *CBF/DREB1* gene family is a small group of transcription factors that play key roles in cold acclimation. The *CBF/DREB1* genes were first identified from the model plant *Arabidopsis*, then from many economically important plant species including grape, tomato, corn, rice, and barley. Overexpression of *CBF/DREB1* genes induces multiple components of cold acclimation and thus increases plant cold tolerance even without cold stimulus. To systematically study the cold resistance mechanism of palms, *CBF/DREB1* orthologs were isolated from various palms, including both cold-resistant and cold-sensitive species. This is the first report of *CBF/DREB1* genes in the Palmae family. Analyses of DNA and putative protein sequences confirmed that the palm CBF/DREB1 has similar structures with known CBF proteins. To further investigate the functions of the palm *CBF/DREB1* genes, the expression patterns of two *Rhapidophyllum hystrix CBF* orthologs, *RhCBF1* and *RhCBF2*, were analyzed with reverse transcription and real-time PCR. The expressions of the two *RhCBFs* were constitutive yet cold-inducible, which provided further evidence of the involvement of these genes in cold acclimation response of palms. The two *RhCBF* genes were transformed into *Arabidopsis*. The transgenic *Arabidopsis* plants showed increased cold tolerance, typical growth retardation of *CBF*-overexpessing plants, and other multiple *CBF*-related cellular changes. The results indicated that *RhCBFs* had functional similarity with *Arabidopsis CBF* genes also.

STUDIES ON COLD RESISTANCE IN PALMS:

ANALYSIS OF *CBF*-LIKE GENES

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DEDICATION

I would like to dedicate this work to my husband and my parents.

ACKNOWLEDGMENTS

There are a number of people giving me tremendous help to my Ph. D study and research at Miami University. I would like to thank my advisors, Dr. Kenneth Wilson and Dr. David Francko, for their help and patience during all these years. I would also thank my committee members, Dr. Susan Barnum, Dr. Nancy Smith-Huerta and Dr. Richard Lee, for their support and guidance. I also owe thanks to Dr. Quinn Li and all his lab members, who gave me very valuable support and suggestions during the research; Mr. Chris Wood for technical assistance; and Maria Lia Molas for helpful discussion. Many colleagues have contributed to my research and I am grateful to every one of them. Finally I want to give special thanks to my parents and my husband for their endless love and support.

CHAPTER 1

INTRODUCTION

Cold stress, cold-induced injuries and cold acclimation of plants

Cold is a major environmental limitation to plant distribution and crop productivity. Sudden frosts can cause large areas of crop damage and the loss of millions of dollars. This has encouraged physiological, structural, and biochemical investigations of cold-induced injuries in plants as well as plant responses to cold stress. In most plants and tissues, freezing-induced injuries result largely from the severe cellular dehydration that occurs upon ice formation (Thomashow 1999). The cellular membrane systems are the primary site of freeze-induced injury and multiple forms of membrane damage can occur as consequence of freeze-induced cellular dehydration (Thomashow 1999). Thus the mechanisms plants use to respond to cold-stress overlap with responses to dehydration and other abiotic or biotic stress (Shinozaki and Yamaguchi-Shinozaki 2000). For example, dehydrins, which may stabilize macromolecules and act as cytoprotectants, are synthesized by cells in response to low temperature, ABA (abscisic acid), or any environmental influence that has a dehydration component, such as drought, salinity, or extracellular freezing (Yordanov et al. 2000)

Cold acclimation is a process whereby some plants increase in cold tolerance upon pre-exposure to low, non-freezing temperature (Thomashow 1999). There are numerous reports of cold acclimation because of its significance in plant biology and agriculture. These reports suggest that cold acclimation is a very complex process that requires many changes in cellular metabolism. These changes include reduction or cessation of growth, reduction of tissue water content, transient increase in ABA levels, changes in membrane lipid composition, accumulation of compatible osmolytes such as proline, betaine, and soluble sugars, as well as increased levels of antioxidants (reviewed by Xin and Browse 2000). Over a thousand genes are involved in these processes in *Arabidopsis* (Kreps et al. 2002). The products of these genes include sucrose phosphate synthase (Guy et al. 1992), alcohol dehydrogenase (Jarillo et al. 1993), dehydrin and dehydrin-like proteins (Gilmour et al. 1992), chitinase-like proteins (Hon et al. 1994), etc. These proteins or their enzymatic products can adjust the osmotic potential of cytoplasm and stabilize the membrane, thus acting as cytoprotectants under freezing conditions. However, there are many other cold-up-regulated genes whose sub-cellular functions

have not been well defined, and none of these cold-related genes can induce full cold acclimation alone, although each of them has some cell-protective functions under cold stress. The small contributions of these genes to cold tolerance pushed scientists to identify the cold-responsive signal transduction pathway (cold STP) and possible regulator genes. Manipulation of the STP could be a powerful method of modifying cold tolerance in plants.

The functions of *CBF* gene family and CBF regulon in cold acclimation

Researchers have identified multiple signal pathways involved in cold acclimation (Xin and Browse 2000; Yang et al. 2005; Zhang et al. 2004a). In one of the pathways, the *CBF/DREB1* genes play a key role in regulating the expression of other cold-related genes and the degree of cold acclimation response (Figure 1). The *CBF/DREB1* genes were first detected in *Arabidopsis* (Gilmour et al. 1998; Liu et al. 1998), then in a number of plant taxa including barley (Skinner et al. 2005), Canola (Jaglo et al. 2001), tomato (Zhang et al. 2004b), rice (Dubouzet et al. 2003), sour cherry, and strawberry (Owens et al. 2002). CBF/DREB1 stands for C-repeat (CRT)-binding factor/dehydration-responsive-element (DRE) binding protein, while CRT/DRE (core sequence: CCGAC) is a *cis*-acting element presenting in promoters of multiple cold-regulated (*COR*) genes (Baker et al. 1994; Jiang et al. 1996). In *Arabidopsis*, there are six *CBF/DREB1* members (Haake et al. 2002) designated as "*AtCBFn*" composing a small gene family. The most studied ones are *AtCBF1/DREB1B*, *AtCBF3/DREB1A*, and *AtCBF2/DREB1C*, which will be referred to by their *CBF* names hereafter.

The CBF proteins contain a conserved AP2 (APETALA2)/ERF (ethylene-responsive element-binding factor) domain which recognizes and binds to the CRT/DRE element, thus regulating the expression of downstream genes (reviewed by Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006). There are over 140 AP2-containing transcriptional factors encoded in the *Arabidopsis* genome. The CBF proteins are distinguished from other members of the AP2 superfamily by the presence of two small conserved CBF signature motifs directly flanking the AP2 domain (Skinner et al. 2005; Van Buskirk and Thomashow 2006). The basic CBF protein structure contains the following major features from amino to carboxy terminal: a variable leader sequence which might contain a nuclear localization signal, the first CBF signature motif, the AP2

DNA-binding domain, the second CBF signature motif, and an acidic C-terminal domain which might have transcriptional activation function (Skinner et al. 2005). Among CBF proteins from different plant taxa, the AP2 domain and the AP2-flanking signature motifs are the main regions that are extensively conserved at the sequence level.

In *Arabidopsis*, the *CBF* gene transcripts appear within 15 min after exposure to low temperature, followed by the increase of transcripts of CRT/DRE-containing target genes at about 2h (Van Buskirk and Thomashow 2006). Constitutive overexpression of *AtCBF1*, *AtCBF2* and *AtCBF3* in transgenic *Arabidopsis* plants leads to significant increases in freezing, drought and salt tolerance (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). These transgenic plants show enhanced expression of multiple *COR* genes and elevated levels of various cryoprotectants such as proline and soluble sugars under warm-temperature growth conditions (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). The target genes that respond to both CBF overexpression and low temperature stimulus have been identified by microarray experiments and defined as a transcription unit "CBF regulon" (Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006). At present, more than 100 genes have been assigned to the CBF regulon, encoding proteins with a wide range of functions such as transcription factors, signal transduction pathway components, biosynthetic proteins, cryoprotectant proteins and other stress-related proteins (Van Buskirk and Thomashow 2006). Thus, the CBF transcription factors and CBF regulon are involved in multiple components of the cold acclimation process. There have been several reports of transformation of *Arabidopsis CBFs* into economically important crops, such as tomato (Hsieh et al. 2002), wheat (Pellegrineschi et al. 2004), and rice (Oh et al. 2005) to increase resistance to low temperature and other abiotic-stresses.

Unexpected cold resistance in palms and Miami University's Cold Hardy Palm Project

Palms (Palmae Juss.) are a large plant family with more than 2000 species in 200 genera and are generally regarded as second only to the grasses (Poaceae Barnhart) in economic importance (Jones 1996). The family includes several well-known members like coconut palm (*Cocos nucifera* L.), oil palm (*Elaeis gunineensis* Jacq. and *Elaeis*

oleifera Kunth), and date palm (*Phoenix dactylifera* L.). Many other species are important ornamentals with great economic value.

As most of the palm species are tropical or subtropical in their distribution, it is easy to assume that they are sensitive to low temperatures. In fact, about 100 palm species can survive freezing down to -7ºC and have been grown for decades in warm-temperate landscapes world-wide (Francko 2004; Gibbons and Spanner 1999). A few species, including *Rhapidophyllum hystrix* Pursh (needle palm), *Trachycarpus fortunei* Hook (Chinese windmill palm), *Sabal palmetto* Walter (cabbage palmetto) and *Sabal minor* Jacq. (dwarf palmetto), are extremely cold resistant by withstanding temperatures below -17.7ºC (0ºF; reviewed by Francko 2003; 2004). *Rhapidophyllum hystrix*, an U.S. native and clearly the most cold tolerant of all palm species, resists foliar damage down to ca. -22ºC and reliably survives short exposure to -30ºC in cultivation once established (Francko 2004).

In contrast, many truly tropical palms, such as the popular ornamental species *Ravenea rivularis* Jum. & Perrier (majesty palm) and *Dypsis lutescens* H. Werdl. (areca palm), are damaged by chilling temperature (4ºC) and killed outright at 0ºC (reviewed by Riffle 1998). At present, most of the research on palm cold resistance is performed by horticulturists and palm enthusiasts, and mainly focuses on the survival of individual plants. Due to the lack of systematic investigation, the physiological and genetic bases for the great difference in cold resistance among palm taxa remain unknown.

In summer 1998 our lab group began a field and laboratory study on cold-hardy palms at Miami University in Oxford, OH (39º 30′ north, 50 km northwest of Cincinnati). The program was aimed at scientific evaluation and public demonstration of available cultivars of warm-climate plants, especially palms, under the extant Zone 6 conditions of southwest Ohio, and the development and commercialization of "next generation" cultivars with even better cold hardiness characteristics (Francko and Wilson 2001). As a part of the project, we sought to isolate key genes functioning in palm cold resistance. The possible *CBF/DREB1* orthologs in palms were selected as one of the main research targets because of their demonstrated involvements in plant cold acclimation and cold resistance.

Chapter 2 of this dissertation presents the identification of *CBF* orthologs from both cold-hardy and cold-sensitive palms, including *R. hystrix*, *S. minor*, *S. palmetto*, *T. fortunei*, *D. lutescens*, *R. rivularis*, *C. nucifera*, *E. guineensis*, *E. olecifera* and *Attalea bassleriana* (Lu et al., submitted). The gene and putative protein sequences of identified palm *CBFs* were analyzed. The palm CBFs showed sequence and structural similarities with known CBF proteins, especially the characteristic AP2 DNA-binding domain and the flanking CBF signature motifs. In addition, the transcript levels of two *R. hystrix CBF* orthologs, *RhCBF1* and *RhCBF2*, were increased greatly after cold stimulus, further indicating that these genes might be involved in the cold acclimation response of palms.

In Chapter 3, functions of the two *RhCBF* genes were analyzed by transforming the palm genes into *Arabidopsis*. The transgenic *Arabidopsis* plants showed increased cold tolerance, accumulated proline and sugars, up-regulated expression levels of *COR* genes, and typical growth retardation of *CBF*-overexpressing plants. The results indicated that *RhCBFs* also had functional similarity with *Arabidopsis CBF* genes.

Chapter 4 summarizes the research on palm *CBF genes* and discusses their likely roles in palm cold resistance. The future directions of study and application of palm *CBFs* are also discussed.

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Figure 1. Transcriptional regulatory network of the genes involved in low temperature and dehydration response (adapted from Yang et al. 2005; Zhang et al. 2004a). ABA-dependent transcriptional factors are shaded in black, while ABA-independent factors are not. Small circles indicate post-transcriptional modification, such as phosphorylation. Transcription factor binding sites are represented as gray rectangles at the bottom of the figure, with the representative binding site-containing promoters listed below. Dotted lines indicate possible regulation. Double arrow lines indicate possible crosstalk. The main components of CBF-regulated cold-responding pathway are marked by red ovals.

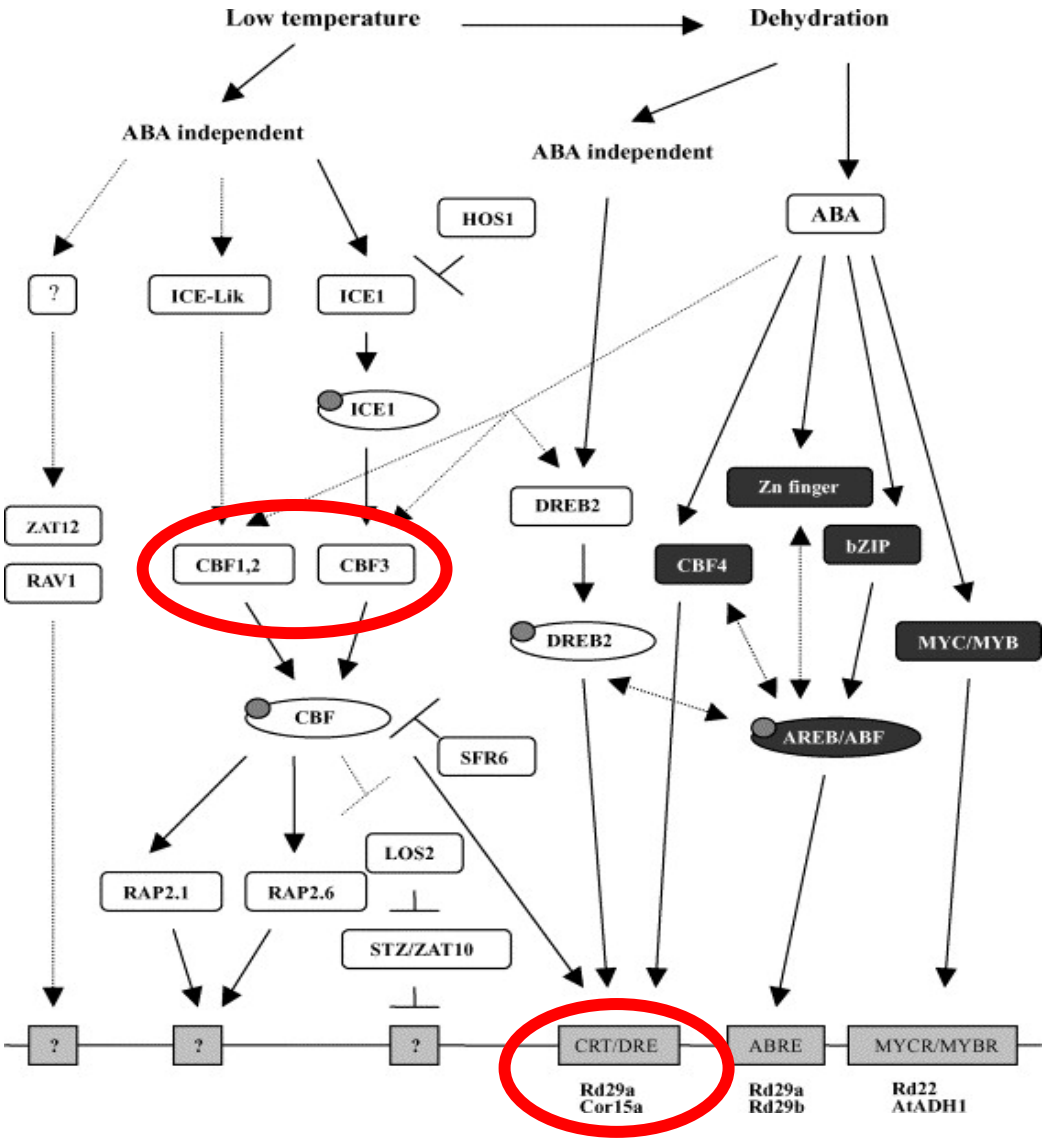
Low temperature
Dehydration
ABA independent
ABA independent
HOS1
ABA
?
ICE-Lik
ICE1
ICE1
DREB2
Zn finger
ZAT12
RAV1
CBF1,2
CBF3
CBF4
bZIP
DREB2
MYC/MYB
CBF
SFR6
AREB/ABF
LOS2
RAP2.1
RAP2.6
STZ/ZAT10
?
?
?
CRT/DRE
ABRE
MYCR/MYBR
Rd29a
Cor15a
Rd29a
Rd29b
Rd22
AtADH1

Figure 1

CHAPTER 2

IDENTIFICATION OF *CBF/DREB1* HOMOLOGS FROM BOTH COLD-HARDY AND COLD-SENSITIVE PALMS

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ABSTRACT

Cold acclimation is a phenomenon that occurs in some plants as an increase in cold tolerance upon exposure to low, non-freezing temperatures. The *CBF/DREB1* gene family is a small group of transcription factors that play key roles in cold acclimation. *CBF/DREB1* genes have been identified from many plant taxa. Here we report the first isolation of *CBF/DREB1* orthologs from various palms, including both cold-resistant and cold-sensitive species. Structural, phylogenetic, and expression pattern analyses indicated the involvement of these genes in cold acclimation response of palms.

Key words: *CBF/DREB1*; cold acclimation; palm.

INTRODUCTION

Cold is a major environmental limitation to plant distribution and crop productivity. Sudden frosts can cause large areas of crop damage and loss of millions of dollars. Cold acclimation is a process whereby some plants increase in cold tolerance upon pre-exposure to low, non-freezing temperature (Thomashow 1999). There are numerous reports on cold acclimation because of its significance in plant biology and agriculture. These reports suggest that cold acclimation is a very complex process and requires many changes in cellular metabolism. These changes include reduction or cessation of growth, reduction of tissue water content, transient increase in ABA levels, changes in membrane lipid composition, accumulation of compatible osmolytes such as proline, betaine, and soluble sugars, as well as increased levels of antioxidants (reviewed by Xin and Browse 2000). Over a thousand genes are involved in these processes in *Arabidopsis* (Kreps et al. 2002). Among these genes, *CBF/DREB1* transcription factors and CBF-targeted genes (the CBF-regulon) play a prominent role (Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006).

CBF/DREB1 stands for C-repeat (CRT)-binding factor/dehydration-responsive-element (DRE) binding protein, while CRT/DRE (core sequence: CCGAC) is a *cis*-acting element presenting in promoters of multiple cold-regulated (*COR*) genes (Baker et al. 1994; Jiang et al. 1996). In *Arabidopsis*, there are six *CBF/DREB1* members (Haake et al. 2002) designated as "*AtCBFn*" composing a small gene family. The most studied ones are *AtCBF1/DREB1B*, *AtCBF3/DREB1A*, and *AtCBF2/DREB1C*, which will be referred to by their *CBF* names hereafter. A conserved AP2 (APETALA2)/ERF (ethylene-responsive element-binding factor) domain in CBF/DREB1 proteins recognizes and binds to the CRT/DRE element, thus regulating the expression of downstream genes. The CBF/DREB1 proteins are distinguished from other AP2-containing proteins by the presence of two small conserved CBF signature motifs directly flanking the AP2 domain (Skinner et al. 2005). The basic CBF protein structure contains the following major features from amino to carboxy terminal: a variable leader sequence about 15 – 40 amino acids, the first CBF signature motif, the AP2 DNA-binding domain, the second CBF signature motif, and an acidic C-terminal domain which might have transcriptional activation function (Skinner et al. 2005).

In *Arabidopsis*, overexpression of *AtCBF1*, *AtCBF2* and *AtCBF3* leads to significant increases in freezing, drought and salt tolerance (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999). Since cold stress pathways overlap with other abiotic stress pathways (Shinozaki and Yamaguchi-Shinozaki 2000), it is not surprising that the transgenic plants overexpressing *AtCBFs* are more resistant to these different stresses. These transgenic plants showed enhanced expression of multiple *COR* genes and elevated levels of various cryoprotectants such as proline and soluble sugars without a low-temperature stimulus. There have been several reports of transformation of *Arabidopsis CBFs* into economically important crops, such as tomato (Hsieh et al. 2002), wheat (Pellegrineschi et al. 2004), and rice (Oh et al. 2005) to increase abiotic-stress resistance.

As *CBF* genes are key regulators of plant cold acclimation and abiotic-stress resistance, there has been tremendous interest in identifying all members of *CBF* family, and searching for orthologs of them in different species. Six *CBFs* in *Arabidopsis* (Haake et al. 2002) and twenty in barley designated as "*HvCBFn*" (Skinner et al. 2005) have been reported with different expression patterns and functions. In *Arabidopsis*, *AtCBF4* has been reported to be inducible by drought and abscisic acid instead of cold treatment (Haake et al. 2002). A more recent study also showed that *AtCBF2* might work as a regulator of *AtCBF1* and *AtCBF3* expression (Novillo et al. 2004) and have more complicated functions in plant abiotic-stress resistance than previously assumed. At the same time, orthologs of *CBF* have been reported from various plant species, including *Brassica napus* (Jaglo et al. 2001), tomato (Zhang et al. 2004), rice (Dubouzet et al. 2003), sour cherry, and strawberry (Owens et al. 2002). Interestingly, this expanding list of *CBF*-containing plants is not just restricted to cold-acclimating or cold-tolerant plants. Thus more detailed studies of the CBF regulon in different plant taxa are needed to explain the great range of plant cold resistance. Here we report the identification of *CBF* homologs from members of the Palm family.

Palms (Palmae Juss.) are a diverse and complex family with more than 2000 species in 200 genera and are generally regarded as second only to the grasses (Poaceae Barnhart) in economic importance (Jones 1996). The family includes several well-known members like coconut palm (*Cocos nucifera* L.), oil palm (*Elaeis gunineensis* Jacq. and

Elaeis oleifera Kunth), and date palm (*Phoenix dactylifera* L.). Many other species are important ornamentals with great economic value.

A common misconception is that palms as a group are intolerant of cold weather. In fact, although most of the palm species are tropical or subtropical in their distribution, perhaps about 100 species can survive freezing down to -7ºC and have been grown for decades in warm-temperate landscapes world-wide (Francko 2004; Gibbons and Spanner 1999). A few species of extremely cold-hardy palms, *Rhapidophyllum hystrix* Pursh (needle palm), *Trachycarpus fortunei* Hook (Chinese windmill palm), *Sabal palmetto* Walter (cabbage palmetto) and *Sabal minor* Jacq. (dwarf palmetto), are capable of surviving temperatures below -17.7ºC (0ºF; reviewed by Francko 2003; 2004). *Rhapidophyllum hystrix*, an U.S. native and clearly the most cold tolerant of all palm species, resists foliar damage down to ca. -22ºC and reliably survives short exposure to -30ºC in cultivation once established (Francko 2004).

In contrast, many truly tropical palms, such as the popular ornamental species *Ravenea rivularis* Jum. & Perrier (majesty palm) and *Dypsis lutescens* H. Werdl. (areca palm), are damaged by chilling temperature (4ºC) and killed outright at 0ºC (reviewed by Riffle 1998). Due to the lack of systematic investigation, the physiological and genetic bases for the great difference in cold resistance among palm taxa are unknown.

In summer 1998 our lab group began a field and laboratory study on cold-hardy palms at Miami University in Oxford, OH (39º 30′ north, 50 km northwest of Cincinnati). The program is aimed at scientific evaluation and public demonstration of available cultivars of warm-climate plants, especially palms, under the extant Zone 6 conditions of southwest Ohio (Francko and Wilson 2001). As a part of the project, we sought to isolate key genes functioning in palm cold resistance. Here, we describe identification of *CBF* orthologs from both cold-hardy and cold-sensitive palms, analyses of their structural similarities and differences with other reported *CBFs*, and determination of their expression patterns following cold stimulation in *Rhapidophyllum hystrix*.

MATERIALS AND METHODS

Plant materials and treatments

Needle palm (*Rhapidophyllum hystrix*) plants 0.5~1 m tall were grown in a growth chamber maintained at 26°C, 60 to 80% relative humidity, and a 18h photoperiod from cool-white fluorescent lights (80-100 μmol $m^{-2}s^{-1}$). To induce expression of cold-related genes in *R. hystrix*, plants were transferred to a chamber maintained at 4°C, 20-30μmol $m^{-2}s^{-1}$ light intensity for 24h.

Other plants used in the study, including *Sabal minor, Sabal palmetto, Trachycarpus fortunei*, *Dypsis lutescens,* and *Ravenea rivularis* were maintained in a greenhouse. Leaf samples of *Attalea bassleriana* Burret Zona*, Cocos nucifera, Elaeis guineensis,* and *Elaeis olecifera* were kindly provided by Fairchild Tropical Botanic Garden (Coral Gables, Florida).

Isolation of palm *CBFs*

Genomic DNA isolations were conducted with DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol, while total RNA isolations were performed with RNeasy Plant Mini Kit (Qiagen). The conserved AP2 domain of *CBF* genes, ~200bp in size, was first amplified by PCR from genomic DNA of *R. hystrix* and sequenced with degenerated primers 5′-AAG TTY CRY GAG ACK CGK CAC C-3′ (forward primer) and 5′-AVG CSG AGT CRG CGA ART TGA G-3′ (reverse primer). All primers used in the experiments were ordered from IDT INC (Coralville, Iowa, USA), and the standard mixbase definitions were: Y= (CT), R=(AG), K=(GT), S=(CG), M= (AC), W= (AT), and V=(ACG).

Based on the sequence of the AP2 domain, gene-specific primers were designed and used to identify full-length cDNAs of *CBF* genes. Total RNA of *R. hystrix* was isolated after 3h of 4°C treatment with RNeasy Plant Mini Kit (Qiagen) and mRNA was purified from total RNA with Oligotex mRNA Mini Kit (Qiagen) according to the manufacturer's instructions. Full-length cDNA was obtained with a GeneRacer Kit (Invitrogen, Carlsbad, California, USA), which ensured that only transcripts containing full-length cDNA ends were amplified. The 3′ end of *Rh-CBF* cDNA was amplified and sequenced using forward gene-specific primer 5′-GAG CCC AAC AAG AAG TCG AGG ATT TG-3′ and GeneRacer 3′ primer (5′-GCT GTC AAC GAT ACG CTA CGT

AAC G-3′, Invitrogen). The 5′ end of *Rh-CBF* cDNA was amplified using reverse gene-specific primer 5′- CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC-3′**,** GeneRacer 5′ primer (5′-CGA CTG GAG CAC GAG GAC ACT GA-3′, Invitrogen) and 5′-nested primers (5′-GGA CAC TGA CAT GGA CTG AAG GAG TA-3′, Invitrogen). The full-length gene sequences were determined by recombining the overlapping cDNA fragments obtained as described above and confirmed by direct RT-PCR and/or PCR with cDNA/genomic DNA of *R. hystrix*. Based on the obtained *R. hystrix CBF* sequences, various primers and nested primers were designed to PCR amplify and sequence *CBF* genes from genomic DNA of nine other palms, including *S. minor, S. palmetto, T. fortunei*, *D. lutescens, R. rivularis, A. bassleriana, C. nucifera, E. guineensis,* and *E. olecifera.* The sequences of isolated palm *CBFs* were deposited in GenBank. The particular accession numbers and related primer information are listed in Table 1.

Structural and phylogenetic analyses with known monocot CBFs

The theoretical pI (isoelectric point) and Mw (molecular weight) of putative CBF proteins were calculated by ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). The region from the first amino acid after the AP2 domain and the second CBF signature motif to the last amino acid was used to calculate the pI of C-terminal domain. The identity and similarity percentage between proteins was analyzed with MacVector 6.5. Available monocot CBF protein sequences were aligned with palm CBFs by ClustalX. The *CBF*s used in the alignment and their GenBank Accession numbers are as following: *HvCBF1* (AY785837); *HvCBF2A* (AY785841); *HvCBF2B* (DQ097684); *HvCBF3* (AY785845); *HvCBF4A* (AY785849); *HvCBF4B* (AY785850); *HvCBF4D* (AY785852); *HvCBF5* (AY785855); *HvCBF6* (AY785860); *HvCBF7* (AY785864); *HvCBF8A* (AY785868); *HvCBF9* (AY785878); *HvCBF10A* (AY785882); *HvCBF10B* (AY785885); *HvCBF11* (AY785890); *HvCBF12* (DQ095157); *HvCBF13* (DQ095158); *HvCBF14* (DQ095159); *OsDREB1A* (AF300970); *OsDREB1B.1* (AY785894); *OsDREB1C* (AP001168); *OsDREB1D* (AY785895); *OsDREB1E* (AY785896); *OsDREB1F* (AY785897); *OsDREB1G* (AP005775); *OsDREB1I* (AP004632); *OsDREB1J* (AP004632); *SbCBF5* (AY785898); *SbCBF6* (AY785899); *ScCBF22* (AF370730); *ScCBF24* (AF370729); *ScCBF31* (AF370728); *TaCBF1* (AF376176); *TaCBF2* (AY785900); *TaCBF5* (AY785902); *TaCBF6*

(AY785903); *TmCBF7* (AY785904); *TaCBF9* (AY785905); *TaCBF11* (AY785906); *TaCBF14* (AY785901); *ZmCBF2* (AF450481). For pseudogene *HvCBF8A*, a theoretical polypeptide sequence was generated based on frame shifts in the sequence (Skinner et al. 2005) and used in the analysis. Phylogenetic analysis was performed with PAUP *4.0b10 using OsDREB2A (AF300971), a closely related monocot AP2-containing protein without the flanking CBF signature motif as the outlier (Skinner et al. 2005). Parsimony analysis was based on 10,000 bootstrap replicates with uninformative characters excluded.

Gene expression analysis with real-time RT-PCR

About 300 mg (fresh weight) of leaf tissue (the youngest, newly expanded leaf) from *R. hystrix* was collected and immediately frozen in liquid nitrogen with 0, 1, 2, 4, 6,10, and 24h of 4°C cold treatment. Total RNA was then isolated with RNeasy Plant Mini Kit (Qiagen) followed the manufacturer's protocol. Genomic DNA contamination was removed from total RNA with DNase I (Qiagen) digestion, cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) using 2~5μg of total RNA. Real-time PCR was performed on Rotor-Gene RG3000 (Corbett Research, Sydney, Australia) using QuantiTect SYBR Green PCR Kit (Qiagen).

The primers for real-time PCR were designed with PrimerQuest Tools of IDT INC (Coralville, Iowa, USA) (http://scitools.idtdna.com/Primerquest/) with some modifications to decrease primer-dimers while obtaining uniform PCR annealing temperature. For *RhCBF1*, the following primers: 5′- TAT ACG CGG ACG AGG AGG TG-3′ (forward primer) and 5′- TTC CTC TTA CGT TGT ACA GGT ACC GC -3′ (reverse primer) were used to amplify a 125bp fragment which had 66bp of 3′-untranslated region. For *RhCBF2*, the following primers: 5′- GCC GAT GAG GAA GAA AGC GAT G -3′ (forward primer) and 5′- ACT CCG GCC CCC ATG ACT AAC C-3′ (reverse primer) were used to amplify an 115bp fragment which had 61bp of 3′-untranslated region.

The eukaryotic translation initiation factor *eIF4A* was used as a house-keeping gene for cold treatment (Haake et al. 2002). Partial *eIF4A* sequence was amplified from genomic DNA of *R. hystrix* and sequenced with primers: 5′- GGA TCC TTG TGA AGC GAG ATG AGC-3′ (forward primer) and 5′- GGT CAA GCA ACA TTA GAT GGG AGC TC-3′ (reverse primer), which were designed based on an available *Elaeis oleifera*

eIF4A fragment (GenBank accession number: AY040227). A fragment ~510bp was amplified and sequenced. The fragment was confirmed to be part of *eIF4A* gene by comparison with *E. oleifera eIF4A*. Based upon the sequence of this *R. hystrix eIF4A* fragment, the following primers: 5′- CTG CCA ACC CAA CCA GAG AAC -3′ (forward primer) and 5′- TGG GAG CTC CTC AAT CAC CAC AT -3′ (reverse primer) were designed to amplify a 153bp fragment during real-time PCR analysis.

The PCR program was set to the following conditions: activation of *Taq* at 95°C for 15 min, 40 cycles of amplification generally, denaturing at 95°C for 10s, annealing at 56ºC for 15s, and extension at 72ºC for 25s. Data were analyzed using Rotor Gene 6.0 software supplied by Corbett Research and quantification of *CBF* transcripts was performed using the "Comparative Quantitation" function of the software (Sinisterra et al. 2005; Warton et al. 2004). Briefly, for each PCR amplification, a "Second Derivative" peak was plotted indicating the maximum rate of fluorescence increase in the reaction. "Takeoff" point, which indicated the end of noise and the transition into exponential phase, was defined as the cycle at which the Second Derivative was at 20% of the maximum level. The average fluorescence increase of four points following the "Takeoff" was calculated as the amplification efficiency of each individual reaction. The "Average Amplification" of all samples was then calculated and the variance was used to provide a measure of error. The relative concentration of a specific gene to a house-keeping gene was determined as Average Amplification ^ (HousekeepingTakeoff — SpecificGeneTakeoff) (Sinisterra et al. 2005; Warton et al. 2004). All real-time experiments were conducted with at least three biological and technical replicates. Melting curve analysis, agarose gel electrophoresis and sequencing were used to verify specific PCR product formation.

RESULTS

Identification of *CBF* genes from a variety of palm species

Two full-length *CBF* homologs, *RhCBF1* (GenBank accession no. DQ497740 and DQ497742) and *RhCBF2* (DQ497741 and DQ497743), were isolated from *R. hystrix*, the most cold-hardy extant palm species (Francko 2004). The length of *RhCBF1* cDNA is ~960bp with a coding region of 636bp. The *RhCBF2* cDNA is ~1100bp with a coding region of 630bp. Both cDNAs have ~80bp of 5′-untranslated region, while *RhCBF2* has a much longer 3′-untranslated region. Like all reported *CBF* genes, *RhCBF1* and *RhCBF2* contain no intron (Skinner et al. 2005). Their putative proteins are very similar to one another with identical AP2 domains (Figure 1) ranging from aa 48 to aa 106. Other CBF protein structure characteristics, such as the leader sequence, the AP2-flanking *CBF* signature motifs, the acidic C-terminal domain, and the conserved LWSY motif near the protein C-terminal end, are also presented in both genes (Figure 1).

Putative *CBF* homologs were isolated from genomic DNAs of nine other palms (Table 1), including *S. minor, S. palmetto, T. fortunei*, *D. lutescens, R. rivularis, A. bassleriana, C. nucifera, E. guineensis,* and *E. olecifera.*. None of these palm *CBF* genes contain an intron. Based on the sequence data and alignment of their putative protein sequences, all of them contain the conserved AP2 domain and are very similar to known CBFs (Figure 2; Table 2). Among palm CBFs, the identity percentage is at least 80%. Between palm CBFs, AtCBFs and HvCBFs, the identities are generally 30% to 56%, while the conserved amino acids are mostly in the AP2 domain.

The palm CBFs are in the HvCBF1-subgroup of monocot CBFs

An earlier study by Skinner et al. (2005) divided known monocot CBF proteins into three major phylogenetic subgroups: HvCBF1-, HvCBF3-, and HvCBF4A-subgroups, each after a representative barley CBF. The isolated palm CBFs clustered with the HvCBF1-subgroup (Figure 3), which contained three distinct barley gene families: HvCBF5, HvCBF7 and HvCBF1/HvCBF11 families (Skinner et al. 2005). The palm CBFs were in the same clade as HvCBF1/HvCBF11 family although the phylogenetic support for this was not strong based on bootstrap (Figure 3). Additional support for grouping palm CBFs and HvCBF1 into one clade is that several blocks of amino acid in the acidic C-terminal domain are conserved specifically among them but

not with HvCBF3 or HvCBF4A (See Figure 1). All palm CBFs fall into two subgroups with each group containing one RhCBF (Fig. 3).

Both *RhCBFs* express constitutively and respond to cold stress

Expression patterns of the two *R. hystrix CBFs* were analyzed with Reverse Transcription and real-time PCR. In all experimental replicates, both genes showed low but detectable expression under warm, pretreatment control conditions. The expression of both genes increased greatly after exposed to cold (4ºC) temperature (Figure 4). Induction became obvious after two hours of cold treatment and peaked at around 10h. After 24h of cold acclimation, the expression levels of *RhCBF1* and *RhCBF2* were still at a relatively high level (Figure 4). During most of the cold treatment period, *RhCBF1* maintained a higher expression level than *RhCBF2* and responded to cold stimulus earlier (at 2h treatment), but it showed a much sharper decrease after the 10h expression peak (Fig. 4).

DISCUSSION

After *CBF/DREB1* genes were first identified in *Arabidopsis* (Liu et al. 1998; Stockinger et al. 1997), many *CBF* orthologs have been identified from different kinds of angiosperms (Dubouzet et al. 2003; Jaglo et al. 2001; Owens et al. 2002; Skinner et al. 2005). In this paper we report the identification of *CBF* genes from palms, a large and important monocot family. A total of twelve *CBF* genes were isolated from ten palm species belonging to four tribes and eight genera. These palms include some of the most cold-hardy species like *R. hystrix*, which is capable of surviving -30ºC freeze events, and some very cold-sensitive species like *D. lutescens*, which is severely damaged by near 0ºC chilling temperatures.

All putative palm CBF proteins share the general primary domain structures of known CBFs, including a leader which might contain nuclear localization signal, an AP2 DNA-binding domain and the flanking CBF signature motifs, and an acidic C-terminal domain which might be important for trans-activation (Skinner et al. 2005). The AP2 domain and the flanking CBF signature motifs are the only regions that are extensively conserved at the sequence level between palm CBFs and the CBF proteins of other plant taxa (Fig. 1). Although RhCBFs and HvCBF1 share several conserved blocks in the C-terminal region (Fig.1), the possible role of these 4~5 aa blocks is unclear. Among the identified palm CBFs, the overall protein sequences are highly conserved regardless of the cold resistance of the palm species (Fig.2). For both protein and nucleotide (data not shown) sequence analysis, the identity percentage is at least 80%. Some genes, like *RhCBF2* and *EgCBF2*, have identical proteins and 99% identity at nucleotide level. This level of conservation is remarkable regarding that *R. hystrix* and *E. guineensis* belong to two different subfamilies (Coryphoideae and Arecoideae, respectively) and differ greatly in sensitivity to cold.

Based on the size of *Arabidopsis* and barley *CBF* families (Haake et al. 2002; Skinner et al. 2005; Zhao et al. 2006), it is likely that there are more *CBF* genes yet to be discovered in the palm genome. In support of this view, we have sequenced a *CBF*-like cDNA fragment with a much shorter C-terminal region from *R. hystrix* (Lu et al., unpublished).

We analyzed the expression patterns of *RhCBF1* and *RhCBF2* with reverse transcription and real-time PCR to further investigate their roles in cold stress. Both genes were constitutively expressed at low levels under warm, control conditions. Melting curve analysis, agarose gel electrophoresis, and sequencing were used to confirm specific PCR amplification. Although phylogenetic analyses put *RhCBFs* and *HvCBF1* into one clade, the expression patterns of *RhCBFs* were unlike the quickly-induced and temporal expression of *HvCBF1* (Skinner et al. 2005) and most of the reported dicot *CBFs* (Liu et al. 1998; Owens et al. 2002; Zhao et al. 2006), which accumulated transcripts as early as 15 min after cold stimulus and reached peak expression before 4h of treatment. For *RhCBFs*, the induction was clearly evident after 2~ 4 h treatments and reached maximum after about 10h (Fig. 4). The expression pattern of *RhCBFs* is more similar with that of HvCBF4-subgroup of barley *CBFs* (Skinner et al. 2005).

The above data may have ecophysiological ramifications for cold resistance in palms. Our field data showed that for many cold-hardy palms, summer foliage was already very cold-resistant before the late fall/early winter natural cold acclimation could occur (Francko and Wilson 2004), suggesting that at least a portion of these plants' ability to withstand freezing may be constitutive. In all species tested, the foliage became more cold-hardy after plants were exposed to the first frost of the season. The constitutive yet cold-responsive expression patterns of the two *RhCBFs* we report here might explain part of the cold-resistance capacity of cold-hardy palms and further confirm their ability to cold acclimate. A low level of *CBF* transcripts and proteins might maintain levels of downstream gene transcripts sufficient to provide relatively high cold (or other abiotic stress) resistance without affecting normal plant growth. In this model, after low temperature inducement, the transcript levels of transcription factors and downstream genes would increase, conferring enhanced cold tolerance on the plant as a whole.

In support of this model, we transformed the two *RhCBFs* into *Arabidopsis* with a double 35S promoter. The transformants showed increased cold resistance along with increased sugar and proline levels compared with non-transformed wildtype *Arabidopsis* (Lu et al., in prep.). The results support the involvement of *RhCBF1* and *RhCBF2* in cold-responding pathways although there is no previous report of palm CRT/DRE containing genes being identified.

If *RhCBF1* and *RhCBF2* can be implicated in cold resistance promotion, then what of the *CBF* like genes we isolated from non-cold-hardy palms? One explanation is that the CBF regulon might be incomplete in some plants (Zhang et al. 2004). While the *CBF* genes are conserved, some of the CBF-regulated genes necessary for full cold resistance might be absent. Another possibility is that some members of *CBF* family might have special functions in cold acclimation despite their similar structures and common involvement in stress resistance. Several reports showed that some *CBF* members might function in feedback regulation of other *CBFs* (Novillo et al. 2004) or be trans-inactive, thus blocking the expression of downstream target genes (Zhao et al. 2006). These *CBF* members might play even more important roles in the regulation and feedback network because of their non-redundant functions. Divergence of these genes might have a large impact on plant stress resistance.

Clearly, much more research is required to clarify the details of CBF system in palms and the contribution of them to the divergent plant cold resistance. A reasonable next step might be analyses the expression patterns of *CBFs* of cold-sensitive palms.

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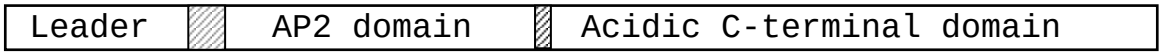
Table 1. Summary of palm *CBF* genes cloned from palm DNA and RNA, the primers used to amplify the genes, and properties of predicted proteins.

GenBank accession numbers	Gene name	Species	Nucleotide length	Primers used in PCR cloning (F: forward, R: reverse)	Predicted protein length	Total protein pI	Acidic C-terminal domain pI
DQ497730	*SpCBF*	*Sabal palmetto*	656bp	F: TCC ACR AGA RAS CSG CAA TGG AGA RCT TCA G R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	210	5.00	3.81
DQ497731	*SmCBF*	*Sabal minor*	656bp	F: TCC ACR AGA RAS CSG CAA TGG AGA RCT TCA G R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	210	5.00	3.81
DQ497732	*TfCBF*	*Trachycarpus fortunei*	686bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	211	5.21	3.92
DQ497733	*RrCBF*	*Ravenea rivularis*	939bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CAC GCA TGC AAA CTG ATT GC	210	5.35	3.99
DQ497734	*EoCBF*	*Elaeis oleifera*	700bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	212	5.34	3.94
DQ497735	*EgCBF2*	*Elaeis guineensis*	1060bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: ACT GTT TAT TGT TAC TGT CAC AGA ATC TAG ATA G	209	5.01	3.88
DQ497736	*EgCBF1*	*Elaeis guineensis*	700bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	212	5.34	3.94
DQ497737	*AbCBF*	*Attalea bassleriana*	1060bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: ACT GTT TAT TGT TAC TGT CAC AGA ATC TAG ATA G	209	5.01	3.88
DQ497738	*DlCBF*	*Dypsis lutescens*	985bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CAC GCA TGC AAA CTG ATT GC	212	5.37	4.07
DQ497739	*CnCBF*	*Cocos nucifera*	697bp	F: GCC CAA MTY CWA RCT CGA ACR MTT CAC R: CCA GCT TCA AAT GGA GTA GCT CCA YAG YGA GAC	211	5.10	3.92
DQ497740	*RhCBF1*	*Rhapidophyllum hystrix*	922bp	F: AAA CAC ACA GTC ACT ACC ACT GC R: CAC GCA TGC AAA CTG ATT GC	211	5.21	3.94
DQ497741	*RhCBF2*	*Rhapidophyllum hystrix*	1088bp	F: GAA AAA CCT CCC AAC GGT CAC TAC R: ACT GTT TAT TGT TAC TGT CAC AGA ATC TAG ATA G	209	5.01	3.88
DQ497742	*RhCBF1 mRNA*	*Rhapidophyllum hystrix*	957bp		211		
DQ497743	*RhCBF2 mRNA*	*Rhapidophyllum hystrix*	1098bp		209		

Note: For the degenerate primers used, the standard mixbase definitions were: M= (AC), W= (AT), Y= (CT), R=(AG), K=(GT), S=(CG), and V=(ACG).

Figure 1. Structure and Sequence analyses of the palm *CBF/DREB* proteins. (A). A diagram of the general domain structure of CBF protein. The leader, AP2 DNA-binding domain, and the acidic C-terminal domain are marked. The two gray boxes are AP2 - flanking CBF signature motif. (B). Alignment of protein sequences of *R. hystrix RhCBF1* (GenBank accession no. DQ497740), *RhCBF2* (DQ497741) with barley *HvCBF1* (AY785837), *HvCBF3* (AY785845), *HvCBF4* (AY785849), *Arabidopsis AtCBF1* (U77378), *AtCBF2* (AF074601) and *AtCBF3* (AF074602). Identical residues are indicated by asterisks, conserved and semi-conserved residues are indicated by colons and dots, respectively. The AP2 domain is underlined. The flanking CBF signature motifs are highlighted with gray background. The LWSY motif near the C-terminal is highlighted with black background. Three amino acid blocks shared between RhCBFs and HvCBF1 are designated by boxes.

(A).



(B).

```
RhCBF1          MEN---FSDYS--MDSPLAQRSASDE--EAYATVSSAPPKRRAGRTKFRETRHPVYKGVR 53
RhCBF2          MES---FSSDS--LDSPVARQSASDE--ETYATVWSVPPKRRAGRTKFRETRHPVYKGVR 53
HvCBF1          MDVGALSSDYSSGTPSPVGADGGNSEGFSTYMTVSSAPPKRRAGRTKFKETRHPVYKGVR 60
AtCBF1          MNS---FSAFSEMFGSDYEP---QGG--DYCPTLATSCPKKPAGRKKFRETRHPIYRGVR 52
AtCBF2          MNS---FSAFSEMFGSDYESPVSSGG--DYSPKLATSCPKKPAGRKKFRETRHPIYRGVR 55
AtCBF3          MNS---FSAFSEMFGSDYESSVSSGG--DYIPTLASSCPKKPAGRKKFRETRHPIYRGVR 55
HvCBF3          MDMG-----LEVSSSSPSSSPVSSSPE---HAARRASPAKRPAGRTKFRETRHPVYRGVR 52
HvCBF4          MDV--------ADIASPSGQ--QKQQG---HRTVSSEPPKRPAGRTKFHETRHPLYRGVR 47
                *:            *       .          :  .*: ***.**:*****:*:***

RhCBF1          RR-NADKWVCEVREPNKK-SRIWLGTFPTAEMAARAHDVAAMALRGR-SACLNFADSAWL 109
RhCBF2          RR-NADKWVCEVREPNKK-SRIWLGTFPTAEMAARAHDVAAMALRGR-SACLNFADSAWL 109
HvCBF1          RR-NPGRWVCEVREPHSK-QRIWLGTFETAEMAARAHDVAALALRGR-AACLNFADSPRR 116
AtCBF1          QR-NSGKWVSEVREPNKK-TRIWLGTFQTAEMAARAHDVAALALRGR-SACLNFADSAWR 108
AtCBF2          QR-NSGKWVCELREPNKK-TRIWLGTFQTAEMAARAHDVAAIALRGR-SACLNFADSAWR 111
AtCBF3          RR-NSGKWVCEVREPNKK-TRIWLGTFQTAEMAARAHDVAALALRGR-SACLNFADSAWR 111
HvCBF3          RRGNTERWVCEVRVPGKRGARLWLGTYATAEVAARANDAAMLALGGRSAACLNFADSAWL 111
HvCBF4          RRGRVGQWVCEVRVPGIKGSRLWLGTFTNPEMAARAHDAAVLALSGR-AACLNFADSAWR 106
                :*  .  :**.*:* *  :  *:****: .:*:****:*.* :** ** :********.
                ____________________________AP2 domain_____________________.

RhCBF1          ----CPVPGS----SNPKDIQRAAVDAAEAFRPQ-------TERVDAAESREDAAMAMIA 155
RhCBF2          ----CPVPSS----SNPKDIQKAAVLAAEAFRPR-------TES-DATESREDAAMAMSA 154
HvCBF1          ----LRVPAV---GASPDEIRRAAVEAAEAFLPA-------PDQSNAPAEEVAAAPTMQF 163
AtCBF1          ----LRIPES----TCAKDIQKAAAEAALAFQDE-------TCDTTTTDHGLDMEETMVE 154
AtCBF2          ----LRIPES----TCAKEIQKAAAEAALNFQDE-------MCHMTTDAHGLDMEETLVE 157
AtCBF3          ----LRIPES----TCAKDIQKAAAEAALAFQDE-------MCDATT-DHGFDMEETLVE 156
HvCBF3          ----LAVPS--AL-SDLADVRRAAVEAVADFQRREAADGSLAIAVPKEASSGAPSLSPSS 165
HvCBF4          MRPVLATTGSFGF-SSTREIKLAVAVAVVAFQQQ-------QIILPVACPSPEAPASPSA 158
                       .      :   ::: *.. *.  *                        :

RhCBF1          RPSLA-AADGPFYMEDGLNFG-----------------MQGYLD-MAAGMLMEPP-PIY 194
RhCBF2          GPSVA-ADD-PFFTEDRLDFG-----------------MQGYLD-MAEGLLIDPP-PMN 192
HvCBF1          ------AGDPYYGMDDGMDFG-----------------MQGYLD-MAQGMLIAPP-PLV 197
AtCBF1          AIYTPEQSEGAFYMDEETMFG-----------------MPTLLDNMAEGMLLPPP-SVQ 195
AtCBF2          AIYTPEQSQDAFYMDEEAMLG-----------------MSSLLDNMAEGMLLPSP-SVQ 198
AtCBF3          AIYTAEQSENAFYMHDEAMFE-----------------MPSLLANMAEGMLLPLP-SVQ 197
HvCBF3          GSDSAGSTGTSEPSANGEFEGPVVMDSEMFRLDLFPEMDLGSYYMSLAEALLMDPPPTAT 225
HvCBF4          ALFYISSGDLLELDEEQWFGG---------------MDAGSYYASLAQGMLVAPPDERA 202
                               :                                :* .:*:  *

RhCBF1          -----ADEEVSDGDVSLWSYSI-- 211
RhCBF2          -----ADEEESDGGVSLWSYSI-- 209
HvCBF1          GPSATAGDGDDDGEVSLWSY---- 217
AtCBF1          WNHNYDGEGDGD--VSLWSY---- 213
AtCBF2          WNYNFDVEGDDD--VSLWSY---- 216
AtCBF3          WNHNHEVDGDDDD-VSLWSY---- 216
HvCBF3          IIHAYEDNGDGGADVRLWSYSVDM 249
HvCBF4          RPENREHSG-VETPIPLWSYLFDC 225
                       .      : ****
```

Figure 1

Figure 2. Alignment of all putative CBF protein sequences from palms. Identical residues are indicated by asterisks, conserved and semi-conserved residues are indicated by colons and dots, respectively. The AP2 domain, the flanking CBF signature motifs and LWSY motif are designated as in Fig.1.

```
SmCBF          MESFSDCSMDSPLAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
SpCBF          MESFSDCSMDSPLAQRSASDEEVYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
TfCBF          MVNFSDYSMDSPLAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
RhCBF1         MENFSDYSMDSPLAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
CnCBF          MESFSSDSMDTPLAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
RrCBF          MESFSSDSMDTPLAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
DlCBF          MESFSSDSMDSPFAQRSASDEEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
EoCBF          MESFSSDSMDSPLASRWASDGEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
EgCBF1         MESFSSDSMDSPLASRWASDGEAYATVSSAPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
EgCBF2         MESFSSDSLDSPVARQSASDEETYATVWSVPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
AbCBF          MESFSSDSLDSPVARQSASDEETYATVWSVPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
RhCBF2         MESFSSDSLDSPVARQSASDEETYATVWSVPPKRRAGRTKFRETRHPVYKGVRRRNADKW 60
               * .**. *:*:*.* : *** *.**** *.****************************

SmCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPRSSSPK 120
SpCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPRSSSPK 120
TfCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPRSSNSK 120
RhCBF1         VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPGSSNPK 120
CnCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWLCPVPRSSNSK 120
RrCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAIALRGRSACLNFADSAWLCPVPRSSSPK 120
DlCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCSVPRSTSPK 120
EoCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSPWLCPVPRSSNPK 120
EgCBF1         VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSPWLCPVPRSSNPK 120
EgCBF2         VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPSSSNPK 120
AbCBF          VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPSSSNPK 120
RhCBF2         VCEVREPNKKSRIWLGTFPTAEMAARAHDVAAMALRGRSACLNFADSAWLCPVPSSSNPK 120
               ********************************:**************.***.** *:..*
                                 AP2 domain

SmCBF          DIQRAAMEAAEAFRPQTES-DAAES-REDAAMAMIAGPSLAATDDPFSMEDGLNFGMQG- 177
SpCBF          DIQRAAMEAAEAFRPQTES-DAAES-REDAAMAMIAGPSLAATDDPFSMEDGLNFGMQG- 177
TfCBF          DIQRAAVEAAEAFRPQTERVDAAES-REDAAMAMIAGPSLAAADDPFYMEDGLNFGMQG- 178
RhCBF1         DIQRAAVDAAEAFRPQTERVDAAES-REDAAMAMIARPSLAAADGPFYMEDGLNFGMQG- 178
CnCBF          DIQRAAVEAAEAFRPQTERVDAAES-REDAAMAMIAGPSLAAADDPFYMEDGLNSGMQG- 178
RrCBF          DIQRAAMEAAEGFRPQTEP-DAAKS-REDATMAMAAGPSLAAADDPFYVEDGLNFGMQG- 177
DlCBF          DIQRAAMEAAEAFRPRTEP-DAAKSTREDAGTSMAAGPSLASADDPFYMEDGLNFGMQG- 178
EoCBF          DIQRAAMEAAEAFRPQTEP-NADKS-REDAAMSMAGGPSLATADDPFYMEDGLNFGMQGY 178
EgCBF1         DIQRAAMEAAEAFRPQTEP-NADKS-REDAAMSMAGGPSLATADDPFYMEDGLNFGMQGY 178
EgCBF2         DIQKAAVLAAEAFRPRTES-DATES-REDAAMAMSAGPSVA-ADDPFFTEDRLDFGMQG- 176
AbCBF          DIQKAAVLAAEAFRPRTES-DATES-REDAAMAMSAGPSVA-ADDPFFTEDRLDFGMQG- 176
RhCBF2         DIQKAAVLAAEAFRPRTES-DATES-REDAAMAMSAGPSVA-ADDPFFTEDRLDFGMQG- 176
               ***:**: ***.***:**  :* :* ****  :* . **:* :*.**  ** *: ****

SmCBF          YLDMAAGLLMEPPPI-YADEEVSDGDVSLWSYSI 210
SpCBF          YLDMAAGLLMEPPPI-YADEEVSDGDVSLWSYSI 210
TfCBF          YLDMASGMLMEPPPI-YADEEVSDGDVSLWSYSI 211
RhCBF1         YLDMAAGMLMEPPPI-YADEEVSDGDVSLWSYSI 211
CnCBF          YLDMASGMLMEPPPI-YADEEVSDGDVSLWSYSI 211
RrCBF          YLDMAAGLLMEPPPT-YADEEVSGSDFSLWSYSM 210
DlCBF          YLDMAEGLLMEPPPILYPDEEVSGGDVSLWSYSI 212
EoCBF          YLDMAAGLLMEPPPILYADEEVSDGDVSLWSYSI 212
EgCBF1         YLDMAAGLLMEPPPILYADEEVSDGDVSLWSYSI 212
EgCBF2         YLDMAEGLLIDPPPM-NADEEESDGGVSLWSYSI 209
AbCBF          YLDMAEGLLIDPPPM-NADEEESDGGVSLWSYSI 209
RhCBF2         YLDMAEGLLIDPPPM-NADEEESDGGVSLWSYSI 209
               ***** *:*::***   .*** *....******:
```

Figure 2

Table 2. Analysis of amino acid identities and similarities of putative CBF proteins from palms, *Arabidopsis* and barley.

	RhCBF1	**RhCBF2** (AbCBF, EgCBF2)	**SpCBF** (SmCBF)	**TfCBF**	**RrCBF**	**EoCBF** (EgCBF1)	**ClCBF**	**CnCBF**	**AtCBF1**	**AtCBF2**	**AtCBF3**	**HvCBF1**	**HvCBF3**	**HvCBF4A**
RhCBF1		87	92	96	90	89	89	95	63	59	61	65	51	51
RhCBF2 (AbCBF, EgCBF2)	81		89	87	89	87	88	88	60	58	58	64	51	50
SpCBF (SmCBF)	91	84		93	92	90	91	93	63	60	61	65	52	51
TfCBF	96	81	92		90	89	89	96	63	60	61	65	50	52
RrCBF	87	81	90	87		92	93	92	63	60	59	64	53	51
EoCBF (EgCBF1)	87	81	89	87	89		92	91	62	59	59	66	50	50
ClCBF	85	81	88	86	90	91		90	63	59	60	65	52	52
CnCBF	94	81	91	96	90	88	86		63	61	61	65	51	52
AtCBF1	49	49	49	49	48	49	51	49		90	92	59	48	52
AtCBF2	45	46	46	46	45	46	47	47	86		91	58	49	51
AtCBF3	49	49	49	50	47	49	50	49	87	87		59	47	51
HvCBF1	56	55	56	56	53	56	55	56	45	44	46		47	47
HvCBF3	36	37	37	36	37	36	38	36	34	34	35	35		49
HvCBF4A	37	36	37	38	36	36	37	37	37	36	37	39	36	

Note: Certain palm CBFs are grouped together because they are identical or nearly identical. EoCBF and EgCBF1 are identical; AbCBF, RhCBF2, and EgCBF2 are identical; SpCBF and SmCBF are nearly identical (1 out of 210 aa is different).

Figure 3. Phylogenic analysis of monocot CBFs using PAUP *4.0b10 Parsimony Analysis. HvCBFn : *Hordeum vulgare* CBF proteins; OsDREB1n: *Oryza sativa* CBF/DREB1 proteins; TaCBFn: *Triticum asetivum* CBF proteins; ScCBFn: *Secale cereale* CBF proteins; ZmCBFn: *Zea mays* CBF proteins. OsDREB2A is an AP2-containing non-CBF protein used as outlier. Vertical bars indicate the defined subgroups of monocot CBFs from Skinner and co-workers' research (2005) with the representative members highlighted. The palm CBF members are designated with a box.

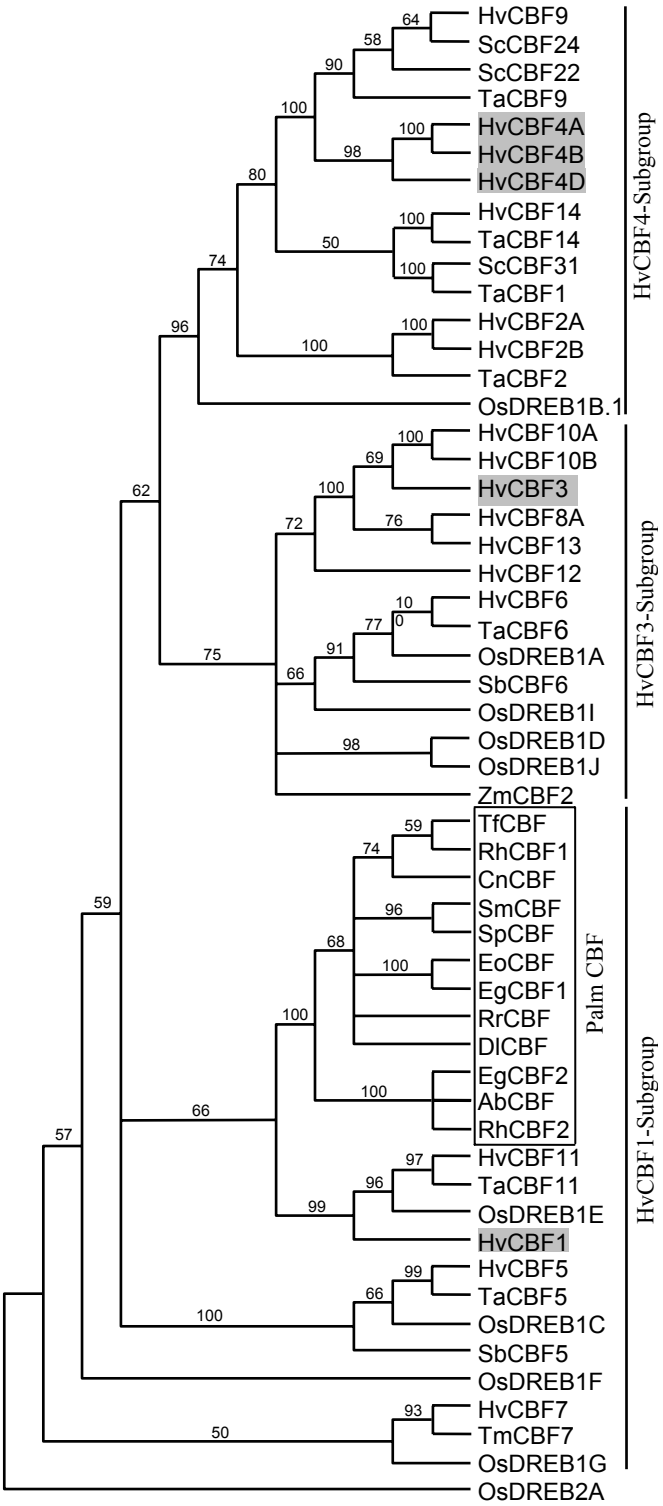

HvCBF9
ScCBF24
ScCBF22
TaCBF9
HvCBF4A
HvCBF4B
HvCBF4D
HvCBF14
TaCBF14
ScCBF31
TaCBF1
HvCBF2A
HvCBF2B
TaCBF2
OsDREB1B.1
HvCBF4-Subgroup
HvCBF10A
HvCBF10B
HvCBF3
HvCBF8A
HvCBF13
HvCBF12
HvCBF6
TaCBF6
OsDREB1A
SbCBF6
OsDREB1I
OsDREB1D
OsDREB1J
ZmCBF2
HvCBF3-Subgroup
TfCBF
RhCBF1
CnCBF
SmCBF
SpCBF
EoCBF
EgCBF1
RrCBF
DlCBF
EgCBF2
AbCBF
RhCBF2
Palm CBF
HvCBF11
TaCBF11
OsDREB1E
HvCBF1
HvCBF5
TaCBF5
OsDREB1C
SbCBF5
OsDREB1F
HvCBF7
TmCBF7
OsDREB1G
HvCBF1-Subgroup
OsDREB2A

Figure 3

Figure 4. Real-time PCR analysis of expression levels of *RhCBF1* and *RhCBF2* in responding to cold treatment using *eIF4A* as internal control. Black bars: *RhCBF1*; dashed bars: *RhCBF2*. The X axis stands for the time of cold (4°C) treatment, 0h stands for control, warm (26°C) condition. The relative expression level of *RhCBF1* to *eIF4A* after 4h of treatment was set as 1.0 (marked by *), all other expression values were normalized as the fold-change relative to this value.

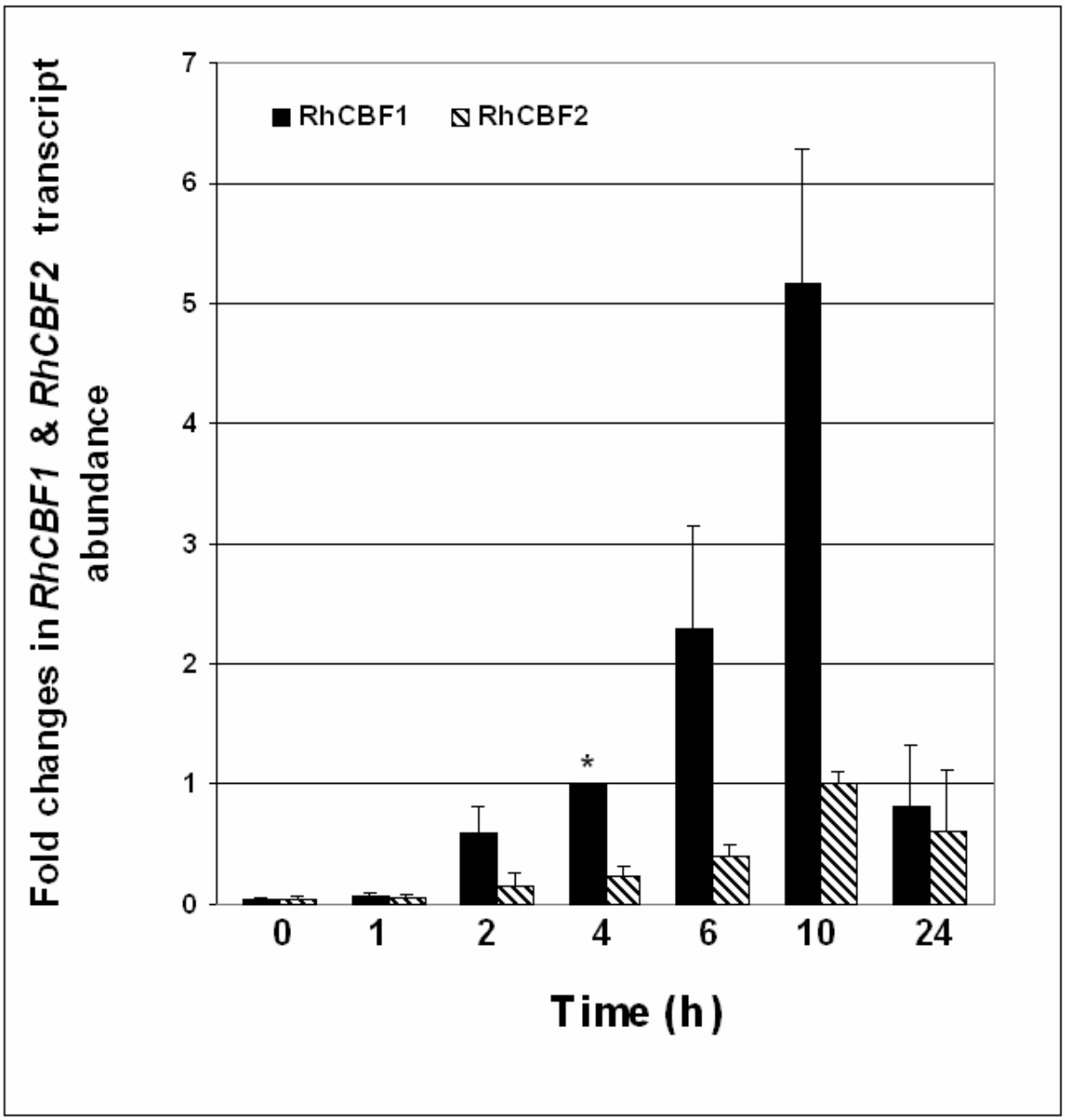

Fold changes in RhCBF1 & RhCBF2 transcript abundance
RhCBF1
RhCBF2
7
6
5
4
3
2
1
0
*
0
1
2
4
6
10
24
Time (h)

Figure 4

CHAPTER 3

IMPROVING COLD RESISTANCE IN *ARABIDOPSIS* BY EXPRESSION OF *CBF* GENES FROM *RHAPIDOPHYLLUM HYSTRIX*

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ABSTRACT

Cold acclimation is a phenomenon that occurs in some plants as an increase in cold tolerance upon exposure to low, non-freezing temperatures. The *CBF/DREB1* gene family is a small group of transcription factors that play key roles in cold acclimation. Overexpression of *CBF/DREB1* genes has been demonstrated to induce multiple components of cold acclimation thus increase plant cold tolerance even without cold stimulus. We previously reported the first isolation of *CBF/DREB1* orthologs from various palms. To further analyze the functions of these palm *CBFs*, two *Rhapidophyllum hystrix CBF* orthologs, *RhCBF1* and *RhCBF2*, were transformed into *Arabidopsis*. The transgenic plants showed increased cold tolerance, typical growth retardation of *CBF*-overexpressing plants, and other multiple *CBF*-related cellular changes. The results indicate that *RhCBFs* have functional similarity to *Arabidopsis CBF* genes.

Key words: *CBF/DREB1*; cold acclimation; cold resistance; *COR*; palm.

INTRODUCTION

Plants encounter a broad range of environmental stresses such as cold, drought and high salinity stress during their life cycles. To better survive possible freezing temperatures, many plants including the model plant *Arabidopsis*, display cold acclimation, which is defined as an increase in cold tolerance after pre-exposure to low, non-freezing temperatures (Thomashow 1999). In *Arabidopsis* ecotype Columbia, the increase in plant freezing tolerance is demonstrated by the decrease of an initial 50% lethal temperature (LT_{50} value) of -4.5ºC to an LT_{50} value of -6.8ºC after 24h of 4ºC cold acclimation (Gilmour et al. 1988). An LT_{50} value of about -9ºC is achieved after more than 10 days cold acclimation (Gilmour et al. 1988). Plant cold acclimation capacity accounts for an important part of the overall cold resistance capacity, and understanding the mechanism of cold acclimation is significant to plant biology and agriculture.

Cold acclimation is a very complex process and requires many changes in cellular metabolism. These changes include reduction or cessation of growth, reduction of tissue water content, transient increase in ABA levels, changes in membrane lipid and protein composition, accumulation of compatible osmolytes such as proline, betaine, and soluble sugars, as well as increased levels of antioxidants (Xin and Browse 2000). Cold acclimation is also associated with changes in gene expression. The transcription levels of large number of genes increase greatly within a few hours of low temperature stimulus and remain elevated during cold acclimation (reviewed by Van Buskirk and Thomashow 2006). These genes are given a variety of names including *COR* (cold-regulated), *LTI* (low-temperature induced), *KIN* (cold-induced), *RD* (responsive to dehydration) and *ERD* (early responsive to dehydration) genes (Van Buskirk and Thomashow 2006) as some of them also respond to dehydration and salt stresses. The expression regulation of these cold-related genes is an important part of the cold acclimation process.

Many of these *COR* genes contain one or more CRT (C-repeat) /DRE (dehydration responsive element) elements in their promoters. The CRT/DRE (core sequence: CCGAC) is a *cis*-acting element that functions in ABA (abscisic acid) - independent gene expression pathways in response to multiple abiotic stresses (Nakashima and Yamaguchi-Shinozaki 2006). Transcription factors binding to the CRT/DRE element have been identified and termed as CBF (CRT/DRE-binding factor;

Gilmour et al. 1998; Stockinger et al. 1997) or DREB1 (DRE-binding protein; Liu et al. 1998). The CBF/DREB1 transcription factors belong to the AP2 (APETALA2)/ERF (ethylene-responsive element-binding factor) family. A conserved AP2 DNA-binding domain in CBF/DREB1 proteins recognizes and binds to the CRT/DRE element, thus regulating the expression of downstream genes. In *Arabidopsis*, there are six *AtCBF/DREB1* members (Haake et al. 2002) composing a small gene family. The most studies ones are: *AtCBF1/DREB1B*, *AtCBF3/DREB1A*, and *AtCBF2/DREB1C*, which will be referred to by their "*AtCBFn*" names hereafter.

Overexpression of *AtCBF1, AtCBF2* and *AtCBF3* leads to significant increases in freezing, drought and salt tolerance (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Liu et al. 1998). The transgenic plants showed multiple changes that are associated with cold acclimation, including enhanced expression of multiple *COR* genes and elevated levels of various cryoprotectants such as proline and soluble sugars (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Liu et al. 1998). The representative *COR* genes that are up-regulated by CBF transcription factors, including *COR6.6/KIN2*, *COR15A*, *COR47/RD17* and *COR78*, encode highly hydrophilic, "boiling soluble" polypeptides which are thought to protect membrane structures under freeze-induced dehydration conditions (reviewed by Thomashow 1999). The polypeptide of *COR15A* is targeted to the stromal compartment of the chloroplasts and functions in decreasing the incidence of deleterious freeze-induced lamellar-to-hexagonal II phase transitions in lipid bilayers (Artus et al. 1996; Steponkus et al. 1998). Overexpression of *COR15A* gene increases the freeze tolerance of both chloroplasts and protoplasts (Artus et al. 1996).

Free proline and soluble sugars are well-documented cryoprotectants during freezing stress in many plants, including *Arabidopsis* (Xin and Browse 2000). During cold acclimation, sugar levels accumulate and correlate with development of freezing tolerance (Wanner and Junttila 1999). The *sfr4* (sensitive to freezing 4) mutant of *Arabidopsis* does not accumulate sucrose and glucose at cold stimulus and is impaired in cold acclimation ability (McKown et al. 1996). In contrast, the *esk1* (eskimo1) mutant constitutively maintains high level of soluble sugars at warm temperatures and is freezing-tolerant (Xin and Browse 1998). The functions of sugars in freezing tolerance

may include stabilizing proteins and biomembrane structures, adjusting osmotic pressure, depressing the freezing point, and preventing excessive dehydration (Xin and Browse 2000). Overexpression of *AtCBF1, AtCBF2* and *AtCBF3* in *Arabidopsis* are reported to increase levels of several sugars such as glucose, fructose, sucrose and raffinose (Gilmour et al. 2004; Gilmour et al. 2000) although the detailed functions of *AtCBF* genes in regulating sugar metabolism are still under investigation.

Accumulation of proline is positively correlated with the levels of freezing tolerance in wheat (Döerffling et al. 1997) and in *Arabidopsis* (Nanjo et al. 1999) although it may not be one of the first responses in cold acclimation (Wanner and Junttila 1999). The freezing-tolerant *esk1* mutant also accumulates high levels of proline at warm temperatures (Xin and Browse 1998). Accumulation of proline is observed in *AtCBF1, 2, 3* -overexpressing *Arabidopsis*, while the levels in non-acclimated transformants approximate those in cold-acclimated control plants (Gilmour et al. 2004; Gilmour et al. 2000). The transcript level of *P5CS2* (Δ^1-pyrroline-5-cgarboxylate synthase) gene, which encodes P5CS, a key enzyme in proline biosynthesis, is also elevated in *AtCBF*-overexpressing plants before and after cold acclimation (Gilmour et al. 2000).

Collectively, the research suggests that *CBF* gene family regulates the activation of multiple components of cold acclimation process. The *CBF* genes, along with the genes that are regulated by both low temperature and CBF-overexpression, are termed as "CBF regulon" (Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006). The CBF regulon constitutes an important part of plant cold acclimation response and in turn cold tolerance.

As *CBF* genes are key regulators of plant cold acclimation and abiotic-stress resistance, there has been tremendous interest in identifying all members of CBF family and searching for orthologs of them in different species. Orthologs of *CBF* have been reported from various plant species, including *Brassica napus* (Jaglo et al. 2001), barley (Skinner et al. 2005), tomato (Zhang et al. 2004), rice (Dubouzet et al. 2003), sour cherry, and strawberry (Owens et al. 2002). Our research team previously identified *CBF* homologs from members of the Palmae family (Lu et al., submitted). All putative proteins of palm *CBF* homologs conserve the general primary domain structures of known CBF proteins, including a leader which might contain a nuclear localization signal,

an AP2 DNA- binding domain and the flanking CBF signature motifs, and an acidic C-terminal domain which might be important for trans-activation (Skinner et al., 2005). Further detailed functional analyses of some of the identified palm *CBFs* were performed and will be reported here.

Palms (Palmae Juss.) are a diverse and complex family with more than 2000 species in 200 genera and are generally regarded as second only to the grasses (Poaceae Barnhart) in economic importance (Jones 1996). The family includes several well-known members like coconut palm (*Cocos nucifera* L.), oil palm (*Elaeis gunineensis* Jacq. and *Elaeis oleifera* Kunth), and date palm (*Phoenix dactylifera* L.). Many other species are important ornamentals with great economic value.

A common misconception is that all palms are tropical and/or subtropical plants and thus are intolerant of cold weather. In fact, about 100 palm species can survive freezing down to -7ºC and have been grown for decades in warm-temperate landscapes world-wide (Francko 2004; Gibbons and Spanner 1999). A few species of extremely cold-hardy palms, such as *Rhapidophyllum hystrix* Pursh (needle palm), *Trachycarpus fortunei* Hook (Chinese windmill palm), *Sabal palmetto* Walter (cabbage palmetto) and *Sabal minor* Jacq. (dwarf palmetto), are capable of surviving temperatures below -17.7ºC (0ºF; reviewed by Francko 2003; 2004). *Rhapidophyllum hystrix*, an U.S. native and clearly the most cold tolerant of all palm species, resists foliar damage down to ca. -22ºC and reliably survives short exposure to -30ºC in cultivation once established (Francko 2004). At present, there has been no systemic investigation for mechanisms of the remarkable cold resistance of these cold hardy palms.

Our lab is investigating the cold-hardy palms under field and laboratory conditions at Miami University in Oxford, OH (39º 30´ north, 50 km northwest of Cincinnati). One of our research goals is to isolate and characterize key genes functioning in palm cold resistance. We identified *CBF* orthologs from both cold-hardy and cold-sensitive palms (Chapter 2; Lu et al., submitted). Further functional analyses reported here were conducted with two *Rhapidophyllum hystrix CBF* orthologs, *RhCBF1* (GenBank accession no. DQ497740) and *RhCBF2* (GenBank accession no. DQ497741) by transforming the two palm genes into *Arabidopsis* and examining the transformants.

MATERIALS AND METHODS

Plant growth

Arabidopsis thaliana (L.) Heynh ecotype Columbia GL. and transgenic plants in the Col. background were grown in growth chambers at 22°C under 16h photoperiod (80-100 μmol $m^{-2}s^{-1}$) in Super Fine Germinating Mix (Conrad Fafard, Inc. Agawam, MA). Plants were subirrigated with deionized water and solution of Miracle-Gro (Scotts Miracle-Gro products, Inc. Marysville, OH) as necessary. Seeds were cold-treated at 4°C for 3d after planting to synchronize germination. Plants were cold acclimated at 4°C with 16h photoperiod (15-20 μmol $m^{-2}s^{-1}$) in an environmental chamber (PERCIVAL I-30VL, PERCIVAL Scientific Inc., Perry, IA) for 7 days if required.

To compare lateral root development, seeds were planted on 1.2% Bacto ™ Agar square plates, containing 1% sucrose and 1x Murashige and Skoog (MS) salts (Sigma-Aldrich, Inc. St. Louis, MO). The plates were vertically placed after removal from 4°C stratification. The plants were harvested two weeks later and photos of the root system were taken. The number and length of roots were analyzed with Image-Pro software.

Constructs and *Arabidopsis* transformation

The complete coding regions of *RhCBF1* (GenBank accession no. DQ497740) and *RhCBF2* (DQ497741) genes were amplified with 5' primer (5'-CTC CTC GAG AAA GCG GCA ATG GAG-3', *XhoI* site underlined) and 3' primer (5'- AAA TTC TAG ACC AGC TTC AAA TGG AGT AGC TC -3', *XbaI* site underlined), then inserted into the *XhoI* and *XbaI* sites of *Agrobacterium*-based transformation vector pKYLX71:35S2 (kanamycin resistant, double CaMV 35S promoter) (Schardl et al. 1987). The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain pGV3850 by electroporation. *Arabidopsis* Columbia GL. plants of appropriate stage were transformed with *CBF*-containing plasmids or the empty transformation vector pKYLX71:35S2 using vacuum infiltration and/or floral dip method (Clough and Bent 1998). Transformed plants were selected by kanamycin resistance and PCR amplification from genomic DNA. Homozygous T3 or T4 generations of plants with single insertion were used for further analysis of stress resistance.

Chilling and freezing tolerance of transgenic *Arabidopsis*

Long-term chilling stress was applied to control and transgenic plants by planting the seeds on MS nutrient medium and incubating them under 4°C with 16h photoperiod (15-20 μmol $m^{-2}s^{-1}$) in an environmental chamber (PERCIVAL I-30VL, PERCIVAL Scientific Inc., Perry, IA). After 8 weeks, germination and survival rates of each plant line were calculated and compared with those grown under a normal, non-chilling temperature regime.

For the whole-plant freezing assay (modified from Liu et al. 1998), control and transgenic plants were grown in pots under warm (22°C) temperature for three weeks. The plants were frozen for 72 h at different sub-zero (°C) temperatures in darkness. The temperature was then adjusted to 4°C and the plants were incubated at 4°C in the dark for an additional 24 h. The plants then were returned to normal growth conditions for recovery and survival rates were recorded after two weeks. The whole-plant freezing assay was also applied to cold-acclimated plants with cold acclimation at 4°C with 16h photoperiod (15-20 μmol $m^{-2}s^{-1}$) in environmental chamber (PERCIVAL I-30VL) for 7 days before freezing.

Proline analysis

Analysis of proline (Pro) levels was performed as described in Gilmour *et al.* (Gilmour et al. 2000) and Bates (Bates 1973) using the acid ninhydrin method. Lyophilized leaf material was extracted with deionized water (1ml water /10mg leaf material) at 80 °C for 15 min. Samples were shaken for 1h at room temperature and then incubated overnight at 4°C. Extracts were filtered with Whatman #1 filter paper, and filtrates were mixed with equal amount of glacial acetic acid and acid-ninhydrin reagent (Bates 1973; Gilmour et al. 2000) and heated at 100°C for 1h. After cooling, the reaction mixture was extracted with toluene, and the absorbance of the organic phase was measured at 515nm using toluene for a blank. Pro concentrations were determined from a standard curve constructed with known amounts of Pro (Sigma-Aldrich, Inc. St. Louis, MO) and calculated on a biomass-specific (dry weight) basis.

Sugar analysis

Analysis of soluble sugar levels was performed using a phenol-sulfuric acid assay (Geater and Fehr 2000; Gilmour et al. 2000). Total soluble sugars were extracted from

lyophilized leaves with 80% (v/v) ethanol (1ml EtOH /10mg leaf material) at 80°C for 15min. Samples were shaken for 1h at room temperature and incubated overnight at 4°C. Extracts were filtered with Whatman #1 filter paper. Chlorophyll was removed by shaking samples with equal amounts of water and chloroform. The aqueous extract was mixed with equal amount of 5% (w/v) phenol. After vortexing, a 2.5 x volume of concentrated sulfuric acid was added to the extract and mixed immediately. The mixture was incubated at 80°C for 30min, and then absorbance of the mixture was measured at 490nm. A blank was prepared by substituting distilled water for the leaf extract. The sugar levels were determined by comparing the absorbance of samples to a standard curve constructed with known amounts of glucose (Sigma-Aldrich, Inc. St. Louis, MO) and calculated on a biomass-specific (dry weight) basis.

Real-time PCR analysis of *RhCBF* and *COR* gene expression levels

Total RNA was isolated from *Arabidopsis* plants with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Genomic DNA contamination was removed from RNA samples with DNase I (Qiagen) digestion, cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using 2~5μg of total RNA. Real-time PCR was performed on Rotor-Gene RG3000 (Corbett Research, Sydney, Australia) using the QuantiTect SYBR Green PCR Kit (Qiagen). The primers for real-time PCR were designed with PrimerQuest Tools (http://scitools.idtdna.com/Primerquest/) of IDT INC (Coralville, IA) with some modifications to decrease primer-dimers while obtain uniform PCR annealing temperature.

To analyze the transcriptional levels of transformed palm *CBF* genes, the following PCR forward primers: 5′- GAC GAG GAG GTG AGC GAC G -3′ and 5′- GCC GAT GAG GAA GAA AGC GAT G -3′ were used for *RhCBF1* and *RhCBF2* respectively. Primer 5′- GGT GTG TGC GCA ATG AAA CTG -3′, which was located on the plasmid pKYLX71:35S2, was used as reverse primer for real-time PCR amplification of both *RhCBF1* and *RhCBF2* genes.

For *Arabidopsis COR15A* (NM_129815) gene, the following primers: 5′- AGG AGA GGC TAA GGA TGC CAC AAA G-3′ (forward) and 5′-TGT GAC GGT GAC TGT GGA TAC CAT -3′ (reverse) were used to amplify a 195bp fragment during real-

time PCR analysis. For *Arabidopsis COR78* (NM_124610) gene, the following primers: 5′- AAG AAA CAG AGT CTG CCG TGA CGA -3′ (forward) and 5′- TCA GTT GTC AGT TTC TCC GCC ACA -3′ (reverse) were used to amplify a 127bp fragment.

Arabidopsis ACTIN 8 (NM_103814) was used as a house-keeping gene (Thomas et al. 2003). The following primers: 5′- CTT TCC GGT TAC AGC GTT TG -3′ (forward) and 5′- GAA ACG CGG ATT AGT GCC T -3′ (reverse) were used to amplify a 91bp fragment during real-time PCR analysis.

PCR program was set to following conditions: activation of *Taq* at 95°C for 15 min, 40 cycles of amplification generally, denaturing at 95 °C for 10s, annealing at 56ºC for 15s, and extension at 72ºC for 25s. Data were analyzed using Rotor Gene 6.0 software supplied by Corbett Research and quantification of *CBF* transcripts was performed using the "Comparative Quantitation" function of the software (Sinisterra et al. 2005; Warton et al. 2004). Briefly, for each PCR amplification, "Second Derivative" peak was plotted indicating the maximum rate of fluorescence increase in the reaction. "Takeoff" point, which indicated the end of noise and the transition into exponential phase, was defined as the cycle at which the Second Derivative was at 20% of the maximum level. The average fluorescence increase four points following the "Takeoff" was calculated as the amplification efficiency of each individual reaction. The "Average Amplification" of all samples was then calculated and the variance was used to provide as a measure of error. The relative concentration of a specific gene to a house-keeping gene was determined as Average Amplification ^ (HousekeepingTakeoff - SpecificGeneTakeoff) (Sinisterra et al. 2005; Warton et al. 2004). All real-time experiments were conducted with at least three biological and technical replicates. Melting curve analysis, agarose gel electrophoresis and sequencing were used to verify specific PCR product formation.

RESULTS

Identification of *Arabidopsis* transformants that express *RhCBF*s

The *Rhapidophyllum hystrix RhCBF1* and *RhCBF2* genes were introduced into *Arabidopsis* under control of a double cauliflower mosaic virus (CaMV) 35S promoter. Transformed plants were selected by kanamycin resistance, the selectable marker carried on the transformation vector pKYLX71:35S2. Eighteen and thirty-three independent transformant lines were identified with one single active T-DNA insertion (showed 3:1 segregation ratio for kanamycin resistance in the T2 generation plants) for *RhCBF1* and *RhCBF2* respectively. Two transformant lines were selected for further analysis of stress resistance for each gene (Rh1-A and Rh1-C for *RhCBF1*; and Rh2-D and Rh2-F for *RhCBF2*). Untransformed *Arabidopsis* GL. plant (WT) and one transgenic line with an empty pKYLX71:35S2 vector lacking *RhCBF* insertion (P22-B) were used as controls during the analysis.

Expressions of *RhCBFs* in transgenic plants grown at normal temperatures were analyzed by Reverse Transcription and real-time PCR (Fig. 1). Gel electrophoresis and sequencing were performed with the PCR results to confirm specific amplification (data not shown). Among the four selected transformants, palm *CBF* genes were successfully expressed in *Arabidopsis* plants while *RhCBF2* had higher expression levels than *RhCBF1*.

Vegetative growth and development of *RhCBF*-expressing plants

Liu et al. (1998) and Gilmore et al. (2004) have reported that transgenic *Arabidopsis* plants overexpressing *AtCBF* genes had a “dwarf” phenotype. Compared with the controls, the transgenic plants showed different levels of growth retardation; the rosette leaves were darker and the petioles were much shorter; and the time to flowering was significantly delayed (Gilmour et al. 2004; Gilmour et al. 2000; Liu et al. 1998). The similar “dwarf” phenotype also appeared in *RhCBF*-expressing plants. After 3 weeks of growth at normal temperature, the dimensions of *RhCBF*-expressing plants were significantly smaller than those of the control plants with less leaf area and shorter petioles (Fig 2A). The time to flowering was also delayed in transgenic plants. The controls plants had first flower buds visible at 23 d of growth (Table 1) and had opened flowers and ripening siliques after 35 d growth (Fig 2B). The Rh1-A & Rh1-C transgenic

plants had flower buds at ~30 d and were about to have the first flower open at 35 d, while Rh2-D & Rh2-F took more than 40 d to develop the first flower bud (Table1, Figure 2B). The *RhCBF*-expressing plants also had more rosette leaves when flowering, the leaves were curled and darker in color than control plants (Table1, Figure 2B).

We also observed that lateral root development in *Arabidopsis* plants was affected by *RhCBF*-expression (Figure 3). Seedlings were grown on vertically-placed petri dishes. After 14 days, ~70% control plants had more than five lateral roots while the median number was eight roots (Fig 3B). For *RhCBFs*-expressing plants, the lateral root numbers of >70% individuals were less than five and the median values were 0, 1 or 1.5 (Fig 3B). The primary root length of Rh2-D was less than those of the controls but for the other three lines the difference was not significant (data not shown).

RhCBF-expressing plants have increased resistance to freezing and long-term chilling stresses

Overexpression of *AtCBF* genes has been reported to increase the freezing, drought and salt tolerance of both non-acclimated and cold-acclimated plants (Gilmour et al. 2004; Gilmour et al. 2000; Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Liu et al. 1998). The *RhCBF* genes have similar functions with *AtCBF*s in plant cold resistance. The controls and *RhCBF*-expressing plants grown in pots were treated at different below-zero (°C) temperatures for three days to completely access cold tolerance. For all conditions examined, the *RhCBF*-expressing plants showed increased cold tolerance and higher survival rates than the controls (Figure 4A, B, and C). For non-acclimated plants, ~ 2/3 of the controls were killed by treatment at -4°C for 3 d while only ~1/3 of *RhCBF1*-expressing plants were killed and *RhCBF2*-expressing plants almost did not suffer any lethal damage. The freezing resistance of *RhCBF2*-expressing plants was generally greater than those of *RhCBF1*-expressing plants as reflected by survival rates, which might be explained by the higher expression levels of *RhCBF2* in transgenic plants (Figure 1).

The *RhCBF*-expressing plants also showed increased chilling resistance. When seeds of controls and *RhCBF*-expressing plants were incubated at 4°C to germinate and develop, the germination and survival rates of the controls were reduced to ~70% after 8

weeks while those of the *RhCBF*-expressing plants were not affected by long-term chilling (Figure 4D).

***RhCBF*-expressing plants have increased proline and sugar levels**

Proline and sugars are important cryoprotectants in many plants including *Arabidopsis* and their levels increase significantly during cold acclimation (Xin and Browse 2000). In *AtCBF*-overexpressing *Arabidopsis* plants, levels of proline and sugars are elevated without cold acclimation (Gilmour et al. 2004; Gilmour et al. 2000). The non-acclimated *RhCBF*-expressing plants showed increased levels of proline and sugars (Figure 5) as well, and the levels rose further after cold acclimation. In Rh2-D & Rh2-F, the levels of proline and sugars of non-acclimated plants exceeded those of cold-acclimated control (WT & P22-B) plants (Figure 5). The accumulation of proline and sugars in *RhCBF1*-expressing lines were not as great as those of Rh2-D & Rh2-F, which was consistent with the freezing resistance of these lines (Figure 4, 5).

***RhCBF*-expressing plants have increased expression of *COR* genes**

Overexpression of *AtCBFs* induced high levels of transcripts of multiple *COR* genes, including *COR6.6*, *COR15*, *COR47* and *COR78* (Gilmour et al. 2004; Gilmour et al. 2000; Liu et al. 1998). We analyzed the expression levels of *COR15A* and *COR78* in non-acclimated *RhCBF*-expressing and control plants using real-time PCR. In all transgenic lines, the transcript levels of *COR* genes were up-regulated at least 5-fold compared with those of the control plants (Figure 6).

DISCUSSION

This study examined the functions of *RhCBF1* and *RhCBF2* in plant cold acclimation process. We transformed the two palm genes into *Arabidopsis* with a doubled CaMV 35S promoter. Transgenic plants with either one of the *RhCBFs* showed constitutive cold resistance (Figure 4), along with accumulation of free proline and sugars (Figure 5), and increased mRNA levels of multiple *COR* genes (Figure 6). All these suggested that the palm *CBFs* not only shared similar sequence characteristics with the *AtCBFs* (Chapter 2; Lu et al., submitted), but also maintained similar functions in cold acclimation and plant cold resistance. The *RhCBF*-expressing plants also showed the typical "dwarf" phenotype which was observed in *AtCBF*-overexpressing plants, giving further evidence that *RhCBF* genes might regulate similar gene sets in *Arabidopsis* as *AtCBFs*.

The proteins of *RhCBFs* share the general primary domain structures of AtCBFs (Lu et al., submitted), but the AP2 DNA-binding domain and the flanking CBF signature motifs are the only regions that are extensively conserved at the sequence level between RhCBFs and AtCBFs. The precise functions of CBF signature motifs are still unknown, but they are widely conserved between CBF proteins from diverse plant taxa and might be involved in the specific binding of CBF proteins to the promoters of downstream genes. At the same time, the sequence-non-conserved C-terminal domain consists of several hydrophobic motifs that have functional redundancy to the trans-activation function of C-terminal domain (Wang et al. 2005) thus ensure the activation of CBF-regulated pathway. This special, highly-ordered structure of CBF proteins might contribute to the functional conservation of them in different plant species.

For the four transgenic plant lines we analyzed, the *RhCBF2*-expressing lines had higher freezing resistance, higher Pro and sugars levels, and greater growth retardation than *RhCBF1*-expressing lines. However, the analyses did not unequivocally support functional differences between *RhCBF2* and *RhCBF1*. It is generally accepted that the degree of freezing resistance, along with the severity of growth retardation of *CBF*-overexpressing plants, are positively related to the expression levels of the *CBF* genes (Gilmour et al. 2000; Kasuga et al. 1999; Liu et al. 1998). It is likely that the difference of freezing resistance we observed in *RhCBF1*-expressing and *RhCBF2*-expressing plants

were due to the different expression levels of the two genes (Fig. 1). Analyses of additional transgenic lines with even lower expression levels of *RhCBF1* or *RhCBF2* showed that they had correspondingly lower cold resistance and less obvious growth retardation (data not shown), thus supporting the above hypothesis.

Although most palms are tropical to subtropical plants, many species, including *R.hystrix*, are capable of surviving freezing temperatures. Our field data showed that for many cold-hardy palms, summer foliage was to a degree constitutively cold-hardy, but became more cold-hardy after plants were exposed to the first frost of the season (Francko and Wilson 2004), suggesting the existence of cold acclimation in these species. The *RhCBF1* and *RhCBF2* genes are constitutively expressed yet cold inducible (Chapter 2; Lu et al., submitted) *in planta*, and they show functional similarity with *AtCBFs*. Thus the CBF/DREB1 cold-responsive pathway might be conserved in palms, at least some of the cold resistant palms although there is no report of downstream *COR* genes in palms at present.

More research is needed to clarify the details of the cold acclimation mechanism in palms and the contribution and detailed functions of *CBF* genes to it. Our lab is performing the isolation of possible CBF-regulated *COR* genes from palms, which is an important part of the study of CBF regulon and will provide more information of *CBF* functions in palms.

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Figure 1. Real-time PCR analysis of expression levels of *RhCBF1* and *RhCBF2* in transgenic *Arabidopsis* plants grown at warm (22°C) temperature using *Actin 8* as internal control. One of the primers used for real-time PCR is located on the transformation vector pKYLX71:35S2 (see Method) to enhance specific amplification. Reactions were performed in 20μl volume using the Qiagen QuantiTect SYBR Green PCR Kit. The relative expression levels of *RhCBFs* to *Actin 8* were calculated by "Comparative Quantitation" function of Rotor Gene 6.0 software (Corbett Research). For control WT (untransformed) and P22-B (transformed without *RhCBF* insertion) plants, no amplification of palm *CBF* fragments was detected (set as zero). For Rh1-A & Rh1-C (*RhCBF1* transformed); and Rh2-D & Rh2-F (*RhCBF2* transformed), the corresponding *RhCBF* transcripts were detected with high expression levels.

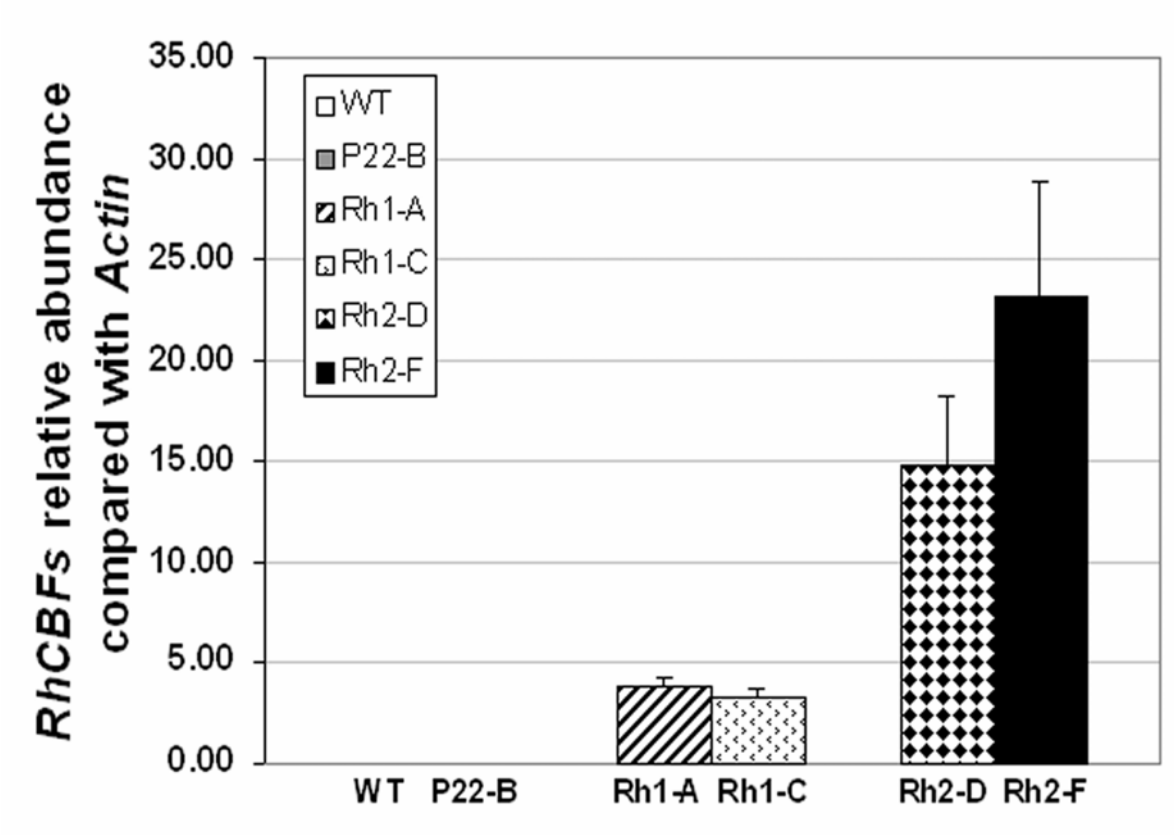

RhCBFs relative abundance compared with Actin
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F

Figure 1

Figure 2. Growth characteristics of *RhCBF*-expressing transgenic *Arabidopsis* plants. Control wild-type (WT) and empty vector transformed (P22-B) plants, *RhCBF1*-transformed (Rh1-A, Rh1-C) plants, and *RhCBF2*-transformed (Rh2-D, Rh2-F) plants were grown for 21 days (A) and 35 days (B) at 22°C on soil. Bars indicate 2 cm.

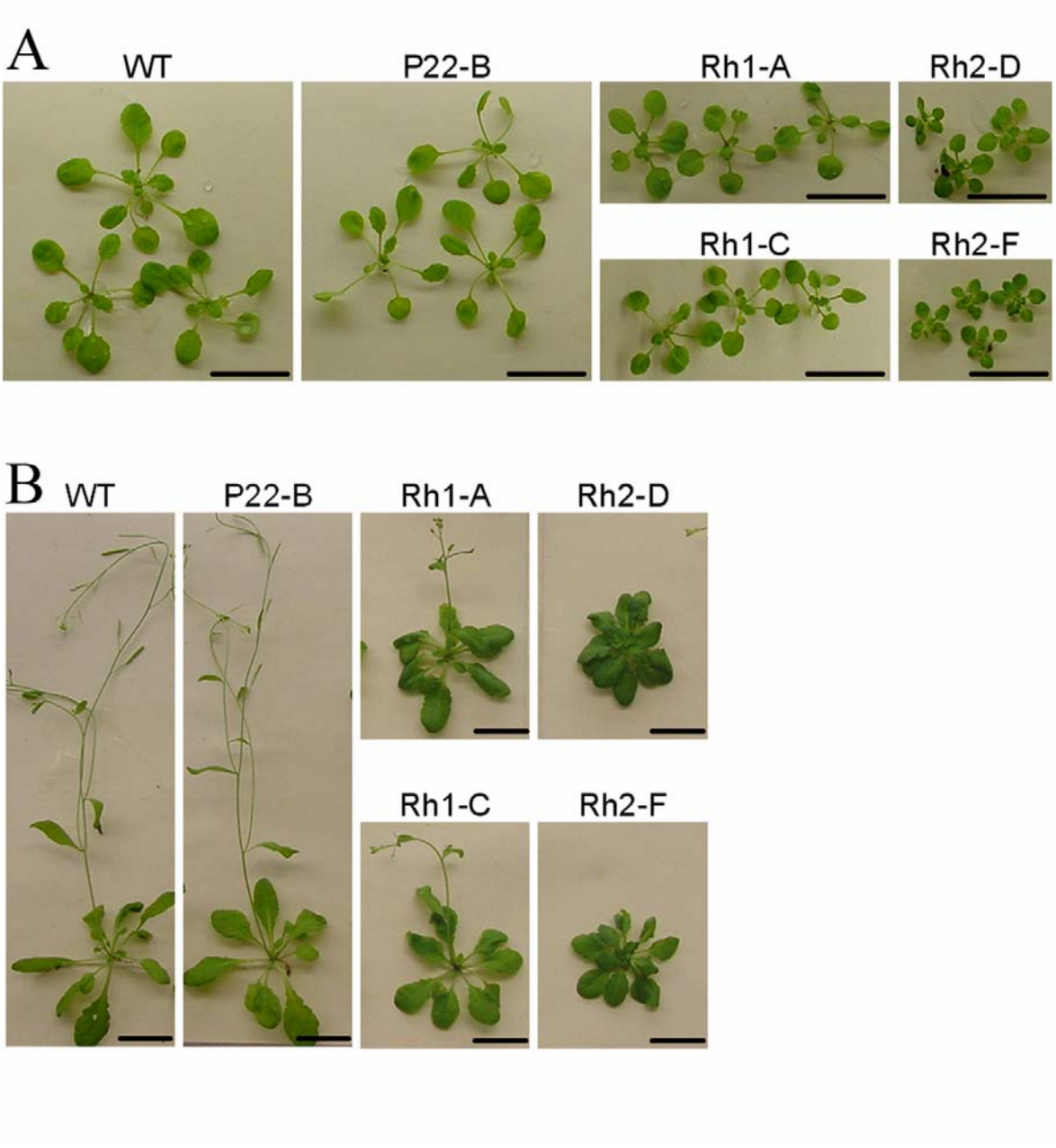


Figure 2

Table 1. Effects of *RhCBF*-expressing on flower production and rosette growth in *Arabidopsis* plants.

Plant line	Time of flowering (days)[a,c]	Rosette leaves per plant [b,c]
WT	23 ± 0.4	12.8 ± 0.4
P22-B	23 ± 0.0	12.8 ± 0.4
Rh1-A	28 ± 0.5	14.7 ± 0.5
Rh1-C	31 ± 0.5	15.8 ± 1.0
Rh2-D	41 ± 1.3	20.8 ± 1.6
Rh2-F	43 ± 1.0	21.0 ± 1.3

[a] Average days from the end of synchronization treatment (4°C) to the appearance of first flower bud. [b] The number of leaves were counted after the appearance of first flower bud. [c] The mean values on the left of ± were determined from ≥ 10 individual plants; the values on the right of ± were the standard deviation.

Figure 3. Lateral root development of control and *RhCBF*-expressing *Arabidopsis* plants. (A). Root systems of control wild-type (WT) and *RhCBF2*-expressing plant Rh2-D after 14 days growth on vertical petri dishes. The roots were stained by Neutral Red. Bars indicate 2 cm. (B). Boxplot of lateral root numbers in controls (WT, P22-B) and *RhCBF*-expressing plants (Rh1-A, Rh1-C, Rh2-D, Rh2-F). Data presented were from three independent experiments, at least 25 individual plants of each line. Outliers are designated by +, medians of each plant line are designated by ⊗ with actual values displayed on the right of the interquartile range boxes.

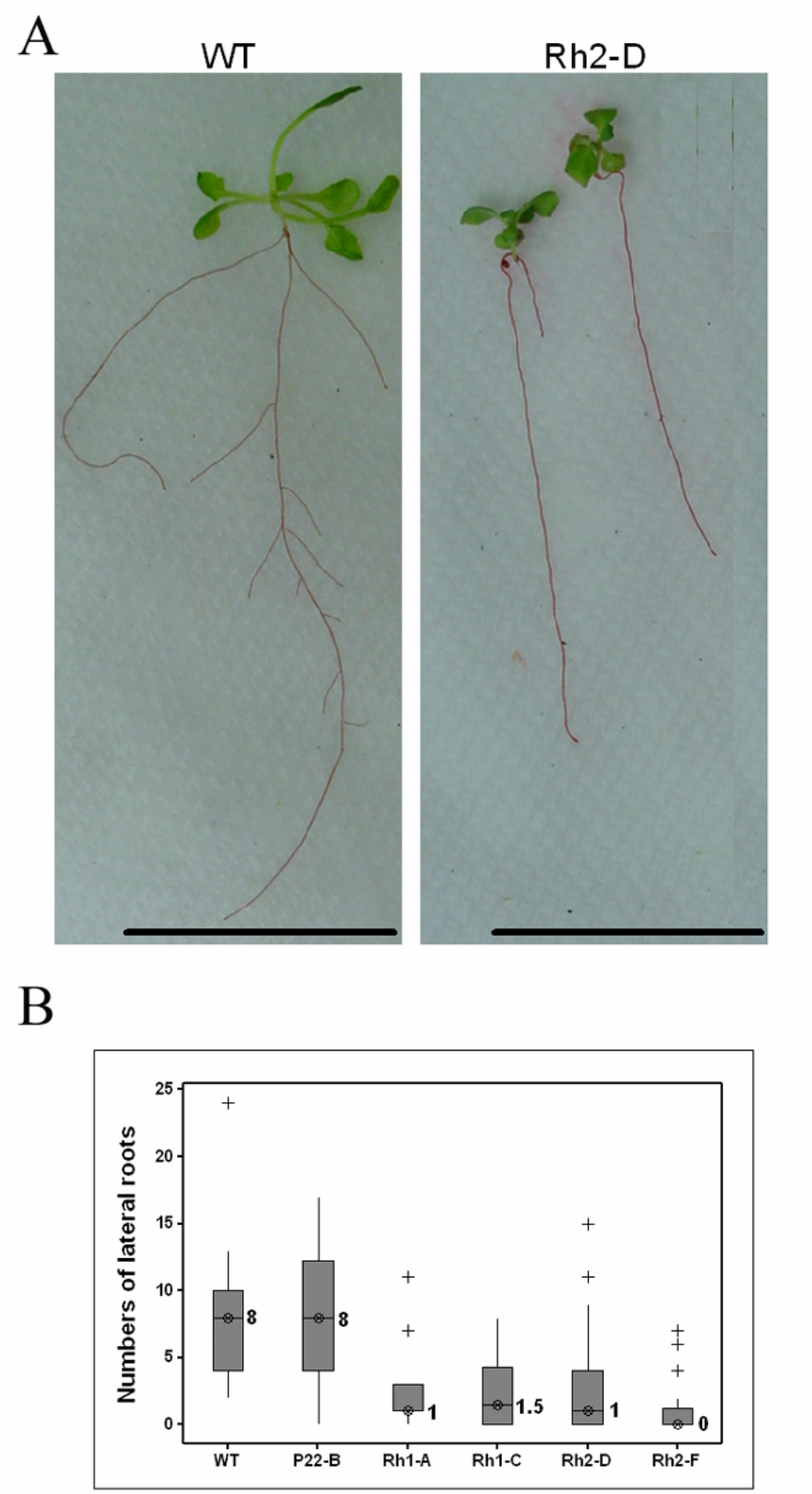

A
WT
Rh2-D
B
Numbers of lateral roots
25
20
15
10
5
0
8
8
1
1.5
1
0
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F

Figure 3

Figure 4. Comparison of cold tolerance and long-term chilling tolerance between controls and *RhCBF*-expressing plants. A. Plants were grown in soil at 22°C for 3 weeks and then cold-treated for 72 h at -4°C in darkness without cold acclimation, followed by 24h at 4°C. The plants were then returned to normal growth conditions for recovery and the photo was taken after 2 weeks. B. Three weeks old plants were exposed to different sub-zero temperatures for 72 h in darkness without cold acclimation, followed by 24h at 4°C. The percentages of survival plants were recorded after 2 weeks recovery. C. Cold acclimation was applied by 7 days incubation at 4°C before cold treatment with sub-zero temperatures. D. Seeds were incubated on petri dishes at 4°C for 8 weeks, then germination and survival rates of each plant line were calculated and compared with those under normal temperature.

A

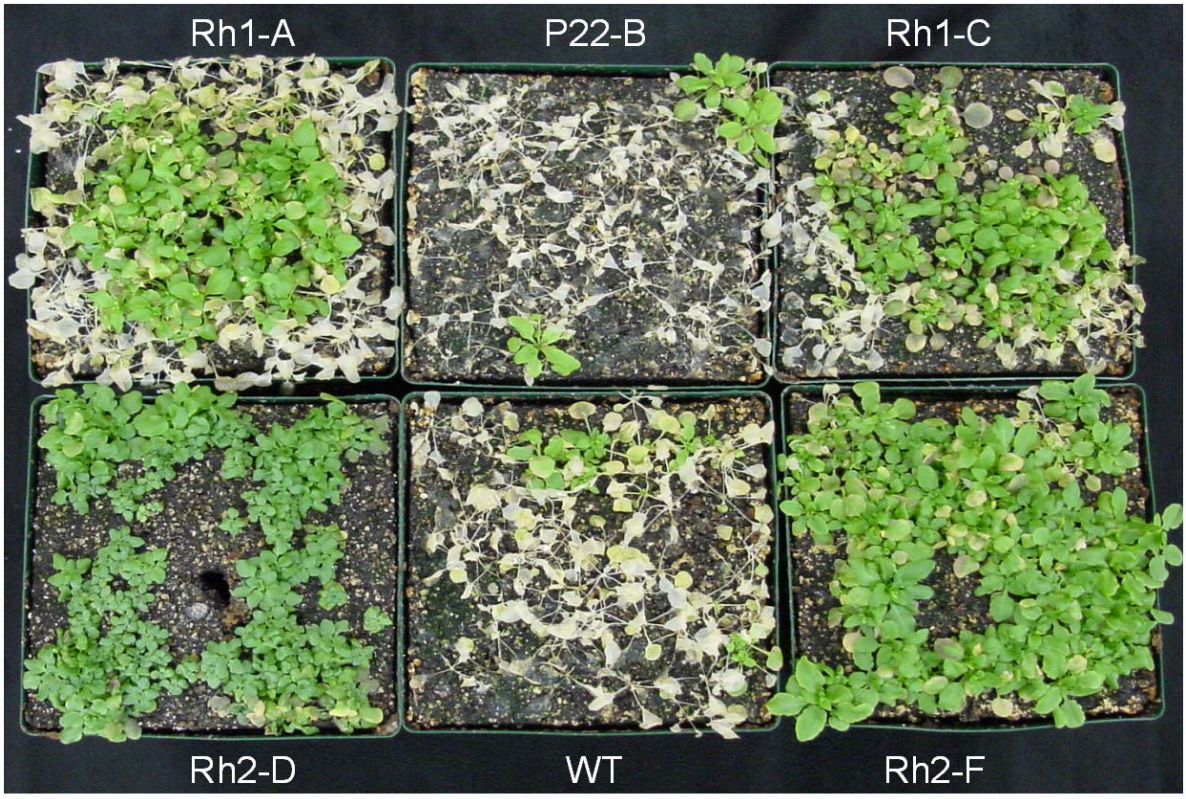
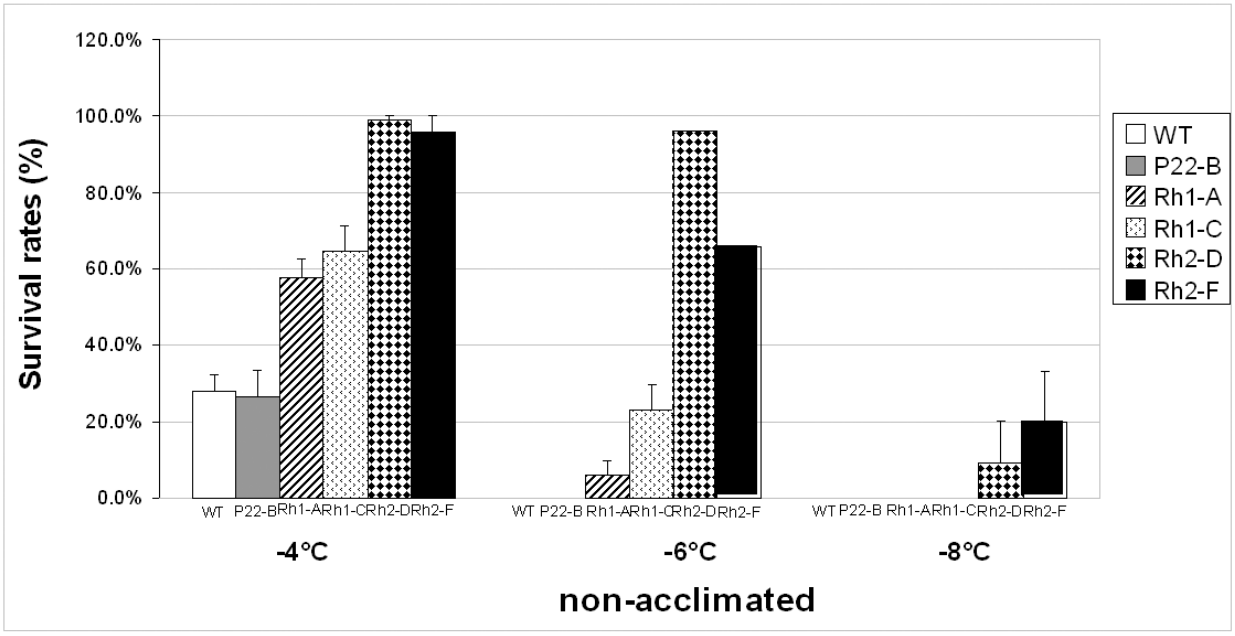


Figure 4

B


120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Survival rates (%)
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
-4°C
-6°C
-8°C
non-acclimated

C

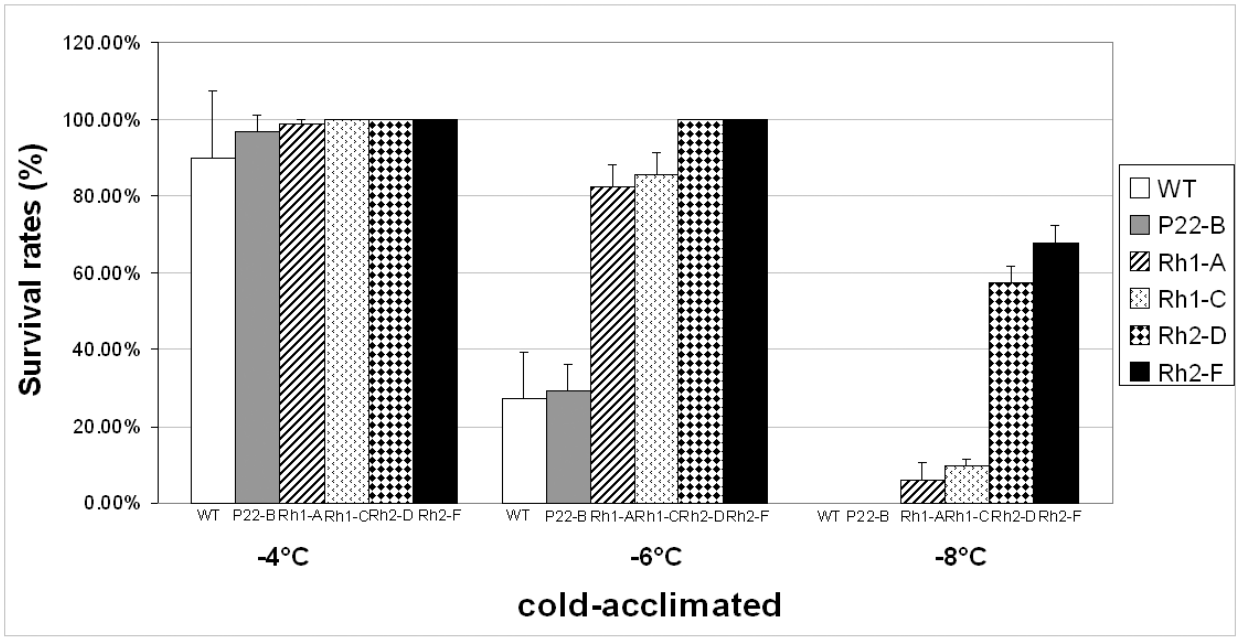

120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Survival rates (%)
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
-4°C
-6°C
-8°C
cold-acclimated

Figure 4

D

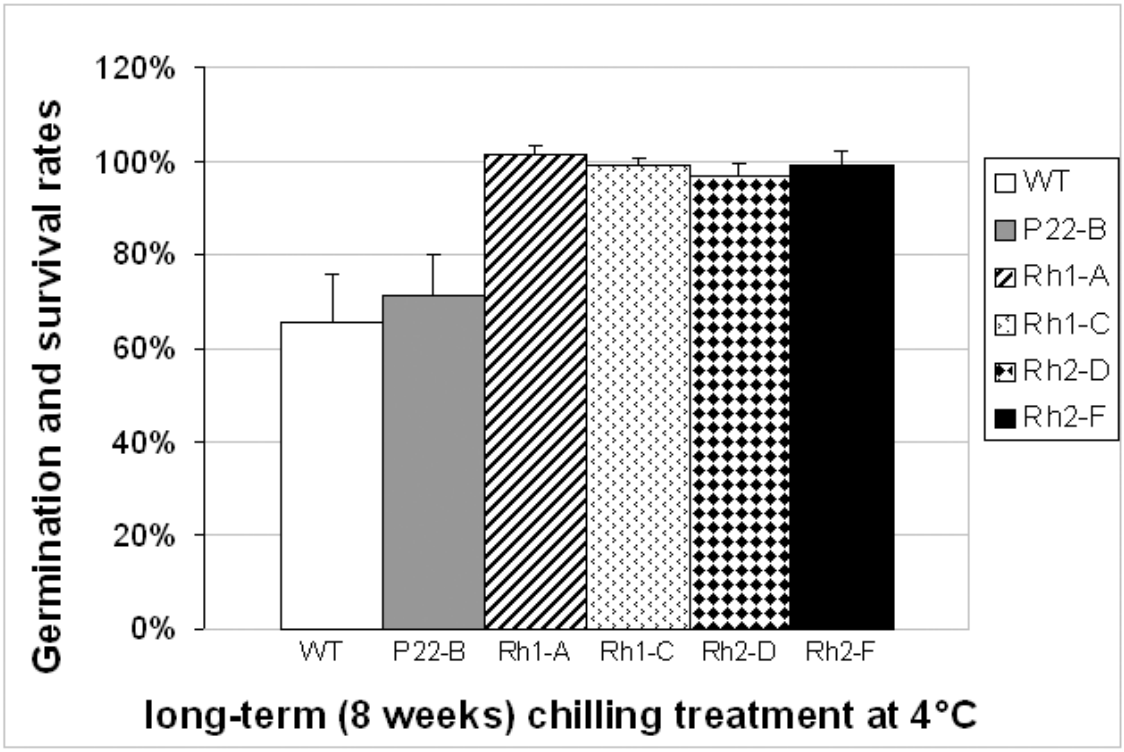
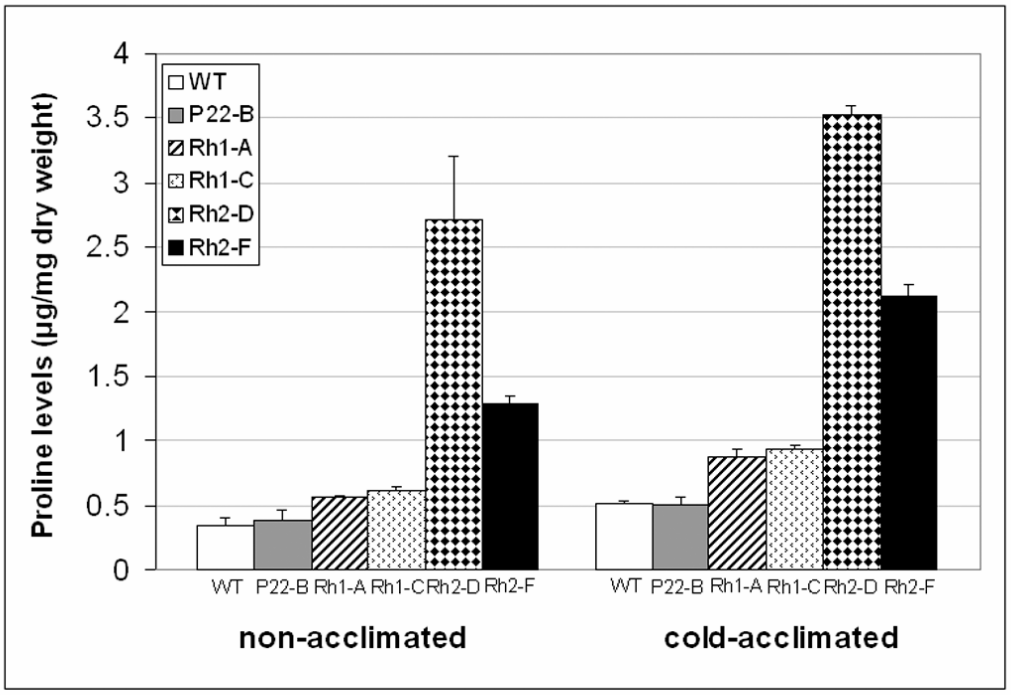

Germination and survival rates
120%
100%
80%
60%
40%
20%
0%
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
long-term (8 weeks) chilling treatment at 4°C

Figure 4

Figure 5. Comparison of free proline levels (A) and total soluble sugar levels (B) between controls and *RhCBF*-expressing plants. Plants were grown in soil at 22°C for 3 weeks. Cold acclimation was applied by 7 days incubation at 4°C.

A


Proline levels (µg/mg dry weight)
4
3.5
3
2.5
2
1.5
1
0.5
0
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
non-acclimated
cold-acclimated

B

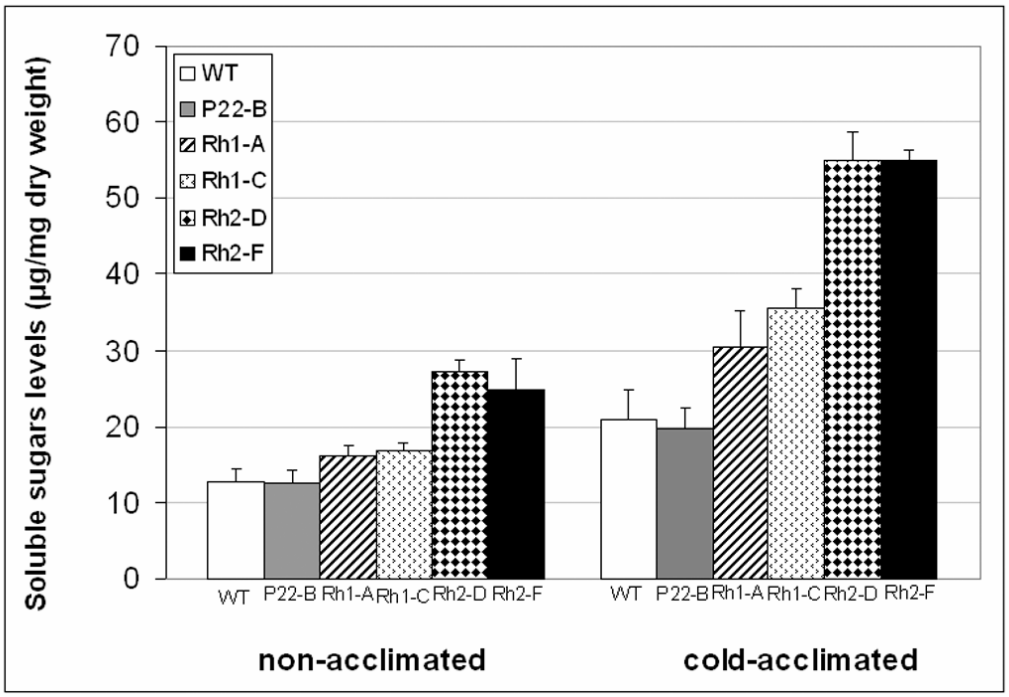
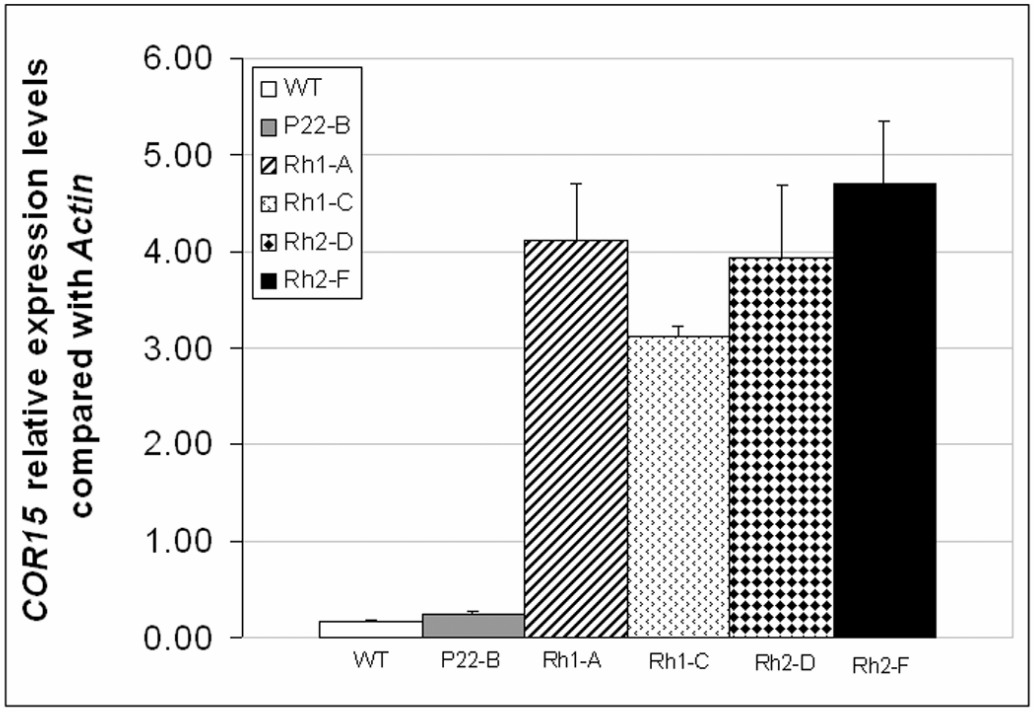

Soluble sugars levels (µg/mg dry weight)
70
60
50
40
30
20
10
0
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
WT P22-B Rh1-A Rh1-C Rh2-D Rh2-F
non-acclimated
cold-acclimated

Figure 5

Figure 6. Comparison of transcript levels of *COR15A* (A) and *COR78* (B) between controls and *RhCBF*-expressing plants by RT and real-time PCR using *Actin 8* as internal control. RNA samples were isolated from 3 weeks plants grown in soil at 22°C without cold treatment. The relative expression levels of *COR* genes to *Actin 8* were calculated by "Comparative Quantitation" function of Rotor Gene 6.0 software (Corbett Research).

A


COR15 relative expression levels compared with Actin
6.00
5.00
4.00
3.00
2.00
1.00
0.00
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F

B

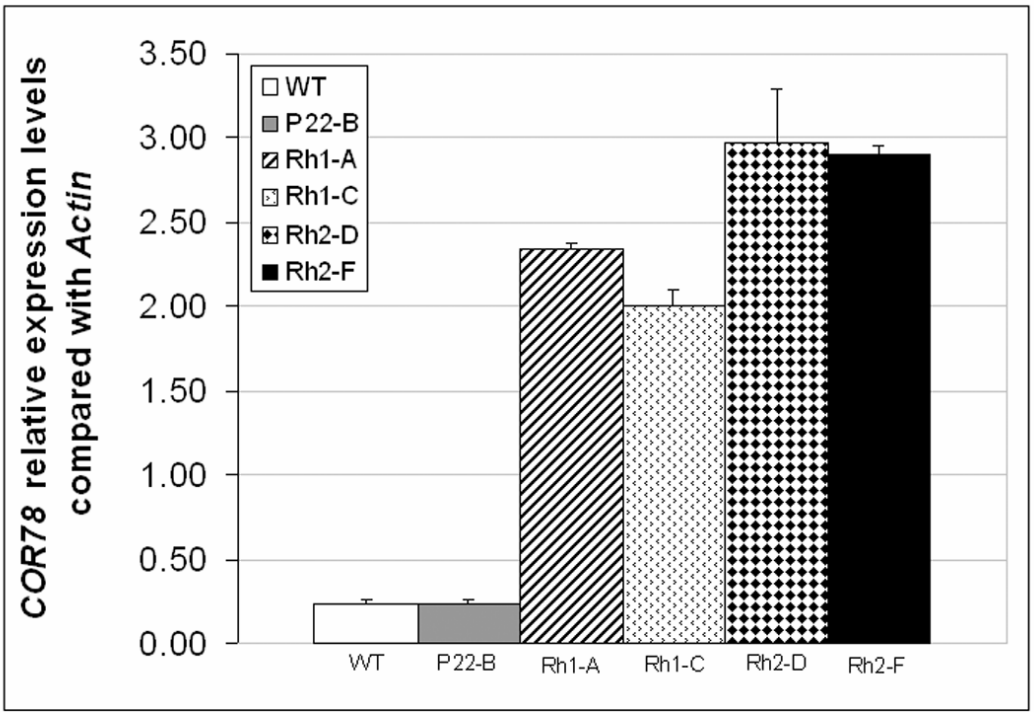

COR78 relative expression levels compared with Actin
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
WT
P22-B
Rh1-A
Rh1-C
Rh2-D
Rh2-F

Figure 6

CHAPTER 4

SUMMARY

The *CBF/DREB1* gene family has attracted tremendous attention ever since the first day of their identification. They are key regulators that control the expression of a group of genes, including several other transcriptional factors, that are involved in plant cold acclimation and low-temperature tolerance (Chinnusamy et al. 2006; Nakashima and Yamaguchi-Shinozaki 2006; Van Buskirk and Thomashow 2006). The *CBF* genes and CBF-regulated cold-response pathway are conserved in various plant species (Dubouzet et al. 2003; Owens et al. 2002; Skinner et al. 2005). The *CBF* genes have been applied to agriculture by overexpressing them in economically important crops to increase low-temperature and other abiotic-stress resistance (Hsieh et al. 2002; Oh et al. 2005; Pellegrineschi et al. 2004) although there might be some drawbacks such as dwarf phenotype and delayed flowering (Gilmour et al. 2004).

As a part of the Miami Cold-hardy Palm Project, which was to investigate the mechanism of cold resistance of general tropical and/or sub-tropical plants, especially palms (Francko and Wilson 2001), the main goals of my doctoral research were to look for possible *CBF/DREB1* homologs from various palms species and investigate whether they are involved in palm cold resistance. The palm *CBF/DREB1* homologs were successfully identified from cDNA and/or genomic DNA of ten different palm species, including *Rhapidophyllum hystrix*, *Sabal minor, Sabal palmetto, Trachycarpus fortunei*, *Dypsis lutescens, Ravenea rivularis*, *Attalea bassleriana, Cocos nucifera, Elaeis guineensis,* and *Elaeis olecifera*. Sequence analyses of the isolated genes and their putative proteins showed that they had high similarity with the known *CBF/DREB1* genes and proteins, especially in the AP2 DNA-binding domain and the AP2-flanking CBF signature motifs (Chapter 2, Figure 1). The putative palm CBF proteins also shared the general primary domain structures of known CBFs (Chapter 2, Figure 1A), which indicated the possibility of conserved functions as well.

To further investigate the functions of these palm *CBF* genes, the expression patterns of two *R. hystrix CBF* genes, *RhCBF1* and *RhCBF2* were analyzed with reverse transcription and real-time PCR. The transcript levels of these two genes were greatly cold up-regulated, further indicating their involvement in plant response to cold stress. The interesting thing was that we also detected a low level of both *RhCBF1* and *RhCBF2*

transcripts under control warm temperature (Chapter 2, Figure 4), which might explain the high cold resistance of *R. hystrix* summer foliage before natural cold acclimation. Finally, *RhCBF*-expressing *Arabidopsis* plants showed increase in cold resistance as expected and confirmed the functions of the two palm *CBF* genes in cold resistance (Chapter 3).

Overall, my research demonstrated that *CBF/DREB1* genes were conserved in palms, and they participated in plant cold resistance as well, at least in some of the cold-hardy palms. Conservation of *CBF/DREB1* genes in palm, which is distant from *Arabidopsis*, is very impressive and provides more circumstantial evidence for the genes' importance. Based on our field data, cold-hardy palms did have cold acclimation in late fall/early winter although the extent of response was not very high (Francko and Wilson 2004). The existence of functional *CBF* orthologs in cold-hardy palms supports that observation. Identification of CBF-regulated cold-related genes in palms will provide further details for the function of palm *CBF* genes *in planta*. It is especially important as the isolated *CBF/DREB1* sequences were quite conserved among different palm species (Chapter 2, Figure 2) although their cold resistances fall in a wide range. Differences in the downstream cold-related proteins might be a reasonable explanation for the cold sensitive, *CBF*-containing species.

Understanding of *CBF* gene family and CBF regulon is still at the early stage although there have been numerous related research articles. One area of special interest is the specificity and/or redundancy in functions of each member of the family (Gilmour et al. 2004; Novillo et al. 2004). At present, from one individual palm species, we just identified two *CBF* genes at most (Chapter 2, Table 1, for *R. hystrix* and *E. guineensis*, two *CBF* genes identified, for other eight palm species, one *CBF* gene in each). From analyses of the two *R. hystrix CBFs*, we cannot provide a distinguishing functional difference between them. Given the size of *CBF* family of *Arabidopsis* and barley (Haake et al. 2002; Skinner et al. 2005), it is highly likely that there are more *CBF* homologs in the genome of palms. Identification and comparison of additional palm *CBFs* will be another imperative aspect of our future research.

This research was an important step of our project in understanding palm cold resistance. It provided evidence of conservation of *CBF* transcriptional factors in palms

and gives explanation to the cold resistance of some cold-hardy species. It is hoped that the research could broaden our understanding to *CBF* gene family and CBF-regulated plant cold-response pathway. The identified *CBF* genes could be applied to agriculture and horticulture in cultivar improvement. A feasible future direction of this project would be the isolation of downstream genes and further comparison between cold resistant and sensitive palms.

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