TRANSCRIPTIONAL REGULATION OF COLD ACCLIMATION IN ARABIDOPSIS THALIANA

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ABSTRACT

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Low temperature is a major environmental factor that affects the yield and quality of food and bioenergy crop plants. To cope with low temperature stress, many plants can acquire freezing tolerance through cold acclimation, in which remodeling of the metabolome and biological pathways leads to physiological adaptation to low Many of these adjustments are brought about by intensively temperatures. reconfiguring the expression of cold-regulated (COR) genes during cold acclimation. Some of the COR genes are regulated by the well-studied CBF pathway and its parallel ZAT12 pathway. However, the regulation and functions of other CBF-independent pathways that are involved in COR gene regulation are not well understood. Freezing tolerance in plants is affected by external signals as well as the growth conditions of plants themselves, but cross talk between cold and other signals has not been fully elucidated. The goal of this dissertation is to understand how freezing tolerance is established during cold acclimation in plants by addressing two key questions: first, how do plants perceive and integrate photoperiod as seasonal signals to modulate the expression of COR genes and subsequent freezing tolerance; second, what are the CBF-independent pathways that have major roles in COR gene expression and their roles in tolerance to low temperatures?

In the first part of the dissertation, the photoperiodic regulation of the coldresponsive CBF pathway and freezing tolerance was demonstrated in *Arabidopsis*. The *CBF* transcript levels in short-day (SD) plants were higher than in long-day (LD) plants. Genetic analysis indicated that phytochrome B (PHYB) functions with two phytochrome interacting factors, PIF4 and PIF7, to down-regulate the CBF pathway and freezing tolerance under LD conditions. Down-regulation of the CBF pathway in LD plants correlated with higher *PIF4* and *PIF7* transcript levels and greater stability of the PIF4 and PIF7 proteins under LD conditions. These findings provide a mechanism of how plants sense and integrate photoperiod as seasonal signals to regulate cold responsive pathways and freezing tolerance.

To address the second question, fifteen early cold-induced transcription factors (TF) sharing similar cold-induction kinetics with *CBFs* and *ZAT12* were identified as regulators of CBF-independent pathways in parallel with the CBF and ZAT12 pathways. These TFs regulate about 29% of the COR genes; they regulate different sets of COR genes but with substantial overlap, which indicates that highly co-regulation of COR genes exists. Functional enrichment analysis of the COR genes regulated by each TF indicated that they are involved in diverse but overlapping biological pathways during cold acclimation. My results shed light on the regulation of COR genes by CBF-independent pathways and also on interactions between different biological pathways in cold acclimation.

In summary, this dissertation provides substantial contributions to the understanding of gene regulatory networks in cold acclimation by integrating photoperiod signals into cold responsive pathways, and by dissecting the coldregulatory pathways. In addition, the knowledge can be potentially applied for crop engineering and improvements. Copyright by CHIN-MEI LEE 2012

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KEY TO ABBREVIATIONS

ABA: abscisic acid

AP2: apetala2

bHLH: basic helix-loop-helix

bp: base pair

bZIP: basic-leucine zipper

CaMV: cauliflower mosaic virus

CAMTA: calmodulin binding transcriptional activator

CBF: c-repeat/ drought-responsive element binding factor

CCA1: circadian clock-associated 1

CFP: cyan fluorescent protein

ChIP: chromatin Immunoprecipitation

CM: conserved motif

COR: cold-regulated

CRT/DRE: c-repeat/drought-responsive element

Cvi: Cape Verde Islands

DEAR1: DREB and EAR motif protein 1

DNA: deoxyribonucleic acid

DREB: dehydration responsive element binding factor

DOF: DNA binding with one finger

EL₅₀: the temperature at which freezing damage results in leakage of 50% of the total

cellular electrolytes

ELA: electrolyte leakage assay

EMAS: electrophoretic mobility shift assay

ERF: ethylene response factor

FR: far-red light

GA: gibberellins

GOLS: galactinol synthase

GUS: beta-glucuronidase

HOS: high expression of osmotically responsive gene

HSF: heat-shock factor

HSP: heat-shock protein

ICE: inducer of CBF expression

ICEr: Inducer of CBF expression region

LD: long-day

LHY: late elongated hypocotyl

HY5: elongated hypocotyl 5

LOS1: low expression of osmotically responsive genes 1

LUC: luciferase

P5CS: delta 1-pyrroline-5-carboxylate synthase

qRT-PCR: quantitative reverse transcription polymerase chain reaction

R: red light

RD29A: responsive to dessication 29a; cor78

PR: plant pathogen-related

PIF: phytochrome interacting factor

PHY: phytochrome

PRR: pseudo-response regulator

PS: photosystem

- QTL: quantitative trait locus
- RIL: recombinant inbred lines
- ROS: reactive oxygen species
- SA: salicylic acid
- SD: short-day
- TAIR: The Arabidopsis Information Resource
- TF: transcription factor
- TOC1: timing of cab expression 1
- TF-OX: overexpression line of transcription factor
- WT: wild type
- ZF: zinc-finger transcription factor, at4g29190
- ZT: zeitgeber time

CHAPTER 1

LITERATURE REVIEW: COLD ACCLIMATION IN PLANTS

Overview of Low Temperature Stress and Cold Acclimation in Plants

Low temperature is one of the environmental factors that regulate multiple developmental processes in plants, but it also has adverse effects on plant growth and survival. In agriculture, chilling (> 0°C) and freezing (< 0°C) stresses cause major economic loss by affecting the production, quality, and biomass of crops. Therefore, understanding how plants cope with low temperature stress has been an important research area for crop improvement.

Under natural selection, many plants have evolved the ability to develop freezing tolerance when exposed to low, non-freezing temperatures, a process known as cold acclimation (1, 2). However, not all plant species have the same ability to survive freezing temperatures after cold acclimation. Fully cold-acclimated red osier dogwood can survive temperatures lower than -50° C, and rye to -30° C, whereas the model plant *Arabidopsis thaliana* is killed at -10° C (3, 4). The freezing tolerance within plant species can also vary, depending on its developmental stage, other environmental signals, and the temperature itself during cold acclimation. The different levels of freezing tolerance result from differences in the transcriptional activity and metabolic changes during cold acclimation (5, 6).

Transcriptional and metabolic adjustments are critical for survival at freezing temperatures (7, 8). The primary site of freezing injury to the plant cell is the plasma membrane, caused by severe cellular dehydration (9). When plant tissues are frozen, water in the extracellular spaces freezes, resulting in lower water potential. As a result, intracellular water moves into the extracellular spaces causing cell dehydration. The

consequences of cellular dehydration and extracellular ice crystal formation are the rupture of membranes, cell wall lesion, and eventually cell death (10). One of the major functions of cold acclimation is to increase the cellular solutes and to stabilize cell membranes to prevent cellular damage. It has been reported that accumulation of cryoprotectants (sucrose, raffinose, and proline), membrane stabilizing proteins (coldregulated proteins (CORs), dehydrins, and heat shock proteins (HSPs)), and induction of plant pathogen-related (PR) proteins prevent dehydration and ice nucleation during cold acclimation (1, 2, 11). In addition, an increase in the unsaturated fatty acid content of membranes, lipid remodeling, and loosening of cell walls occur to maintain membrane integrity at low temperatures (12-14). Secondary freezing damage is induced by cellular dehydration and changes in temperature-sensitive reactions in cells (1, 15). Part of the cold acclimation process is to relieve the stresses that accompany cold. Drought-responsive and ABA-responsive genes are induced to cope with water deprivation (16, 17). Stress caused by cold-induced reactive oxygen species (ROS) can be relieved by increases in the antioxidation enzymes (superoxide dismutase, glutathione peroxide, and glutathione reductase) and antioxidants (glutathione and ascorbic acid) (11, 18). Accumulation of flavonoid and anthocyanin, reduction in components of the light harvesting complexes (LHC) and plastocyanin can mitigate photo-oxidative stress induced by excessive excitation of Photosystem II (PSII) and excessive electron transfer to PSI under low temperatures (19, 20).

Plants also establish physiological adaptation to long-term cold. Remodeling of the photosynthetic capacity has been shown to be an important factor for perennial plants to overwinter (21, 22). Maintaining energy homeostasis by reconfiguration of

energy flow and growth cessation is part of the strategy for plant survival (22, 23). Low temperatures also modulate hormone signaling pathways, such as those of gibberellins (GA), salicylic acid (SA), and auxin, to regulate plant growth (24-26).

CBF Pathway in Cold Acclimation

Many of aforementioned metabolic adjustments and physiological adaptations are brought about by reprogramming the transcription of cold-regulated (COR) genes during cold acclimation (27-30). The CBF (CRT/DRE Binding Factor) pathway is the best studied gene regulatory pathway in cold acclimation (31). In *Arabidopsis*, *CBF1*, *CBF2*, and *CBF3* genes (also known as *DREB1B*, *DREB1C*, and *DREB1A*, respectively) form a tandem repeat on chromosome 4, and their transcripts are induced within 15 minutes of exposure to cold (32). The *CBF* genes encode members of AP2/ERF transcriptional activators, and specifically interact with the CRT/DRE (C-repeat/droughtresponsive element, RCCGAC) elements in the promoters of their target genes (33, 34).

Overexpression of *CBF* affects the expression of approximately 140 COR genes, termed the "CBF regulon", under warm conditions, and the transgenic lines confer freezing and drought tolerance (35, 36). Results from the metabolomic analysis of the CBF-overexpressing lines agree with transcriptome data in that CBF affects multiple key pathways in cold acclimation (5). Some COR genes directly regulated by *CBFs*, such as *COR6.6*, and *COR15a*, encode membrane stabilizing proteins (33, 37). Other *CBF* target genes, such as *GALACTINOL SYNTHASE 3* (*GOLS3*) and *DELTA 1-PYRROLINE-5-CARBOXYLATE SYNTHASE* (*P5CS*) encode key enzymes in raffinose and proline biosynthesis for cryoprotectant production (35, 38).

CBF pathways are conserved among many plant species (39-42). Ectopic expression of *Arabidopsis CBFs* in chilling-sensitive species, such as potato, or rice, increases their freezing tolerance (40, 41, 43). Quantitative trait locus (QTL) analysis of recombinant inbred lines (RIL) from two parental lines with different freezing tolerance ability in *Arabidopsis*, barley and wheat identified a major locus responsible for freezing tolerance, which is co-localized to the CBF locus (44, 45). In *Arabidopsis* Cape Verde Islands (Cvi), an accession originating from the subtropics, lower freezing tolerance is exhibited compared to other accessions. Cvi has a deletion of 1.6 kbp in the *CBF2* promoter, which greatly decreases the cold induction of *CBFs* and hence the CBF regulon (5, 44). In addition, the changes in metabolites induced by overexpression of *CBF3* are largely depleted in Cvi (5). These studies suggest a prominent role of the CBF pathway in cold acclimation.

Regulation of the CBF Pathway in Response to Low Temperature

It is still not understood how plants perceive low temperature, but extensive studies in both cis-acting elements and trans-acting regulators have shed light on the regulation of *CBFs* in response to low temperatures (28, 31, 46). The transcripts of *CBFs* are induced within minutes at 4° C, peak at around 3 h, and then drop off again in cold. Moreover, the transcripts of *CBFs* are degraded rapidly soon after returning to warm temperature (47). Promoter deletion analysis identified a 125 bp promoter region in the *CBF2* promoter that is sufficient to impart cold-induction of *CBF2* expression (47). The 125 bp *CBF2* promoter region contains cold-responsive elements, ICEr1 and ICEr2 (Inducer of *CBF* expression region 1 and 2), which are conserved among *Arabidopsis*

CBF1, *CBF2*, and *CBF3* (47, 48). Further analysis of this promoter region defined 7 conserved motifs (CM1-7) shared between *CBF2* and another cold-induced transcription factor, *ZAT12* (49). CM6 and CM7 partially overlap with ICEr1, and CM2 partially overlaps with ICEr2. CM6 and CM4 have negative regulatory activities, and CM2 functions as both a positive and negative regulatory element. These results indicate that *CBF2* is tightly regulated through different elements.

The trans-acting regulators of CBFs were largely identified by a genetic screen of the *CBF* promoter fused to a *LUC* reporter (50). INDUCER OF CBF EXPRESSION 1 (ICE1), a MYC-like bHLH transcription factor, was identified as interacting with the CANNTG element in the *CBF3* promoter by this strategy (51). Mutation in *ICE1* affects cold induction of *CBF3* but not of *CBF1* and *CFB2*. The transcriptional activation activity of ICE1 is post-translationally regulated by HOS1 and SIZ1 (52, 53). HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1) encodes a RING type E3 ligase for ubiquitination of K403 in ICE1 and its degradation in cold (52, 54). A SUMO E3 ligase, SIZ1, on the other hand, functions as a positive regulator of ICE1 via sumoylation of ICE1 to antagonize ubiquitination of ICE1 by HOS1 (53). ICE1 is also found in wheat and *Camellia sinensis*, suggesting it is a conserved regulator of cold acclimation (55, 56). An ICE1 homolog, ICE2, appears to affect the expression of *CBF1*, but has been less studied (57).

In addition to ICE1, MYB15 has been identified from a mutant screen as a coldinduced transcription factor that negatively regulates *CBF1*, *CBF2*, and *CBF3* by interacting with their promoters (58). Rather than being involved in cold induction of the *CBFs*, the *myb15* mutant showed higher transcript levels of the *CBFs* than WT after 6h

in cold, indicating that MYB15 down-regulates the CBFs after cold induction. There is crosstalk between MYB15 and ICE1 in regulating the *CBFs*. At the protein level, MYB15 interacts with ICE1, and the MYB15 protein is more stable in the *ice1* mutant (58). In addition, disruption of SIZ1-mediated ubiquitination of ICE1 increases the expression of *MYB15* (59).

Another positive regulator of *CBF1* and *CBF2* is *CALMODULIN BINDING TRANSCRIPTIONAL ACTIVATOR 3* (*CAMTA3*) (49). CAMTA3 interacts with the CG-1 element (CGCG) in the CM2 motif of the *CBF2* promoter. In the *camta3* mutant, the expression of *CBF1*, *CBF2*, and *GOLS3* were reduced about 50% at 0°C with no obvious effect on freezing tolerance. The *camta1/ camta3* double mutant, however, is compromised in freezing tolerance. This finding provides a potential link between the CBF pathway and calcium signaling, which is thought to be an early signaling transduction pathway in cold (49, 60).

The transcription of CBFs is tightly regulated. Mutation of *CBF2* enhances the expression of *CBF1* and *CBF3* in the cold, suggesting that *CBF2* has a role in the negative regulation of *CBF1* and *CBF3* (61). Surprisingly, freezing tolerance was found to be decreased in the *cbf2* mutant. Constitutive expression of several cold-induced transcription factors has been shown to negatively regulate *CBFs*. ZAT12 is a cold-induced transcription factor sharing similar expression profile to the *CBFs* in cold. Overexpression of *ZAT12* showed repression of *CBFs* in the cold (62). *DEAR1* is another cold-induced AP2 transcription factor that negatively regulates the *CBFs* (63). The function of these negative regulation could be similar to that of MYB15 in that they prevent overexpression of *CBFs*, which gives rise to stunted plants (35, 36).

Interplay between Cold and the Circadian Clock

The main trigger for cold acclimation is low temperature, but other environmental factors have impacts on acquired freezing tolerance (3, 4). Harmer et al. (64) showed that the expression of *CBFs* is circadian regulated at warm temperatures. The transcript levels of *CBFs* oscillate with a peak around 8h after dawn (zeitgeber time 8; ZT8) and a trough around ZT20. It has been demonstrated that the peak expression pattern is regulated by two central clock regulators, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which bind to the promoters of the *CBFs* at ZT8 (65). The oscillation of *CBF1* and *CBF3* were eliminated in the *cca1/ lhy* double mutant. The cycling expression of *CBF2* is largely reduced in the *cca1/ lhy* double mutant, but with some residual activity. The trough is partially regulated by PHYTOCHROME INTERACTING FACTOR 7 (PIF7), a bHLH transcription factor which binds to the G-box, and which overlaps with the CM6 motif in the CBF2 promoter. The expression of *CBFs* is elevated around ZT15 in the *pif7-2* mutant under circadian conditions (66).

The cold responsiveness of the *CBFs* is "gated" by the circadian clock (67). This means that the CBF transcript levels are time-dependent during the day; for example, cold treatment given at ZT4 induces higher *CBF* expression than at ZT16. Mutation in both *CCA1* and *LHY* disrupts the gating effects on the *CBFs* and impairs freezing tolerance (65). Another line of evidence supporting the importance of the clock on freezing tolerance was obtained using the *prr5/prr7/prr9* triple mutant (*d975*) (68). In the *d975* mutant, the oscillated expression of *CCA1* and *LHY* is disrupted, but with

intermediate expression levels. Up-regulation of the CBF pathway and accumulation of galactinol, raffinose, and proline in this mutant lead to an increase in freezing tolerance.

Conversely, low temperature has an impact on circadian rhythm (69, 70). Transcription profiling of plants undergoing cold stress and other abiotic stresses showed that the cold-responsive genes lost their oscillating expression patterns in the cold, which is a unique phenomenon among abiotic stresses (71). In chestnut, the expression of several central clock regulators, including CCA1, LHY, TOC1, and PRRs stop their oscillated expression pattern in winter (70, 72). Recently, Bieniaawska et al. (69) surveyed the clock genes in Arabidopsis and showed that the oscillation of many central clock regulators are dampened in the cold under diurnal conditions, and that they are arrhythmic in the cold under continuous light. The exception to this is LUX ARRHYTHMO (LUX), whose expression continues to cycle under both conditions. Transcriptome and metabolome analyses further showed that cold affects major clockregulated metabolic pathways, such as carbohydrate metabolism and amino acid biosynthesis (30). In the cold under diurnal conditions, approximately 80% of the tested metabolites are still cycling but their phase is shifted. The oscillated expression of genes encoding enzymes involved in biosynthetic pathways of the selected metabolites is largely dampened. Most of the tested metabolites and the expression of genes regulating their biosynthesis are arrhythmic at low temperature under continuous light conditions. Mutations in both CCA1 and LHY have a negative impact on freezing tolerance; however, the biological significance of disrupting the cycling expression of central clock regulators is not understood (73). The circadian clock regulates major metabolic pathways (64, 74), and disruption of the clock may provide an efficient

remodeling of metabolism in the cold. Some metabolites, however, still cycle in the cold, and this finding raises a question as to whether the circadian clock is totally disrupted by the cold. There may be another set of regulators for maintaining the circadian clock in the cold.

Regulation of Freezing Tolerance by Light

Light can regulate freezing tolerance in many different ways. High light treatment has been shown to increase freezing tolerance and induces COR genes in winter wheat and rye without cold treatment (75, 76). This phenomenon is due to photoinhibition, a process whereby light energy absorbed in the photosynthetic light processes exceeds the energy demand of the dark processes, which then leads to over-reduction of PS II and subsequent inhibition of the photosynthetic capacity (77). Photoinhinition and the ROS stress that accompanies it are common mechanisms shared by cold acclimation and high light acclimation (22).

Cold, in conjunction with light treatment, is required for full-strength freezing tolerance in *Arabidopsis* (78). Transcriptome profiling has shown that twice as many genes are cold-induced in the light as in the dark (79). The major differences in up-regulated genes in the light and dark are the oxidative stress-related genes, photosynthesis-related genes, ABA biosynthesis pathway genes, and genes involved in the production of protective molecules, such as phenylpropanoids. How does the light signal integrate into the cold pathway to regulate COR genes? Recently, Catala et al. (80) identified HY5, a master regulator of light signaling, as being involved in regulating about 100 COR genes independent of the CBF pathway under low temperature

conditions in the light. The expression of *HY5* is induced by cold, and low temperature stabilizes the HY5 protein by preventing its proteasome-mediated protein degradation. The *hy5* mutant displays a freezing sensitive phenotype, which is due to a decrease in anthocyanin and antioxidant production.

Regulation of Freezing Tolerance by Seasonal Signals in Plants

In nature, plants perceive seasonal signals in addition to drop in temperature before the onset of winter. Annual changes in light quality, in particular red (R) and farred (FR) light, occur during the twilight period when the solar elevation is less than 10°. A decrease in R light, or low R/FR ratio, in autumn has been suggested to be a signal in which plants anticipate winter (81). In addition, shortening day-length in autumn has been reported to prepare plants for overwintering (3). It is well-established in woody species that the acquired freezing tolerance is obtained first by a shortening of photoperiod, and thereafter by a decrease in temperature (82, 83).

Plants sense light signals through photoreceptors. R and FR light are perceived through a set of phytochrome photoreceptors, PHYTOCHROME A to E (PHYA to PHYE) (84, 85). The phytochromes are dimers of two chromoproteins consisting of polypeptide subunits that carry a tetrapyrrole chromophore in each chromoprotein. They exist as two inter-convertible forms: the biologically inactive R-absorbing Pr and the active FR-absorbing Pfr. R light converts Pr to Pfr, whereas FR converts Pfr back to Pr. Phytochromes regulate a wide range of biological pathways through interacting with other proteins, including PHYTOCHROME INTERACTING FACTORS, or PIFs, a subfamily of bHLH transcription factors. The functions of phytochromes are more than

just sensing changes in light quality; PHYA and PHYB have also been shown to be involved in photoperiodic regulation of flowering time, dormancy, bud set, and tuberization in plants (86).

Historically, the effects of photoperiod on freezing tolerance were first documented in woody species. It has been demonstrated that a short-day (SD) photoperiod and low temperature are more effective in establishing freezing tolerance than are long-day (LD) and cold in many woody species, including red osier dogwood, aspen, and birch (3, 87). The SD-induced increase in ABA content and the subsequent induction of dehydrins have been shown to enhance freezing tolerance (88, 89). Interestingly, the effects of SD on freezing tolerance were hampered by 15 minutes of R light treatment, and these inhibitory effects are reversible by subsequent FR light treatment (87, 90, 91). These results suggest that phytochromes are involved in photoperiodic regulation of freezing tolerance in woody species. It has been shown that overexpression of oat PHYA in aspen leads to a SD-insensitive phenotype, including loss of dormancy and growth cessation (88). However, overexpression of PHYA in Arabidopsis has both positive and negative effects on pathways mediated by other phytochromes, including PHYB (92). In potato, overexpression of PHYA resulted in a photoperiod-insensitive tuberization response, which was caused by disruption of the circadian clock. Therefore, the mechanism of how plants sense photoperiod through phytochrome in regulating freezing tolerance is far from being completely understood.

The effect of SD on freezing tolerance is not limited to woody species. Similar phenomena have been reported in spring wheat, barley, *Arabidopsis* and *Gaura coccinea* (44, 93-95). Studies in barley and wheat suggested that SD- and cold-induced

vernalization can affect expression of *CBFs* or *COR* genes, which may be responsible for the freezing tolerance phenotype (93, 96). QTL mapping of RILs from Ler (Landsberg *erecta*) and Cvi showed that the QTLs responsible for freezing tolerance are different under SD and LD conditions, except for the CBF locus (44). The photoperiodic regulation of freezing tolerance was observed in both woody species and herbaceous species. Is this response regulated by the same mechanism? How does photoperiod regulate freezing tolerance? These important questions remain to be elucidated.

Franklin and Whitelam first demonstrated that light quality alone (12h light and 12h dark photoperiod, a neutral day-length condition) affects freezing tolerance in *Arabidopsis* at ambient temperature (81). They showed that the expression of several CBF regulon genes, *COR15a*, *COR47*, *COR78*, and *KIN1*, are induced under low R/FR ratio at 16°C but not at 22°C, a response mediated by PHYB and PHYD. Furthermore, low R/FR ratio induced higher expression of *CBF1-3* than high R/FR ratidid o under circadian conditions. Low R/FR ratio treatment at 16°C is sufficient to increase freezing tolerance in wild type *Arabidopsis* (81). These results showed that light quality regulates the CBF pathway and therefore freezing tolerance. It further indicated that the CBF pathway could be an integration point for light signals.

CBF-independent Pathways in Cold Acclimation and Freezing Tolerance

Freezing tolerance in plants is regulated by multiple pathways in addition to the CBFs (38). Overexpression of CBF at warm temperatures is sufficient for development of freezing tolerance, but it cannot lead to full-strength freezing tolerance induced by cold acclimation (35, 36). In addition, transcription profiling has shown that

overexpression of CBF affects about 140 COR genes, while there are thousands of COR genes that are affected by cold (28, 38, 62). These findings indicate that other cold-regulatory pathways in addition to CBF pathway exist.

Several regulators have been identified which regulate CBF-independent pathways. The esk1 (eskimo1) mutant was identified through a mutant screen. esk1 showed constitutive freezing tolerance, which is due to the accumulation of high levels of proline (97). Transcriptome analysis indicated that about 130 COR genes were affected in esk1, and around 40 of them overlap with the CBF regulon (98). ESKMO1 encodes a DUF231 domain protein of unknown function; it is currently not understood how ESKMO1 regulates COR genes (98). The hos9 mutant was identified as constitutively expressing RD29A in mutant screens of cold-induced RD29A promoterreporter lines, and the mutant displayed an increase in freezing tolerance (99). HOS9 encodes a putative homeodomain transcription factor. Transcription profiling has shown that *hos9* affects around 40 COR genes, none of which overlaps with the CBF regulon. As described in the previous section, HY5 mediates the light regulation of around 100 COR genes, but these genes do not overlap with the CBF regulon either (100). A coldinduced nuclear protein, GIGANTEA (GI), has pleiotropic effects on the circadian clock and flowering (101, 102). The *gi* mutant exhibits a freezing sensitive phenotype, but expression of the CBFs or the CBF regulon are not affected in the mutant (103). Further analysis showed that failure to accumulate soluble sugar as a cryoprotectant in the gi mutant may account for the phenotype (104). These results reveal that the regulation of COR genes is interdigiteted between CBF-dependent and CBFindependent pathways.

Gene Regulatory Network of Cold Acclimation

Plant response to low temperature is a complex process: integration of multiple external signals to establish freezing tolerance, intensive interactions between biological pathways in cold acclimation, and multiple levels of regulation within a pathway. Understanding the dynamics of cold responses in plants needs to be in the context of systems biology. Ideker et al. (105) proposed systems biology as a framework for modeling biological systems by integrating genome-wide measurements to understand the properties of biological systems: from gene to protein and metabolites, their functions, pathways and then their response to changing environments. For the first step of the systems approach, the components of the interaction within the network have to be identified and characterized in a global manner. The next step is to perturb the system and monitor its responses on a genome-scale. Finally, the data are collected to model the system and novel interactions can be inferred and examined (105).

Top-down strategies have accumulated a wealth of genomic-scale data, such as transcriptome, proteome, and metabolome analyses in wild type and mutants, which facilitate the discovery of complex biological pathways in cold stress responses. Comparison of cold-responsive genes to those regulated by other conditions has revealed novel interaction between pathways, such as cold and the clock (106). Association network modeling based on the co-expression analysis of microarray data across various conditions has predicted several regulatory modules in different biological processes (107, 108). Whether the predicted regulators and pathways are involved in cold stress responses still awaits experimental validation. Vogel et al. (62)

analyzed the expression kinetics of COR genes with cold transcriptome data and identified *ZAT12* as a cold-induced transcription factor in addition to the CBF pathway. Overexpression of *ZAT12* confers freezing tolerance in a whole plant freeze test, which may result from an increase in tolerance to ROS stress (109). Chawade et al. (110) combined the time-dependent expression information and consensus transcription factor binding sites to model gene regulatory networks in the cold. This strategy may give several false positive interactions. For example, not all the motifs in the promoter are involved in controlling cold responses. Additionally, the consensus binding sites can be bound by multiple transcription factors within the same family or in different transcription factor families.

The bottom-up strategy to identify components of gene regulatory networks is limited, partly due to the lack of systematic strategies to capture the cold responses and to identify the components in these responses. Mutant screens have been a popular genetic strategy to identify regulators in biological pathways. However, current methods, such as survival tests and electrolyte leakage assays are laborious and not quantitative. Ehlert and Hincha (111) reported a new strategy that measures chlorophyll florescence. It has been shown that chlorophyll florescence is correlated to leaf damage. With this method, a large-scale screen would be feasible, but this method cannot capture all the cold responses. Insights have been gained into regulation of the CBF pathway by targeted genetic screens with the *CBF* or *RD29A* promoter fused to a reporter gene, but fewer advances have been made with the CBF-independent pathways (50).

From transcription profiling experiments, more than a thousand COR genes have been identified (38, 62, 112-115). The expression of these COR genes revealed a

series of transient waves, suggesting transcriptional regulation cascades (28, 38, 62). Our understanding of the regulation of the COR genes by early cold-induced transcription factors is limited to CBF1-3 and ZAT12 (62). To understand the regulation of biological pathways and the interactions between pathways during cold acclimation, new regulators for each pathway in cold acclimation will need to be identified, and their effects on COR genes will have to be studied systematically.

CHAPTER 2

PHOTOPERIODIC REGULATION OF THE CBF COLD ACCLIMATION PATHWAY AND FREEZING TOLERANCE IN ARABIDOPSIS THALIANA

Abstract

The CBF pathway has a major role in plant cold acclimation, the process whereby certain plants increase in freezing tolerance in response to low nonfreezing temperatures. In Arabidopsis thaliana, this pathway is characterized by rapid cold induction of CBF1, CBF2 and CBF3, which encode transcriptional activators, followed by induction of CBF-targeted genes that impart freezing tolerance. At warm temperature, CBF transcript levels are low, but oscillate due to circadian regulation with peak expression occurring at 8 h after dawn (ZT8). Here we establish that the CBF pathway is also regulated by photoperiod at warm temperature. At ZT8, CBF transcript levels in short-day (SD; 8 h photoperiod) plants were 3-5 fold higher than in long-day plants (LD; 16 h photoperiod). Moreover, the freezing tolerance of SD plants was greater than that of LD plants. Genetic analysis indicated that phytochrome B (PHYB) functions with two phytochrome interacting factors, PIF4 and PIF7, to down-regulate the CBF pathway and freezing tolerance under LD conditions. Down-regulation of the CBF pathway in LD plants correlated with higher PIF4 and PIF7 transcript levels and greater stability of the PIF4 and PIF7 proteins under LD conditions. Our results indicate that during the warm LD growing season, the CBF pathway is actively repressed by PHYB, PIF4 and PIF7 thus mitigating allocation of energy and nutrient resources towards unneeded frost protection, and that this repression is relieved by shortening day-length resulting in up-regulation of the CBF pathway and increased freezing tolerance in preparation for coming cold temperatures.

Introduction

Plants vary greatly in their ability to survive freezing temperatures. Whereas plants from tropical and subtropical regions are generally killed by the slightest freeze. plants from temperate regions exhibit varying degrees of freezing tolerance (4). For instance, Arabidopsis thaliana (hereafter referred to as Arabidopsis) and wheat have a maximum freezing tolerance of about -10° C and -20° C, respectively, and hardy deciduous trees can survive freezing below -50°C. However, the freezing tolerance of frost hardy plants is not a constant property; it changes over the course of the year in response to changing environmental conditions. The primary factor is low temperature (3, 4). When winter rye is grown at warm temperature, plants are killed upon freezing at about -5°C, but upon exposure to low nonfreezing temperatures, they can survive freezing below -20°C. The molecular basis for this phenomenon, known as cold acclimation, is not completely understood, but includes changes in membrane cryobehavior, the production of cryoprotective proteins, and the biosynthesis of low molecular weight cryoprotectants such as sucrose, raffinose and proline (1, 116).

Many of the biochemical and metabolic changes that occur in response to low temperature and contribute to an increase in freezing tolerance involve changes in gene expression (28, 29, 117). The best understood cold regulatory pathway with a role in freezing tolerance is the CBF pathway of Arabidopsis (28, 31, 118). *CBF1*, *CBF2* and *CBF3* (also known as *DREB1B*, *DREB1C*, and *DREB1A*, respectively) encode closely related members of the AP2/ERF family of DNA binding proteins that recognize the CRT/DRE DNA regulatory element, RCCGAC (33, 34). Within minutes of transferring

Arabidopsis plants to low temperature, *CBF1*, *CBF2*, and *CBF3* are induced followed at about 3 h by induction of CBF-targeted cold-regulated (COR) genes, referred to as the CBF regulon (28). Constitutive overexpression of *CBF1*, *CBF2*, or *CBF3* at warm temperature leads to constitutive expression of the CBF regulon and a marked increase in freezing tolerance (34, 36, 119). Although the CBF pathway is not as well studied in other plant species, it has been established that cold-inducible *CBF* genes are highly conserved among higher plants and that *CBF* overexpression increases the freezing tolerance in plants ranging from closely related canola to distantly related poplar and wheat (120-123).

Photoperiod is another environmental factor that regulates freezing tolerance, a phenomenon that is well documented in woody deciduous trees (3, 83). As summer turns to fall, these plants sense the shortening day-length and initiate developmental programs that result in the cessation of growth and an increase in freezing tolerance that can be more than 10°C in some hardy species. As the season continues to progress and the temperatures become cold, the plants sense the low nonfreezing temperatures and increase an additional 40°C or more in freezing tolerance (3, 124).

The molecular basis for photoperiodic regulation of freezing tolerance is not well understood. However, the increase in freezing tolerance that occurs in response to short-day in red-osier dogwood and other perennial woody tree species is prevented if the plants are briefly exposed to red (R) light during the nighttime, but not if the R light exposure is followed by brief exposure to far-red (FR) light (87, 125). These are classic indicators of a phytochrome-mediated response (126). Phytochromes are proteins that have a tetrapyrrole chromophore that exists in two interchangeable forms covalently

linked to their N-terminal end: the R-light absorbing form, designated Pr, and the FRlight adsorbing form, designated Pfr. The Pr form, which is inactive, is converted to the active Pfr form by exposure R light, and is converted back to the inactive Pr form by exposure to FR light. The fact that the freezing tolerance of short-day grown red-osier dogwood is reduced when the plants are exposed to R light during the night suggests that an active Pfr phytochrome represses freezing tolerance.

Whereas photoperiodic regulation of freezing tolerance is recognized as a fundamental feature of cold acclimation in woody plants, there is little evidence for the phenomenon in herbaceous plants (3). Pietsch et al. (95) found that the freezing tolerance of Gaura coccinea, a perennial herbaceous species, increases about 3°C when plants are exposed to short-day photoperiods, but beyond this, photoperiodic regulation of freezing tolerance at warm growth temperature is poorly documented in perennial and annual herbaceous species. However, similar to what has been reported in woody plants, Franklin and Whitelam (81) showed that phytochromes have a role in regulating freezing tolerance in Arabidopsis. When plants were grown at 16°C (but not at 22°C) under a 12 h photoperiod, the freezing tolerance and transcript levels for three CBF target genes—COR15a, COR15b and KIN1—were greater in plants exposed to a low R/FR light ratio than if they were exposed to a high R/FR light ratio. Also, transferring plants to constant light and exposing them to a low R/FR light ratio for 2 h in the morning resulted in increased transcript levels for CBF1, CBF2 and CBF3. These results suggested that a Pfr form of one or more phytochromes repressed the expression of the CBF pathway. Indeed, at 16°C, under high R/F light, both phyB and

phyD mutations resulted in increased transcript levels for *COR15a*, a CBF target gene, and the *phyD* mutation resulted in greater freezing tolerance (the effects of a *phyB* mutation on freezing tolerance were not reported).

Arabidopsis has proven to be a powerful model plant to study the regulation of freezing tolerance by low temperature (28, 127). Here we show that it is also a powerful model to study photoperiodic regulation of freezing tolerance. Our results indicate that Arabidopsis plants increase in freezing tolerance in response to a short-day photoperiod, that this regulation involves photoperiodic regulation of the CBF pathway, and that this regulation is mediated by the PHYB photoreceptor and two PIF transcription factors with which PHYB physically interacts, PIF4 and PIF7 (128, 129).

Results

Freezing Tolerance is Regulated by Photoperiod

To determine whether the freezing tolerance of Arabidopsis (Col-0) is regulated by photoperiod, we grew plants under short-days (SD; 8 h light, 16 h dark) and longdays (LD; 16 h light, 8 h dark) and compared their freezing tolerance using the electrolyte leakage assay. The results indicated that the freezing tolerance of the SD plants was greater than that of the LD plants; whereas the EL₅₀ (the temperature at which freezing damage results in leakage of 50% of the total cellular electrolytes) of the LD plants was about -3°C, the SD plants had an EL₅₀ of about -5.5°C (Fig. 2.1*A*).

In these experiments, the SD and LD plants were tested at the point that they each had about 8 true leaves. However, to produce this number of leaves, the SD and
LD plants were grown for 5 weeks and 3 weeks, respectively. To address the possibility that differences in age were the cause of the observed differences in freezing tolerance, we grew plants under SD or LD conditions and then switched their photoperiod and tested their freezing tolerance; all plants again had about 8 true leaves. When plants were grown under SD for 3 weeks and transferred to LD for 2 weeks, they had the same freezing tolerance as plants grown for 3 weeks under LD (Fig. 2.1*B*). When plants were grown under LD for 2 weeks and transferred to SD for 2 weeks, they had the same freezing tolerance as plants grown under SD for 5 weeks (Fig. 2.1*B*). Thus, regardless of the direction of the day-length shift or total age of the plants, the freezing tolerance of the plants was determined by the final 2-week treatment; SD to LD produced the same freezing tolerance as constant LD treatment, and LD to SD produced the same freezing tolerance as constant SD treatment. From these results, we concluded that the freezing tolerance of Arabidopsis is regulated by photoperiod.

The CBF Pathway is Regulated by Photoperiod

Given the prominent role of the CBF pathway in cold acclimation, we asked whether the *CBF* genes were expressed at different levels in SD and LD plants. Previous studies (64, 66, 130) established that *CBF1*, *CBF2* and *CBF3* are regulated by the circadian clock and that the transcript levels for each gene peaks at about 8 h after dawn, a time referred to as ZT8 (zeitgeber time 8). Our results were consistent with these findings; the transcript levels for each *CBF* gene peaked at about ZT8 under both SD and LD conditions (Fig. 2.2). However, the *CBF* transcript levels at ZT8 were about 3 to 5 fold higher in the SD plants as compared to the LD plants. The transcript levels

for two CBF regulon genes, *COR15a* and *GOLS3*, also oscillated, having peak expression between ZT8 and ZT12, and at their peak the transcript levels for these two genes were about 5 fold higher in the SD plants (Fig. 2.2).

To address the possibility that differences in plant age accounted for the differences in CBF and CBF regulon gene transcript levels in the SD and LD plants, we transferred LD plants to SD conditions, and SD plants to LD conditions, and determined their transcript levels. First, we determined the transcript levels of CBF2 at ZT8 at 0, 1, 3, 5, and 7 d after the switch in photoperiod. The results indicated that over the course of the week, the lower level of CBF2 transcripts initially observed in the LD plants rose to the level observed in the SD plants, and that the higher level of CBF2 transcripts initially observed in the SD plants, decreased to the level observed in the LD plants (Fig. 2.3A). We then determined the transcript levels of CBF1, CBF2, CBF3 and two CBF regulon genes, COR15a, and GOLS3, at ZT8 in SD and LD plants, in SD plants transferred to LD for 7 d, and in LD plants transferred to SD for 7 d. The results indicated that with each gene, the lower transcript levels initially observed in the LD plants rose to those observed in the SD plants after transfer to SD, and that the higher transcript levels initially observed in the SD plants, fell to those observed in the LD plants after transfer to LD (Fig. 2.3B). These results indicated that the CBF pathway is regulated by photoperiod and that the greater freezing tolerance of the SD plants was due, at least in part, to greater expression of the CBF pathway under SD conditions. The results also showed that the difference in CBF pathway expression produced by SD and LD conditions was reversible during vegetative growth.

Photoperiodic Regulation of *CBF*2 Involves a G-box Motif within the *CBF*2 Promoter

The photoperiodic control of CBF transcript levels could involve either transcriptional or posttranscriptional regulatory mechanisms or both. To determine whether transcriptional mechanisms were involved, we asked whether the CBF2 promoter included DNA regulatory elements that were responsive to photoperiod. Previous studies showed that the region of the CBF2 promoter from -189 to -35 relative to the transcription start site (this region is numbered -207 to -53 in the current TAIR10 database; hereafter we use TAIR10 designations for sequence locations) included elements that could impart both cold (47, 49) and circadian (67) regulation when fused the GUS reporter gene. We therefore tested this region for photoperiodic regulation. We fused the CBF2 promoter region from -207 to +134 to the GUS reporter gene (WTpro) (Fig. 2.4A), transformed the construct into Arabidopsis, and determined the level of GUS transcripts in SD and LD grown plants (Fig. 2.4B). The results indicated that the WT-pro construct produced peak levels of GUS transcripts at ZT8 in both SD and LD grown plants, but that the peak was about 3 fold greater in the SD plants. These results were consistent with the -207 to + 134 *CBF2* promoter fragment including a regulatory motif that was responsive to photoperiod.

Previous studies showed that the *CBF2* promoter region between -207 and -53 bp included a G-box motif, CACGTG (-112 to -107) that imparted negative regulation in plants that were grown at warm temperature and in plants that were exposed to low temperature (49, 66). Therefore, we asked whether this motif was also involved in photoperiodic regulation. We mutated the G-box sequence within the WT-pro construct

(Gmut-pro) (Fig. 2.4*A*), transformed the construct into Arabidopsis, and determined the GUS transcript levels under SD and LD conditions. As with the WT-pro construct, the GUS levels for the Gmut-pro construct peaked at ZT8 in both SD and LD grown plants (Fig. 2.4*B*). However, whereas the GUS transcript levels produced by the WT-pro construct were greater in SD plants, the levels produced by the Gmut-pro construct were approximately the same in the SD and LD grown plants (Fig. 2.4*B*). These results were consistent with the G-box having a role in photoperiod regulation of *CBF*2.

To confirm this result, we determined the GUS transcript levels at ZT8 for the WT-pro and Gmut-pro constructs in 8 independent transgenic lines grown under SD and LD conditions (Fig. 2.4*C*). The results indicated that the GUS transcript levels obtained with the WT-pro construct were, on average, 2-fold higher in the SD plants as compared to LD plants, and that this difference was eliminated when the G-box was mutated (Fig. 2.4*D*). In addition, the results indicated that mutation of the G-box resulted in higher-level expression of the reporter gene in both SD and LD grown plants (Fig. 2.4*E*), a finding that was consistent with the element having a repressive effect.

PIF4 and PIF7 Repress Expression of the CBF Pathway under LD Conditions

Kidokoro et al. (66) found that the PIF7 transcription factor binds *in vitro* to the Gbox within the *CBF2* promoter (the G-box present in the WT-pro GUS fusion described above) and represses expression of the *CBF* genes during the subjective night phase in circadian regulation experiments (i.e., the plants were shifted from a day-night cycle to constant light and gene expression was determined). Thus, we considered PIF7 to be a candidate for mediating photoperiodic control of *CBF2* expression. In addition, we

considered PIF4 a candidate as it has been reported to physically interact with PIF7 (66) and like other PIFs, it binds to G-box and related E-box (CANNTG) motifs (128).

To test whether PIF4 or PIF7 were involved in photoperiodic regulation of *CBF2*, we asked whether *pif4* or *pif7* null mutations affected the patterns of *CBF2* expression under LD or SD conditions. Our results indicated that neither of the single mutations had an effect; *CBF2* transcript levels peaked at ZT8 in WT, *pif4* and *pif7* plants and were about 3-fold higher in SD plants than in LD plants (Fig. 2.5 *A* and *B*). However, the *pif4 pif7* double mutation eliminated the photoperiodic regulation of *CBF2*; at ZT8, *CBF2* transcript levels in *pif4 pif7* double mutant plants grown under LD conditions were the same as in WT plants grown under SD conditions (Fig. 2.5*C*). The *pif4 pif7* double mutation also eliminated the differences in transcripts levels at ZT8 observed for *CBF1*, *CBF3*, *COR15a* and *GOLS3* in WT plants grown under SD and LD conditions (Fig. 2.5*D*). These results indicated that PIF4 and PIF7 function redundantly to repress expression of the CBF pathway under LD conditions.

The lower level of CBF expression under LD conditions could have resulted from higher-level expression of *PIF4* and *PIF7* in plants grown under LD. Indeed, we found that the transcript levels for *PIF4* and *PIF7* oscillated over the course of the day under both LD and SD conditions, peaking at ZT8 and ZT4 respectively, and that the levels for both genes were higher under LD conditions (Fig. 2.6*A*). In addition, the steady-state accumulation of the PIF4 and PIF7 proteins was slightly greater under LD conditions. This was determined by examining the accumulation of TAP-tagged PIF4 and CFP-tagged PIF7 in transgenic plants carrying these protein fusions placed under control of the CaMV 35S promoter. Whereas the transcript levels for the two transgenes were not

affected by photoperiod (Fig. 2.6*C* and *D*), the protein levels of both PIF4-TAP and PIF7-CFP at ZT8 were about two-fold higher in LD plants as compared to SD plants (Fig. 2.6*B*). Additional experiments indicated that PIF4-TAP and PIF7-CFP proteins were functional repressors; in the higher expressing lines, both PIF4-TAP and PIF7-CFP reduced *CBF2* transcript levels by about 50% at ZT8 under SD conditions (Fig. 2.7 *A* and *B*); similar results were obtained testing *CBF2* expression over a 24 h growth period (Fig. 2.7*C*-*F*). Under LD conditions, PIF4-TAP also reduced *CBF2* transcript levels by about 50%, whereas PIF7-CFP had little or no effect suggesting that the endogenous PIF7 levels were saturating in regard to *CBF2* repression under LD conditions.

Finally, using the electromobility shift assay we established that both PIF4 and PIF7 could bind to G-box motifs in the *CBF1* and *CBF2* promoters and the E-box motif in the *CBF3* promoter (Fig. 2.8). Moreover, the results of chromatin immunoprecipitation (ChIP) experiments indicated that the PIF7-CFP protein bound at the CBF locus at ZT8 in LD plants. In test experiments using antibody against CFP, significant enrichment of PIF7-CFP was detected throughout the CBF locus (Fig. 2.9*A*); in mock experiments using non-immune serum, no enrichment was observed (Fig. 2.9*B*).

PHYB is Required for Photoperiodic Regulation of the CBF Pathway and Freezing Tolerance

PHYB is known to physically interact with both PIF4 and PIF7 (131, 132). Thus, we asked whether PHYB was required for photoperiodic regulation of the CBF pathway.

Our results indicated that it was. Under LD conditions, the transcript levels for *CBF2* were about 3-fold higher at ZT8 in plants carrying a *phyB* null mutation than they were in WT plants, and matched the *CBF2* transcript levels observed in WT plants grown under SD (Fig. 2.10*A*). This was also true for *CBF1*, *CBF3* and the CBF regulon genes *COR15a* and *GOLS3*; under LD conditions at ZT8, the transcript levels for these genes were about 3-fold higher in *phyB* plants than in WT plants and approximated the transcript levels in WT plants grown under SD (Fig. 2.10*B*). Consistent with these results, the *phyB* mutation eliminated the photoperiodic regulation of freezing tolerance; the freezing tolerance of *phyB* plants grown under LD conditions was equal to that of WT plants grown under SD (Fig. 2.10*C*).

Discussion

Here we show that Arabidopsis, like woody perennial trees, can sense shortening day-length as a harbinger of coming cold temperatures and respond by increasing in freezing tolerance. Although the increase that we observed, about 2° C, is modest in comparison to the increase that typically occurs in woody tree species, it is about the same as that reported for the perennial herbaceous species *G. coccinea* (95). Moreover, it is a considerable portion of the maximum increase in freezing tolerance that occurs in Arabidopsis in response to low temperature, which is about 6° C (133, 134). Thus, it is likely that the SD-induced increase in freezing tolerance that occurs in Arabidopsis has adaptive value in nature protecting plants against sudden early autumn

frosts. It will be of interest to determine whether there is significant natural variation in

photoperiodic regulation of freezing tolerance among Arabidopsis accessions and if there is, to understand the relationship of these differences to the environmental conditions that characterize the geographical locations from where the accessions originate. It will also be of interest to determine whether photoperiodic regulation of freezing tolerance has been overlooked as a common feature of cold acclimation in frost hardy herbaceous plants.

Our results indicate that the increase in freezing tolerance that occurs in response to SD conditions is due to a loss of negative regulation of freezing tolerance under LD conditions. In particular, our results show that PHYB mediates repression of freezing tolerance under LD conditions and that this is due, at least in part, to PHYBmediated repression of the CBF pathway. The key findings supporting these conclusions are two-fold. First, whereas the freezing tolerance of LD WT plants was about 2°C less than that of SD WT plants, the freezing tolerance of LD *phyB* plants was equal to that of SD WT plants (Fig. 2.10C). Second, whereas the transcript levels for CBF1, CBF2, and CBF3 and downstream CBF regulon genes were lower in LD WT plants than they were in SD WT plants, they were about the same in LD phyB plants and SD WT plants (Fig. 2.10A and B). This down-regulation of the CBF pathway under LD conditions could have adaptive value in at least two ways. One is that it would diminish the allocation of energy and nutrient resources towards unneeded frost protection during the active growing season. In addition, it would mitigate CBF-induced retardation of growth during the growing season. Achard et al. (24) have shown that activation of the CBF pathway results in up-regulation of *gibberellin 2-oxidase* genes causing a decrease in the levels of active gibberellins. This, in turn, results in an

increase in the levels of DELLA proteins, a small family of regulatory proteins that inhibit growth (135). Achard et al. (24) have presented evidence that the CBF-programmed repression of growth caused by the DELLA proteins contributes to the increase in freezing tolerance that occurs with cold acclimation.

How does PHYB mediate repression of the CBF genes? A simple working model is suggested from what is known about phytochrome signal transduction and our results indicating a role of PIF4 and PIF7 in regulating the CBF pathway. It is well established that exposure of plants to white light converts the inactive Pr form of PHYB to the active Pfr form, which rapidly moves from the cytoplasm into the nucleus where it interacts with PIF transcription factors to alter gene expression (128, 129). We propose that PHYB is converted to the Pfr form in the morning, moves into the nucleus where it interacts with either homo- or heterodimers of PIF4 and PIF7 (or both), and that the PHYB-PIF4/PIF7 complexes bind to the promoters of the CBF genes at the G-box and E-box motifs and down-regulate their transcription. This model (Fig. 2.11) is supported by multiple findings. First, it has been shown that PIF4 and PIF7 form homo- and heterodimers (66, 136) and that they physically interact with the Pfr form of PHYB (131, 132). Second, our results indicate that PIF4 and PIF7 repress expression of the CBF genes; whereas the peak transcript levels for CBF1, CBF2, and CBF3 and downstream CBF regulon genes were about 3-5 fold lower in LD WT plants than in SD WT plants, they were about the same in LD *pif4 pif7* double mutant plants and SD WT plants (Fig. 2.5). Single *pif4* and *pif7* mutations did not affect expression of the CBF genes indicating that PIF4 and PIF7 act redundantly to repress expression of the CBF pathway (Fig. 2.5). This conclusion is further supported by our observations that: constitutive

overexpression of PIF4-TAP and PIF7-CFP reduced the transcript levels of *CBF2* at ZT8 in SD plants (Fig. 2.7); both PIF4 and PIF7 bind *in vitro* to G-box and E-box motifs within the *CBF* locus (Fig. 2.8); the G-box at position -112 to -107 of the *CBF2* promoter is involved in photoperiod-regulated transcription of *CBF2* (Fig. 2.4); and PIF7 can bind *in vivo* throughout the CBF locus at ZT8 under LD conditions (Fig. 2.9).

Although this working model is strongly supported by our results, a key aspect of it—that the Pfr form of PHYB interacts with PIF4 to form a complex that represses expression of the *CBF* genes—would appear to be in conflict with results indicating that the interaction of PHYB-Pfr with PIF4 results in degradation of PIF4 (137). It is true that the interaction of PHYB-Pfr with PIF7 does not lead to degradation of PIF7 (131), and thus, interaction of PHYB-Pfr with heterodimers of PIF4 and PIF7 might result in stable protein complexes. However, this would not explain our observed repression of the *CBF* genes in the *pif7* mutant (Fig. 2.5). In our model, this repression would involve interaction of PHYB-Pfr with PIF4. A detailed analysis of the PHYB and PIF protein complexes that are physically present at the *CBF* locus under SD and LD photoperiods will be required to resolve this issue.

Whereas our results clearly establish a role for PHYB, PIF4 and PIF7 in repressing the expression of *CBF1*, *CBF2* and *CBF3*, they only provide a partial explanation for why the CBF pathway is repressed to a greater extent under LD conditions than under SD conditions. Our results indicate that *PIF4* and *PIF7* transcripts peak at about a two-fold higher level under LD conditions (Fig. 2.6*A*) and that the PIF4 and PIF7 proteins are about two-fold more stable under LD conditions (Fig. 2.6*B*). These results suggest that greater levels of the PIF4 and PIF7 proteins account,

at least in part, for the greater repression of the CBF pathway under LD conditions. However, the molecular basis for the greater expression of *PIF4* and *PIF7* under LD conditions is unknown. Additional study will be required to address this issue.

A final note regards the near overlap in peak expression of *PIF4* and *PIF7* with that of the CBF genes. If PIF4 and PIF7 were expressed at high levels when expression of the CBF genes was low (at ZT16, for instance), their effects on the CBF pathway would be minimal. A key facet of PIF4 and PIF7 control of CBF expression is the concordant expression of these genes. The CBF genes are regulated by the circadian clock in plants exposed to normal warm growth temperature (64, 66, 130) and their peak expression is driven largely by the Myb transcription factors CCA1 and LHY (65), key components of the core circadian regulatory loop (138, 139). The CCA1 and LHY proteins, which have peak levels in the morning, bind to the Evening Element (EE) and related DNA regulatory motifs present within the CBF locus and induce high-level expression of the CBF genes at ZT8 (65). One simple model would be that PIF4 and *PIF7* are also circadian-regulated and timed to peak in the morning hours. Indeed, the oscillation in PIF4 transcript levels is disrupted by constitutive overexpression of CCA1 (140), a classic indicator of circadian regulation. In addition, the promoters of *PIF4* and *PIF7* genes have EE motifs that could potentially drive their circadian regulation. Future experiments will be directed at testing this hypothesis and how output from the clock is integrated with photoperiodic regulation of the CBF pathway to condition freezing tolerance.



Figure 2.1 Arabidopsis freezing tolerance is regulated by photoperiod. *(A)* WT plants were grown under SD or LD conditions for 5 weeks and 3 weeks, respectively, and tested for freezing tolerance using the electrolyte leakage assay. *(B)* Plants grown under four conditions were tested for freezing tolerance: SD for 5 weeks (SD); LD for 3 weeks (LD); SD for 3 weeks and transferred to LD for 2 weeks (SD to LD); and LD for 2 weeks and transferred to SD for 2 weeks (LD to SD). The results are mean values from three independent experiments (error bars indicate SEM). (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation)



Figure 2.2 The CBF pathway is regulated by photoperiod. WT plants were grown under SD or LD conditions and the transcript levels for *CBF1*, *CBF2*, *CBF3*, and two CBF regulon genes, *COR15a* and *GOLS3*, were determined at the indicated times. The expression values were normalized with internal control gene, *IPP2*. The results are mean values from three independent experiments (error bars indicate SEM).



Figure 2.2 (Continued)



Figure 2.3 The CBF pathway is regulated by photoperiod. (*A*) Plants were grown under SD or LD conditions, further grown under the same photoperiod or shifted from SD to LD or LD to SD for the indicated number of days, and the transcript levels for *CBF2* were determined at ZT8. (*B*) Plants were grown as in (*A*) and the transcript levels for *CBF1*, *CBF2*, *CBF3*, *COR15a*, and *GOLS3* were determined at ZT8 (photoperiod shift was for 7 d). The results are mean values from three independent experiments (error bars indicate SEM).



Figure 2.4 A G-box motif within the *CBF2* promoter confers photoperiod-regulated gene expression. (*A*) Diagram of *CBF2::GUS* reporter fusions used to test role of the G-box (-112 to -107 bp) in photoperiod-regulated gene expression. (*B*) Transgenic plants carrying the WT-pro and Gmut-pro constructs were grown under SD or LD conditions and GUS transcript levels were determined at the indicated times. (*C*) The relative GUS transcript levels normalized against *IPP2* were determined at ZT8 in 8 independent transgenic lines for each construct are presented. The results are means from two independent experiments (error bars indicate SEM). (*D*) Ratio of GUS transcript levels at ZT8 in transgenic plants carrying the WT-pro (WT) or Gmut-pro (Gmut) constructs grown under SD or LD conditions. Values are mean ratios from 8 independent transgenic lines in (*C*) (Student's t-test, p<0.001) (*e*) Relative expression levels of the WT-pro and Gmut-pro constructs in the experiments described in (*D*) (Student's t-test, p<0.001).



Figure 2.4 (Continued)



Figure 2.5 *PIF4* and *PIF7* are required for repression of the CBF pathway under LD conditions. (*A*, *B*, *C*) Plants were grown under SD or LD conditions and the transcript levels for *CBF2* were determined at the indicated times in WT plants and in *pif4*, *pif7*, and *pif4 pif7* mutant plants as indicated. (*D*) Plants were grown under SD or LD conditions and the transcript levels for *CBF1*, *CBF2*, *CBF3*, *COR15a*, and *GOLS3* were determined at ZT8. The results are mean values from three independent experiments (error bars indicate SEM).



Figure 2.5 (Continued)



Figure 2.6 *PIF4* and *PIF7* are expressed at higher levels, and their proteins are more stable under LD conditions. (*A*) WT plants were grown under SD or LD conditions and the transcript levels for *PIF4* and *PIF7* were determined at the indicated times. (*B*) The steady-state accumulation of PIF4-TAP (PIF4-OX) and PIF7-CFP (PIF7-OX) in SD or LD transgenic plants at ZT8 were detected with anti-myc or anti-GFP antibodies respectively. Histone H3 protein was used as loading control and detected using rabbit anti-Histone H3 antibodies. n.s. indicates non-specific signals. The results presented are representative of three experiments. (*C abd D*) The relative transcript levels for the PIF4-TAP (PIF4OX-1 and -2) and PIF7-CFP (PIF7OX-1 and -2) gene fusions were determined at ZT8 in transgenic plants grown under SD or LD conditions. The expression levels were normalized to *IPP2*. Transcripts for the transgenes were non-detectable (ND) in WT plants. The results are mean values from three independent experiments (error bars indicate SEM).



Figure 2.7 Overexpression of *PIF4* or *PIF7* represses *CBF2* expression. Relative levels of *CBF2* transcripts in a 24 hour period (*C to F*) and at ZT8 (*A and B*) in transgenic plants overexpressing PIF4-TAP or PIF7-CFP grown under LD and SD conditions were determined. The results are mean values from three independent experiments (error bars are SEM). (Student's t-test, p<0.05; p<0.01)



Figure 2.7 (Continued)



Figure 2.8 PIF4 and PIF7 bind to *CBF1*, *CBF2* and *CBF3* promoters through G-box and E-box motifs. The production of GST-PIF4 and GST-PIF7 recombinant proteins and information of how the EMSAs were performed are described in the Materials and Methods. The probes used are indicated at the top of the gels. The observed binding was specific as it decreased in response to addition of 20-fold and 100-fold G-box (CACGTG) or E-box (CATGTG) sequence as competitor, but not mutated G-box (ggtacc). The sequences of competitors are listed in Table 2.2.



Figure 2.9 PIF7 binds at the *CBF* locus. The CBF locus is comprised *CBF*1, *CBF3*, and *CBF2* genes in tandem repeat, and the white boxes represent transcribed regions. The location of G-box (CACGTG, circle) and E-box (CANNTG, triangle) were labeled across CBF locus. WT plants and transgenic plants overexpressing PIF7-CFP were grown under LD conditions, tissue was harvested at ZT8, and ChIP assays were performed using *(A)* anti-GFP antibody (IP) or *(B)* IgG. Precipitated DNA sequences were quantified using primer sets across *CBF* locus (boxes A through N). DNA sequences from ACTIN7 and UBQ10 were used as negative controls. The fold enrichment of precipitated DNA for each primer set in PIF7-OX (PIF7-IP in *(A)* or PIF7-mock in *(B)*, black bars) samples are relative to the level in the WT samples (WT-IP in *(A)* or WT-mock in *(B)*, open bars). The locations and sequences of primer sets are listed in Table 2.1. Data are presented as mean \pm SEM; n=4 (* p<0.05 and ** p<0.01, paired t-test). The location of G-box (CACGTG, circle) and E-box (CANNTG, triangle) motifs are indicated.



Figure 2.10 *PHYB* is required for photoperiod regulation of the CBF pathway and freezing tolerance. (A) WT and *phyB* plants were grown under SD or LD conditions and the relative transcript levels for *CBF2* were determined at the indicated times. (B) WT and *phyB* plants were grown as in (A) and the relative expression levels of *CBF1*, *CBF2*, *CBF3*, *COR15a*, and *GOLS3* were determined at ZT8. (C) WT and *phyB* plants were grown under SD and LD and tested for freezing tolerance using the electrolyte leakage assay. The results are mean values from three independent experiments (error bars indicate SEM).



Figure 2.10 (Continued)



Figure 2.11 Model of photoperiodic regulation of the CBF pathway and freezing tolerance.

Name	Locus	primer (Fw or Rv)	primer sequence	position
qRT-PCR				
CBF1	AT4g25490	Fw	CCGCCGTCTGTTCAATGGAATCAT	+ 734
	-	Rv	TCCAAAGCGACACGTCACCATCTC	+ 774
CBF2	AT4g25470	Fw	CGACGGATGCTCATGGTCTT	+ 562
		Rv	TCTTCATCCATATAAAACGCATCTTG	+ 630
CBF3	AT4g25480	Fw	TTCCGTCCGTACAGTGGAAT	+ 694
		Rv	AACTCCATAACGATACGTCGTC	+ 741
COR15a	AT2g42540	Fw	GAAAAAAACAGTGAAACCGCAGAT	+ 704
		Rv	CCACATACGCCGCAGCTT	+ 750
GOLS3	AT1g09350	Fw	CTGACGAGCGAGGTTCTTGTC	+ 1090
		Rv	AACAAATTCTAAGTAAACATCACCAGTT	+ 1137
IPP2	AT3g02780	Fw	ATTTGCCCATCGTCCTCTGT	+ 115
		Rv	GAGAAAGCACGAAAATTCGGTAA	+ 155
PIF4	AT2g43010	Fw	TCTCCGACCGGTTTGCTAGA	+ 1360
		Rv	CGCGGCCTGCATGTGT	+ 1397
PIF7	AT5g61270	Fw	CAAGTGCGAGTGGTACCAATATG	+ 484
		Rv	TTCAAGCTCCGACCGGATT	+ 523
GUS		Fw	TGGCCTGGCAGGAGAAACT	
		Rv	CGTATCCACGCCGTATTCG	
TAP (mvc)		Fw	TGCAGCCTAGGGATTACGATATC	
(,		Rv	GGCCCCTGGAACAGAACTTC	
CFP		Fw	GTCCGCCCTGAGCAAAGA	
		Rv	TCCAGCAGGACCATGTGATC	

Table 2.1 List of primers for qRT-PCR and ChIP assays.

Name	Locus	primer (Fw or Rv)	primer sequence	position
ChIP				
А	AT4g25490	Fw	TGCTTTCAAGGCCGAATGAT	- 1312
		Rv	CGTTCTCATTCCACGTGTGATG	- 1247
В	AT4g25490	Fw	TTACCACTCTTTTTTTCCCTCTTTG	- 845
		Rv	CTCGCTCTCACGTTATTGACATTT	- 801
С	AT4g25490	Fw	TCTTTACAAGGGTCAAAGGACACA	- 186
		Rv	GCGAAGCAATCCCACGAT	- 142
D	AT4g25490	Fw	CCGCCGTCTGTTCAATGGAATCAT	+ 734
		Rv	TCCAAAGCGACACGTCACCATCTC	+ 774
E	AT4g25480	Fw	AGTTCTATCGGACTAATTCTTGGCTTA	- 1859
		Rv	GATGATCAAGCGTAATTGCTTTGT	- 1752
F	AT4g25480	Fw	TGACTAAGGACGTGGTGGTTGA	- 1235
		Rv	AGCGCACTTCCTTCTCACTCA	- 1178
G	AT4g25480	Fw	TGTTACATTTGATCATTCACCCAAA	- 604
		Rv	CGTATATAAGCACGTAAGTCACCAAGT	- 550
Н	AT4g25480	Fw	CGTGGCATTACCAGAGACACA	- 124
		Rv	GCGGAAGATATTTTAGAGGCAAAA	- 83
I	AT4g25480	Fw	TTCCGTCCGTACAGTGGAAT	+ 694
		Rv	AACTCCATAACGATACGTCGTC	+ 741
J	AT4g25470	Fw	CAAGAGAGCACTGTCCGTAGCTT	- 1851
		Rv	TGGTTACAAGAGGAGCCACGTA	- 1811
K	AT4g25470	Fw	TTTGCCGGAAAACTCAACTCA	- 1147
		Rv	CCTTCTTTTGGTCTGAAA	- 1108
L	AT4g25470	Fw	GAGAGATGCTGGAAATTGTGATCA	- 943
	-	Rv	AAATATGGTAAGTGGTTAGGCGAAA	- 897
Μ	AT4g25470	Fw	GGGTCAAAGGACACATGTCAG	- 201
	-	Rv	GAACGCGGAGTTTCTGTCTC	- 102

Table 2.1 (Continued)

Name	Locus	primer (Fw or Rv)	primer sequence	position
ChIP				
Ν	AT4g25470	Fw	CGACGGATGCTCATGGTCTT	+ 562
	-	Rv	TCTTCATCCATATAAAACGCATCTTG	+ 630
Actin7	AT5g09810	Fw	CGTTTCGCTTTCCTTAGTGTTA	+ 54
		Rv	AGCGAACGGATCTAGAGACTC	+ 167
UBQ10	AT4g05320	Fw	TCCAGGACAAGGAGGTATTCCTCCG	+ 1616
		Rv	CCACCAAAGTTTTACATGAAACGAA	+ 1796

Table 2.2 List of primers for cloning and EMSA

Name	Locus	Primer (Fw or Rv)	Primer sequences	position
Cloning				
CBF2-pro	AT4g25470	Fw	CAAGATGGGTCAAAGGACACATGTCAGATT	- 189
		Rv	TGATCAGAAGAGTACTCTGTTTCAAGAAACTGGA	- 1
CBF2-pro-Gmut	AT4g25470	Fw	TTAGCTGTTTCTTATCggtaccGCATTCACAGAGACAGA	- 133
		Rv	TCTGTCTCTGTGAATGCggtaccGATAAGAAACAGCTAA	-95
PIF4	AT2g43010	Fw	ATGGAACACCAAGGTTGGAGTTTTGAGGAGAA	+ 234
		Rv	CGCGGCCTGCATGTGT	+ 1397
PIF7	AT5g61270	Fw	CAAGTGCGAGTGGTACCAATATG	+ 484
		Rv	TTCAAGCTCCGACCGGATT	+ 523
EMSA				
CBF1-pro	AT4g25490	Fw	AAGAACTCATAAAGGTTAACGAGTGAAGAGTCAAAAG	- 189
	C C	Rv	TGTGTAGTTAGTATAAAAAGTGAGAGTGAGAATTGGT	- 1
CBF2-pro	AT4g25470	Fw	CAAGATGGGTCAAAGGACACATGTCAGATT	- 224
	C C	Rv	TGATCAGAAGAGTACTCTGTTTCAAGAAACTGGA	- 1
CBF3-pro	AT4g25480	Fw	ACGGTTACCCTACACCTAGTACACTAAATCCT	- 316
	Ū	Rv	ACGGAGTTTGTGTCTCTGGTAATGCCACGT	- 96
6X G-box			CACGTGCACGTGCACGTGCACGTGCACGTGCACGTG	
6X E-box			CATGTGCATGTGCATGTGCATGTGCATGTG	
6X G-box mutated			GGTACCGGTACCGGTACCGGTACCGGTACC	

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana Columbia-0 WT and mutant derivatives were used in all experiments. Plants carrying the *pif7-1*, *pif4-2*, *pif4-2 pif7-1*, and *phyb-9* null mutant alleles were kindly provided by Dr. Peter Quail (University of California, Berkeley, CA) (131). Seeds were stratified for 3 to 5 d at 4°C in the dark and then grown under either SD (8h light, 16h dark) or LD (16h light, 8h dark) conditions. For gene expression studies, plants were grown under SD (~12 d) or LD (~10 d) conditions on sterilized Gamborg's B5 medium (Caisson Laboratories). For freezing tolerance studies, plants were grown in soil as described (49) under SD (~ 5 weeks) or LD (~ 3 weeks) conditions unless indicated otherwise. All plants were grown at 22°C under ~100 µmol m⁻² s⁻¹ fluorescent white light.

Determination of Transcript Levels

Transcript levels were determined using real-time quantitative RT-PCR (qRT-PCR). Plant sample collection, RNA extraction, and qRT-PCR were performed as described (49), except that 200 ng of total RNA were used for 20 µl reverse transcription reaction, and 2 µl of 10-fold diluted cDNA was used as template in a 10 µl reaction for qRT-PCR. In the SD and LD experiments, the 8 h and 16 h time points, respectively, were taken during the light phase; in both experiments, the 24 h sample was taken during the light phase. *IPP2* (*ISOPENTENYL PYROPHOSPHATE-DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2*) was used as the reference gene. Primers are listed in Table 2.1.

CBF2 Promoter Reporter Lines

The *CBF2* promoter fragment from -207 bp to + 134 (just upstream of the ATG) was first cloned into pCR®8/GW/TOPO® vector (Invitrogen). The G-box at -112 to -107 bp, CACGTG, was converted to GGTACC by site-directed mutagenesis using the Quick Change kit (Stratagene). The WT and mutagenized promoter fragments were fused to the *GUS* reporter in the pMDC164 vector (141) using a recombination reaction (Invitrogen), and then transformed to WT *Arabidopsis* using the floral dip method (142). Transgenic lines in the T3 generation were used for experiments. The primers used for cloning are listed in Table 2.2.

Transgenic Lines Overexpressing PIF7 or PIF4

For 35S::PIF7-CFP-HA, the open-reading frame (ORF) sequence of PIF7 was cloned into the pCR®8/GW/TOPO® vector (Invitrogen), transferred to the plant binary vector pEarleyGate102 (143) by recombination, and then transformed into WT *Arabidopsis* plants. The cloning primers are listed in Table 2.2. For 35S::PIF4-TAP lines, a plant binary vector containing the ORF of PIF4 fused to the TAP-tag (DKLAT2G43010) was obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu) and transformed into WT *Arabidopsis* plants.

Electromobility Shift Assay (EMSA)

To prepare recombinant PIF4 and PIF7 protein for EMSA, the ORF of PIF4 or PIF7 was first cloned into pCR®8/GW/TOPO® vector (Invitrogen), and then moved to pET-60-DEST vector (Stratagene) through recombination reactions (Invitrogen). The

recombinant proteins were induced in E. coli BL21-Gold (DE3) strain by addition of 0.1 mM of IPTG at 30°C for 6 h. The crude protein was extracted with B-PER Protein Extraction Reagent (Pierce), and quantified with BCA Protein Assays (Pierce).

The probes for EMSA were prepared by amplification of *CBF1* (-224 to -1), *CBF2* (-189 to -1), and *CBF3* (-316 to -94) promoter regions by PCR from the genomic DNA, and then end-labeled with gamma ³²P. The WT and G-box mutated competitors were made by annealing of G-box (CACGTG), E-box (CATGTG) or mutated G-box (ggtacc) primers. The primers are listed in Table S2. The EMSAs were performed as described (49), except 20 ng of crude protein extract, 4 fmole of probes, and 20-fold and 100-fold competitors were used in the binding reactions. The samples were resolved on 5% polyacrylamide as described (144).

Protein Extraction and Immunoblots

Protein from *Arabidopsis* seedlings was obtained by heating samples at 70°C for 10 minutes in extraction buffer (60 mM Tris-HCl, pH8.5; 2% SDS; 2.5% Glycerol; 0.13 mM EDTA, pH8.0; protease inhibitor cocktail (Roche)). The soluble protein was quantified with *DC* Protein Assay (Bio-Rad). 100 μ g of total soluble protein with 5% β -mercaptoethanol was separated on 4-12% NuPAGE® SDS-PAGE (Invitrogen) followed by western blotting analysis. Immunodetection of PIF4-TAP and PIF7-CFP-HA was done using rabbit anti-myc monoclonal antibodies (71D10, Cell Signaling) and mouse anti-GFP antibodies (11814460001, Roche), respectively. Histone H3 was detected with rabbit anti-Histone H3 antibodies (07-690, Millipore). Corresponding secondary

antibodies conjugated with horseradish peroxidase (Thermo Scientific) and SuperSignal West Pico or Femto Chemiluminescent Substrate kits (Thermo Scientific) were used for detection.

Chromatin Immunoprecipitation (ChIP)

The ChIP assays were performed as described previously (65) with minor modifications. The 35S::PIF7-CFP-HA line and wild type (WT) 14-day-old seedlings were grown under LD and harvested at ZT8. For each biological replicate, IP and mock samples were normalized to the total input for each line, and the fold enrichment was relative to WT. Paired t-test was applied to test the statistical significance of fold enrichment for each primer sets. The primers for qRT-PCR are listed in Table 2.1.

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CHAPTER 3

TRANSCRIPTIONAL REGULATORY NETWORKS OF COLD ACCLIMATION IN ARABIDOPSIS THALIANA
PREFACE

The work presented in Chapter 3 was in collaboration with Dr. Sunchung Park. Dr. Park was responsible for the cluster analysis of cold stress transcriptome data and selection of cold-induced transcription factors for further experiments. The expression kinetics of *CBF1*, *CBF2*, *CBF3*, *ZF*, *CZF1*, *RAV1*, *CZF2*, *MYB73*, *ZAT12*, *DOF1.10*, *DOF1.3*, *DEAR2*, *ZAT10*, *ANAC62*, *AZF2*, and *RVE2* with qRT–PCR was examined by the author of this dissertation, and Dr. Park was responsible for *HSFC1*, *DEAR1*, *MYB44*, *ERF5*, *ERF6*, *CRF2*, *CRF3*, *WRKY33*, *ATHB2*, *AXR5*, *WRKY22*, *WRKY40*, *ERF11*, and *MYB7*. For generation of transgenic lines, characterization of phenotypes, and microarray experiments, the author of this dissertation was responsible for the overexpression lines of *CZF1*, *CZF2*, *MYB73*, *RAV1*, *ZAT10*, *ANAC62*, and *ZF*, and Dr. Park worked on the overexpression lines of *HSFC1*, *MYB44*, *DEAR1*, *ERF5*, *WRKY33*, *ERF6*, *CRF3*, and *CRF2*. Further the transcriptome analysis, functional analysis and verification with qRT-PCR in the *ZF*-OX lines were performed by the author of this dissertation.

Abstract

Low temperatures profoundly affect growth, development, and survival of plants. Many temperate plants can acquire freezing tolerance via exposure to low, non-freezing temperature, a process known as cold acclimation. Extensive alterations of biochemical and metabolic pathways occur during cold acclimation that help plants adapt to low temperatures. Many of these changes are brought about through reprogramming transcription. The expression of thousands of cold-regulated (COR) genes are affected by cold, and their expression occurs in successive waves, suggesting hierarchical regulation. The well-studied CBF pathway and its parallel ZAT12 pathway, however, account for the expression of less than 10% of these COR genes. In this study, fifteen transcription factors (TF) sharing a similar expression pattern to that of the CBFs and ZAT12 in the cold were identified. Additionally, transcriptome analysis was performed to understand the regulation of the COR genes. The results showed that these TFs likely regulate about 29% of the COR genes, and they regulate different sets of COR genes with substantial overlap, which indicated that extensive crosstalk and functional redundancy in COR gene expression. Functional enrichment analysis of the COR genes regulated by each TF indicated that they regulate diverse, but overlapping, biological pathways in cold acclimation. Among these pathways regulated by these cold-induced TFs, the COR genes that are regulated by a zinc-finger transcription factor (ZF, AT4G29190) exclusively enriched in starch metabolism and circadian rhythm pathways, which have been established to be strongly affected by cold. These results shed light on the regulation of COR genes by CBF-independent pathways and their potential roles in freezing tolerance.

Introduction

Low temperature is one of the adverse environmental factors that limit land usage and affect crop production. To cope with cold stress, many temperate plants have evolved the ability to acquire freezing tolerance through exposure to low, nonfreezing temperatures, a process known as cold acclimation (1). This process is associated with the alteration of biochemical processes and metabolic pathways (27, 28, 145). Several major metabolic adjustments not only prevent cell damage from ice formation, but also allow the development of resistance to other stresses, such as drought, osmotic, oxidative, and photo-oxidative stresses associated with cold stress (7, 11, 29). Long-term adaptations, such as changing plant architecture by modulating hormone pathways and adjustment of energy utilization are also established during cold acclimation (22, 25, 117). Recently, it was demonstrated that low temperatures have global effects on major metabolic pathways through regulating the circadian clock; however, the mechanism for this is not well understood (30, 64).

The metabolic adjustments and physiological adaptations associated with cold acclimation are largely caused by extensive reprogramming of transcription during cold acclimation (27-30). Hundreds to thousands of cold-regulated (COR) genes were identified as changing their expression during cold acclimation depending on the conditions of plant growth and treatments and the expression profiling platforms used (38, 62, 112-115). Although the functions of the COR genes are not fully understood, Gene Ontology (GO) analysis of COR genes and co-expression analysis have revealed that COR genes are involved in diverse biological pathways (38, 113, 115, 146). The expression kinetics of the COR genes reveals successive waves in time-series

expression profiling experiments, suggesting hierarchical regulation of the COR genes, i.e., the early cold-induced genes regulate the ones that respond later (28, 38, 62).

In *Arabidopsis*, a group of early cold-induced *C-REPEAT BINDING FACTORS*, or *CBF1*, *CBF2*, and *CBF3*, play prominent roles in regulating COR genes and cold acclimation (5, 32). They encode transcriptional activators of the AP2/ERF transcription factor family and bind specifically to CRT/DRE (RCCGAC) elements in the promoters of their target genes. (33, 34, 62). Overexpression of *CBFs* at warm affects expression of about 140 COR genes, or the "CBF regulon", confers freezing and drought tolerance and results in small stature of the plants (36, 62, 119). The functions of CBF regulon genes are not fully understood. Some of them encode enzymes involved in biosynthesis of sugars or proline and membrane stabilizing proteins (35, 147). Others encode late cold-induced transcription factors, such as *RAP2.1*, which negatively regulates some CBF regulon genes involved in cold or associated osmotic stresses (62, 148)

Transcriptome and quantitative trait locus (QTL) studies have revealed multiple cold regulatory pathways besides the CBF pathway (44, 98, 99, 149). *ZAT12* represents a cold regulatory pathway parallel to the CBF pathway, but is much less well-studied (62). *ZAT12* encodes a C_2H_2 zinc-finger transcription factor with a expression pattern similar to that of the *CBFs* in the cold under continuous light. Overexpression of *ZAT12* increases resistance to cold and oxidative stress (62, 150). Transcriptome analysis of transgenic lines overexpressing *ZAT12* showed that *ZAT12* regulates around 70 COR genes, termed the "ZAT12 regulon", which has a small overlap with the CBF regulon (62). Interestingly, *ZAT12* negatively regulates the *CBFs*,

which indicates multiple pathways and a complex interaction between pathways in the regulation of COR genes during cold acclimation. In addition to *ZAT12*, *GIGANTEA* (*GI*) is the only cold-induced transcription factor identified as regulating a CBF-independent pathway, although the transcriptome was not examined (103).

Deciphering transcriptional regulatory networks that regulate cold acclimation provides information for the molecular basis of cold acclimation. This knowledge has the potential to help find molecular tools for engineering crops to improve crop quality (151). Chawade et al. applied computational modeling based on co-expression analysis and transcription factor binding sites to predicted transcription factors (TF) regulating COR genes in *Arabidopsis thaliana* (110). Several cold-regulated TFs were identified as putative regulators; however, there is largely a lack of experimental validation. One limitation in validating the predicted results and identifying new regulators is the functional redundancy of TFs in *Arabidopsis*.

The goal of this study was to systematically identify early cold-upregulated TFs that regulate COR genes in CBF-independent pathways, and to dissect the biological pathways regulated by these TFs. Fifteen selected TFs were ectopically expressed in *Arabidopsis thaliana* and subjected to transcriptome analysis with Arabidopsis ATH1 GeneChips. The regulons of seven tested TFs, *ZAT10*, *HSFC1*, *CZF1*, *ZF*, *MYB44*, *DEAR1*, and *ERF5*, were compared to the CBF and ZAT12 regulons, and the results showed considerable overlap of COR genes, suggesting the COR genes are tightly regulated by multiple regulators. Functional analysis of these TFs indicated that they regulate different biological and metabolic pathways in cold acclimation, with some overlap of functions. In the transgenic plants overexpressing a zinc-finger transcription

factor (*ZF*, *AT4G29190*), the expression of genes associated with the circadian clock and carbohydrate metabolic pathways were affected. The function of *ZF* in cold acclimation is discussed.

Results

Expression Kinetics of COR Genes Reveals Successive Waves of Transcription in Cold Acclimation

From previous studies, the number of the cold-regulated (COR) genes can vary depending on the experimental settings (38, 62, 112-115). To define the COR genes in this study, *Arabidopsis* low temperature time course expression profiling experiments (GSE5621 and GSE5620) (113) from ATGenExpress were analyzed. In these datasets, the WT *Arabidopsis* plants were grown in long-day conditions (16 h light and 8 h dark), and the tissue was harvested 3 h after dawn either with or without cold treatment for 0, 0.5, 1, 3, 6, 12, and 24 h. The expression of 2397 probe sets, which corresponds to 2455 genes based on the TAIR10 database (Appendix Table A1), was found to be changed more than 4-fold (FDR< 0.01) at one or more time points at 4°C by comparison of cold-treated samples (GSE5621) and warm samples (GSE5620).

These COR genes were clustered into 6 groups (G1 to G6, Fig. 3.1*A*) based on their temporal expression patterns in the cold with the k-mean clustering (k=6) algorithm. The expression of these 6 groups (G1 to G6) of COR genes revealed successive waves of transcription, with 1091 genes mostly down-regulated (G1 and G2) and 1364 genes up-regulated (G3 to G6) sequentially in a 24h cold-treatment period.

The expression in cold samples was compared to that in warm samples at each time point rather than only at 0h because it has been demonstrated that COR genes are regulated by the circadian clock and have daily cycling expression patterns at warm temperatures (69, 71). However, it is possible that the COR genes selected by these criteria may be "apparently" affected by cold rather than "actually" regulated by cold. For example, the expression of genes possibly remains constant in the cold, but the expression cycles at warm temperature, thus resulting in up- or down-regulation when cold and warm samples are compared. To test this possibility, the expression of coldregulated genes at each time point compared to 0h for the COR genes defined in the previous section (cold/warm) were plotted (Fig. 3.1*B*), and this comparison was defined as "cold/0h". The overall expression patterns of the previously defined cold/warm and cold/0h are very similar (Fig. 3.1 A and B). The primary differences are in G3, G4 and G6. The induction levels of many genes at 1, 3, 6, 12, and 24h in G3 and at 6 and 12h in G4 in cold/0h are much lower compared to that in cold/warm. Conversely, expression is considerably repressed at 3, 6, and 12h in G6. To test whether these differences are due to diurnal expression in the warm, the expressions of warm samples compared to Oh for the COR genes defined by cold/warm was plotted (Fig. 3.1*C*), and this comparison was defined as "warm/0h". Indeed, the expression level of G3 genes at 1, 3, and 6h and that of G4 genes at 6 and 12h were decreased compared to 0h (Fig 3.1C). In G6, the peak expression of some genes at 3, 6, and 12h in warm/0h resulted in repression at 3 and 6h without much induction at 12h in cold/warm compared to cold/0h (Fig 3.1 A-C). Although the expression levels are not identical in cold/warm and cold/0h,

it does not change the general expression kinetics for each cluster. Therefore, the cold/warm comparison was used to define COR gene sets for further analysis.

Identification of Early Cold-induced TFs in the Same Cluster as CBFs and ZAT12

The sequential waves of expression patterns of the COR genes suggested hierarchical regulation. To identify regulators parallel to the CBF and ZAT12 pathway, we first identified 174 transcription factors up-regulated in cold from G3 to G6 in Fig. 3.1*A* based on Gene Ontology (GO) (Table 3.1). These 174 TFs were subjected to kmean clustering analysis (k=7) to find the TFs sharing similar expression kinetics to that of *CBF*s and *ZAT12*. Among seven clusters (TFC1 to TFC7 in Fig. 3.2), *CBFs* and *ZAT12* are members of TFC3; there are 26 additional transcription factors in the same cluster.

We first confirmed that these 26 transcription factors are induced in cold. Plants were grown under the same photoperiodic conditions and harvested at the same time points as in the ATGenExpress experiments (113), and the transcript levels were examined by quantitative RT-PCR (qRT-PCR). The expression kinetics of the TFs in the cold were compared to the expression in the warm (cold/warm, blue solid lines in Fig 3.3) or at 0h (cold/0h, red dashed lines). Of the 26 transcription factors examined, 24 of which were induced to different levels in cold/warm, which agrees with the results from transcription profiling experiments, except for *MYB7* and *ERF11*. However, the expression levels were generally lower than those observed in the array experiments. A possible cause for this is the difference in growth conditions, such as light intensity and growth media.

The general expression patterns of TFs in the TFC3 peak at 3 or 6h after cold treatment, and even persist at 12 and 24h (Fig. 3.2). The *CBF*s were highly induced at 3h in the cold and then the expression dropped (Fig. 3.2). Some TFs, including *ZF*, *RAV1*, *MYB73*, *ZAT10*, *DEAR1*, *ERF5*, *CRF2*, *WRKY33*, *ERF6*, *CRF3*, *RVE2*, and *WRKY 40*, showed a similar expression pattern to the *CBF*s. The expression of *ZAT12* peaked at 3h cold, decreased at 6h and then increased again at 12 and 24h, which is different from a previous report under continuous light (62). The differences between the expression patterns of the *CBFs* and *ZAT12* is likely due to the diurnal conditions in this experiment; this is supported by comparing the cold/warm to cold/0h patterns in Fig. 3.3. Several TFs, including *DOF1.10*, *DOF1.3*, *CZF1*, *CZF2*, *HSFC1*, *MYB44*, and *ANAC62* followed a similar expression pattern to *ZAT12*. When the patterns of cold/warm and cold/0h were compared, *CBF1*, *CBF2*, *CBF3*, *ZF*, *CZF1*, *RAV1*, *MYB73*, *DOF1.10*, *DEAR2*, *DOF1.3*, *AZF2*, *RVE2*, *AXR5*, *ATHB2*, and *WRKY40* showed very different patterns, which was due to strong cycling of these TFs under warm conditions.

Finally, TFs with similar expression patterns to those of the *CBF*s or *ZAT12* were chosen to study the regulation of the COR genes. Transgenic lines ectopically expressing these TFs driven by the CaMV 35S promoter were generated (listed in Table 3.2) with the exception of *WRKY40*, *RVE2*, *DOF1.3*, and *DOF1.10*, which we failed to generate lines for further experiments.

Early Cold-induced TFs Regulate Approximately 29% of COR Genes

To identify the COR genes regulated by the 15 early cold-induced TFs described in the section above, two independent transgenic lines overexpressing each TF along with WT were grown under continuous light at 22°C for 14d and their RNA hybridized to Arabidopsis ATH1 GeneChips. The continuous light condition was chosen to reduce any circadian or diurnal effects. We first identified differentially expressed genes (DEG) in TF-overexpressing lines (TF-OX) by comparing transgenic lines to WT using a criterion of more than 2-fold change (p-value< 0.01) (Table 3.2). The DEG were subsequently compared to the defined COR genes to find the COR genes regulated by each TF, which were defined as their regulons (Table 3.2 and Table A1). Combined with the previously published transcription profiling datasets of CBF2-OX and ZAT12-OX lines (62), the regulons of these TFs covered around 29% of the COR genes defined in this study. Of these 17 TF-OX lines, some affected expression of more than 50 genes as well as COR genes, whereas the rest of the TF-OX lines had mild effects on gene expression; this result could be due to the low expression of transgenes for the CZF2-OX and MYB73-OX lines (data not shown), or the requirement for posttranslational modification and/or cofactors at low temperature. Transgenic lines overexpressing CBF2, ZAT12, HSFC1, ZF, ZAT10, CZF1, MYB44, DEAR1, and ERF5, were selected for further analysis because the COR genes are over-represented in their DEG (p-value< 1E-8, fisher's exact test), suggesting these TFs have significant roles in regulating the COR genes.

The regulons of each TF are significantly distributed in multiple groups, except for the CBF regulon. The majority of the CBF regulon falls into G5 (Fisher's exact test, p < 0.01), whose expression patterns are mainly induced after 12h and persist at 24h, consistent with the role of the *CBF*s functioning as activators. The known CBF direct targets, including *COR15a*, *COR78*, *COR47*, and *GOLS3* (35, 152), belong to G5. In

contrast, the rest of the TF-OX lines affect both down- (G1 or G2) and up-regulated (G3 to G6) genes (Table 3.2). The regulons for each TF are mainly found in G1, G2, G5, and G6 (p-value< 0.01), whose expression patterns are either repressed or induced after 12h, similar to the induction kinetics of *CBF* targets. Some DEAR1 regulon genes were significantly distributed in G4, and their expression was repressed by overexpressing *DEAR1*, which suggests *DEAR1* may have role in negatively feedback regulation of these COR genes.

We further examined whether the COR genes up-regulated by the TFs are also up-regulated in cold, and vice versa. The correlation of the expression patterns (upand down- regulation) of TF regulons to those of the COR genes at different time points were analyzed (Fig. 3.4). The expression patterns of the CBF, HSFC1, and ZAT10 regulons show a general positive correlation at most of the time points. The strongest positive correlation is only at 12 and 24h for the CBF regulon (spearman's correlation coefficient, rho> 0.8, p-value < 0.01). The positive correlations suggest *CBF*, *HSFC1*, and *ZAT10* may have a positive effect in regulating COR genes and therefore freezing tolerance. In the case of *MYB44*, *ERF5*, *DEAR1*, and *ZF*, however, only a mild negative correlation was found, suggesting that overexpression of these TFs resulted in misregulation of the COR genes.

Early Cold-induced TFs Co-regulate a Substantial Number of COR Genes

Previous studies have suggested that multiple pathways regulate COR genes (44, 98, 99, 149); we therefore investigated the relationships of the TF regulons identified

here. The TF regulons were visualized with Cytoscape (http://www.cytoscape.org/) (153) shown in Fig. 3.5*A*. Each TF apparently regulates a different set of COR genes represented by an individual circle connected to each TF, while they also co-regulate COR genes with other TFs. Of 696 COR genes regulated by the TFs in this study, 70% of them were regulated by a single TF (Fig. 3.5*A* and *B*). However, this number should be interpreted with caution. In this study, only 17 cold-induced TFs were examined, while there are 174 TFs induced in cold. The number of COR genes co-regulated by multiple TFs may increase when more TFs are examined. In addition, several co-regulated genes could be missed in this study if they require more than one TF to activate or repress their expression.

Another result which supports the high likelihood of co-regulation of COR genes is that around 50% of the TF regulon genes are also regulated by other TFs (Fig. 3.5 *C*). In the ERF5 and DEAR1 regulons, there are close to 80% co-regulated COR genes. The CBF regulon has fewer co-regulated genes, which is around 35% of its regulon (Fig 3.5C). These results indicate the regulation of COR genes is tightly coordinated among these TFs. Among the co-regulated genes in these TF regulons, there is a significant number of COR genes co-regulated by *ZF* and *HSFC1* (more than 20%, p-value< 0.01, fisher's exact test) (Fig. 3.5*D*). It is possible that they are involved in regulating the same biological pathways, such as carbohydrate metabolism shown in Table 3.3.

Phenotypic Analysis of the TF-overexpressing Lines

It has been demonstrated that low temperatures can modulate the GA and SA pathways to inhibit plant growth (24, 26). Accumulation of SA and its glucosylated

derivatives is associated with growth inhibition at low temperatures, although the mechanism has not been studied (26). Overexpression of CBF1 induces expression of GA-inactivating GA-2 oxidase (GA2ox), which leads to the accumulation of the growth inhibiting DELLA proteins and subsequent growth retardation (24). Similar to the CBFoverexpressing lines, when the ZAT12 was overexpressed in Arabidopsis, the plants also exhibited stunted growth (36, 62). We therefore set out to study the growth phenotype and freezing tolerance of the TF-OX lines. WT and two transgenic lines for each TF were grown at 22°C in long-day (16h light and 8h dark) for 19 days. Similar to CBF-OX and ZAT12-OX, most of the TF-OX lines showed dwarf phenotypes except for ERF5-OX (Fig. 3.6). To test whether the dwarfism was caused by changing the GA and/or SA pathways, the expression of genes encoding enzymes involved in SA biosynthesis or its glycosylation, including ICS1, ICS2, PAL1, PAL2, PAL3, PAL4, UGT74F1, and UGT74F2/SGT1, and biosynthesis and inactivation of GA, such as GA200x, GA30x, and GA20x, were investigated in the TF-OX lines. The expression of genes encoding GA inactivating enzymes, GA2ox2 (AT1G30040) and GA2ox6 (AT1G02400), and enzymes in the SA biosynthetic pathway, PAL2 (AT3G53260) and PAL3 (AT5G04230), were down-regulated in HSFC1-OX; whereas no significant difference in other TF-OX lines was observed. These results indicate that the dwarfism could be due to the adverse effects of overexpressing TFs rather than alteration of GA and/or SA pathways.

Overexpression of *CBFs* or *ZAT12* enhances freezing tolerance of plants. To test the freezing tolerance of the TF-OX lines, electrolyte leakage assays (ELA) were performed, in which damage to leaves caused by freezing is measured by ion leakage

from the leaves. Only HSFC1-OX was slightly increased in freezing tolerance compared to WT in both non-acclimated (Fig. 3.7) and cold-acclimated (Fig. 3.8) conditions. These results are consistent with the positive correlation of expression patterns of COR genes and the HSFC1 regulon (Fig. 3.4). Most of the transgenic lines, however, showed different levels of decrease in freezing tolerance, except for the ERF5-OX under non-cold acclimated condition. The CZF1-OX and DEAR1-OX showed significant reduction in freezing tolerance in both conditions, and chilling sensitive phenotypes were observed as the cold-acclimated transgenic lines had higher ion leakage even without freezing treatments. These phenotypes can be partially explained by negative regulation of CBFs and/or their downstream targets by overexpressing CZF1 or DEAR1 in the cold (63, 154). Another possible reason could be a growth defect when TFs are constitutively expressed. The expression of COR genes is regulated coordinately and expression changed during cold acclimation (Fig. 3.1A). Constitutive induction or repression when TFs were overexpressed may affect the normal expression pattern of COR genes and the freezing tolerance of the plants (Fig 3.4).

Gene Ontology Analysis Reveals Diverse Pathways Regulated by Early Coldinduced TFs

To investigate the functions of the early cold-induced TFs in the complex cold acclimation process, we analyzed the enrichment of the Gene Ontology (GO) categories of COR genes and the TF regulons (Table 3.3). The GO analysis of COR genes reveals that GO categories of abiotic stresses, biotic stresses, hormone responses,

carbohydrate metabolism, circadian rhythm, cell wall modification, pigment metabolism, and chlorophyll metabolism are significantly enriched (p-value< 10E-2, Fisher's exact test). Although the regulons for each TF are over-represented in different GO categories, they have substantial overlaps between TFs. The CBF regulon is significantly enriched in response to abiotic stresses and ABA responses, which are largely associated with osmotic and drought stresses along with cold stress. Among the other regulons, the HSFC1 and ZAT10 regulons are also enriched in response to abiotic stresses; however, they are associated with water deprivation stress.

It may appear contradictory to observe that the GO categories of abiotic stress and defense responses are over-represented in the COR genes; however, previous research has shown that low temperatures induce a set of defense responsive genes, including *PATHOGEN-RELATED GENE* 1 (*PR1*), *PR2*, and *PR5*. In rye, several *PR* genes encode short cysteine-rich peptides that may function as antifreeze proteins (155), but the function of the *Arabidopsis PR* genes in cold acclimation has not been studied. *Arabidopsis PR1*, *PR2* and *PR5* are also induced by SA (156). The induction of PR genes is observed within 24 h, suggesting the regulation of these genes is by *CBF*, *ZF*, and *HSFC1* as they accumulate early in the cold response, rather than by SA, which does not accumulate until after 7 days in the cold (26).

Both the ZAT10 and ZF regulons are enriched in genes involved in pigment biosynthesis; however, they regulate different pathways. ZAT10 has reduced expression of *TOCOPHEROL CYCLASE* (*AT4G32770*), a key enzyme in the tocopherol biosynthetic pathway (157). The rapid reduction of *TOCOPHEROL CYCLASE* expression has been associated with a decrease in tocopherol content in leaf tissue and

accumulation of anthocyanin in low temperatures, which may explain the purple color in the adxial leaf of *ZAT10*-OX lines (158). Overexpression of *ZF* repressed several genes involved in chlorophyll biosynthesis, including *HEMA1* (*AT1G58290*), *GUN4* (*AT3G59400*), and *CHLOROPHYLL A OXYGENASE* (*AT3G59400*). It has been suggested that reduction of chlorophyll biosynthesis has a protective role in relieving photo-oxidative stress at low temperatures (29).

The GO enrichment of carbohydrate metabolism in COR genes has long been recognized as being involved in the production of sugars which have a cryoprotective function in freezing tolerance (5, 11, 29). Accumulation of galactinol and raffinose occurs as a result of induction of a group of galactinol synthase genes (*GOLS*) (38, 159). Taji et al showed that *Arabidopsis GOLS3* (*AT4g23990*) is induced by cold through the CBF pathway, while *GOLS1* (*AT2g47180*) and *GOLS2* (*AT1g56600*) are induced by salt and drought stresses (159). However, transcriptome analysis in previous studies (8, 38) and our study showed that *GOLS1* and *GOLS2* were also induced in cold. Overexpression of *HSFC1* increases the expression of *GOLS1* and *GOLS2*, but not *GOLS3*. This result may explain the increase of freezing tolerance in *HSFC1*-OX.

The Role of ZF in Starch Degradation and Circadian Rhythm

Starch degradation provides a source of sucrose during cold acclimation (160). Starch degradation is initiated by phosphorylation of amylose and amylopectin in starch granules by phosphoglucan water dikinase (PWD) and glucan water dikinase (GWD). It is then further linearized to glucans by α -amylase (AMY) and isoamylase (ISA) (Fig. 3.9*A*). The linear glucans can be converted to maltose through the action of β -amylase

(BAM) or glucose by disproportionating enzyme (DPE1). Glucose and maltose are transported to the cytosol, and maltose can be converted to glucose through transglucosidase (DPE2). It has been demonstrated that BAM2, BAM3, ISA3, DPE2 are induced by cold (161). In addition, a mutation in the cold-inducible GWD1 was found to impair freezing tolerance (160). Interestingly, overexpression of ZF increased transcript levels of GWD1, GWD3, ISA3, DPE1, and DPE2 (Table 3.4). We further verified these results by qRT-PCR. Consistent with previous reports (162) these genes showed diurnal expression at warm temperature (Fig. 3.9*B*) in a 12h light and 12h dark photoperiod with peak expression at ZT8 (or 8h after dawn), prior to starch degradation, which occurs at night. When cold treatment was started at 4h after dawn, the expression of all the genes was induced except for DPE1. We then verified the effects of overexpressing ZF by examining the expression of these genes at ZT8 in both WT and two ZF-OX lines (Fig. 3.10). Consistent with the array data, the transcript levels of these genes induced 3 to 4-fold in the ZF-overexpressing lines. The enrichment of starch degradation genes in the ZF regulon is unique in the TFs we tested (Table 3.3), which suggested that ZF might play an important role in regulating the starch degradation pathway in cold acclimation. Further analysis of starch metabolism and expression of the genes involved in this pathway in ZF mutants or knockdown lines in the cold may elucidate the function of *ZF* in cold acclimation.

Another unique GO term enriched in the *ZF*-OX lines is circadian rhythm (Table 3.3). In plants, the central clock components form three interlocking loops to regulate each other (Fig. 3.11) (139, 163, 164). Briefly, the morning-phased *CCA1* and *LHY* are induced in the morning when they repress the evening-phased *TOC1*, and accumulation

of TOC1 at night in turn represses CCA1 and LHY. TOC1 can also activate the expression of CCA1 and LHY before dawn, possibly through interacting with other regulator(s). CCA1 and LHY also induce PRR7 and PRR9 in the morning and their proteins form a negative feedback loop to regulate CCA1 and LHY. The stability of the TOC1 protein is post-translationally regulated by PRR5 and PRR3 to prevent ZTLmediated degradation. GI indirectly affects the stability of the TOC1 protein by stabilizing the ZTL protein in the light. Recently, an evening-phased JMJD5 was identified as positively regulating CCA1 and LHY. It has been established that low temperatures either dampen or disrupt cycling patterns of many central clock genes, but the regulators linking the cold signal to the clock have not been identified (69). Overexpression of ZF repressed the morning-phased genes, CCA1 and LHY, while it induced evening-phased genes, including TOC1, PRR5, PRR3, GI, and JMJD5 (Table 3.4). This tendency was extended to all the ZF regulon (Fig 3.12). The phase enrichment analysis of the ZF regulon revealed that up-regulated genes are enriched around ZT12, while down-regulated genes are enriched around ZT2. This result suggests that overexpression of ZF may have global effects on the cycling patterns of circadian- regulated genes through regulating central clock components. To test this hypothesis, WT and ZF-OX lines were entrained in 12h light and 12h dark for 12d and then moved to continuous light at ZT4 for free-run experiments. Samples were harvested at 4h intervals for 48h, and the transcript levels were examined (Figure 3.13). The rhythm of the central clock genes, including CCA1, LHY, PRR9, PRR7, PRR5, PRR3, TOC1, JMJD5, and GI, and a classic clock output gene, CAB2, were largely unaffected in ZF-overexpressing lines. However, the transcript levels of PPR3 and

CAB2, are significantly induced and repressed, respectively, in *ZF*-OX plants. In addition, overexpression of *ZF* has mild effects on repression of *CCA1* and *LHY a*fter dawn (ZT24 and ZT28), and induction of *GI*, whereas the expression of *PRR9*, *PRR7*, *TOC1*, and *JMJD5* was not affected. This result supports the hypothesis that *ZF* regulates central clock genes. Further global analysis of circadian-regulated genes under circadian conditions could help elucidate the function of *ZF* in regulating the circadian clock during cold acclimation.

Discussion

Cold acclimation is a process that brings about complex remodeling of biological and metabolic pathways through extensive changes in gene expression. The objective of this study was to determine how the expression of COR genes is configured by novel regulators in addition to the previously identified CBF and ZAT12 pathways of cold acclimation. To this end, 15 early cold-induced TFs with similar expression patterns to those of the *CBF*s or *ZAT12* were chosen for ectopic expression and transcriptome analysis. The results presented here increase our understanding of the regulation of about 29% of the COR genes defined in this study (Table 3.2). The actual size of the regulon could be larger than we observed in this study in which the effects of overexpression lines were examined in individual TF-OX lines. It is possible that plants have a requirement for regulating COR genes coordinately by more than one TF in the cold, or for post-translational modifications to activate their functions (165). In addition, the repressive effects on the cold-induced COR genes by TFs in the cold could have

been missed from our data (63). Further transcriptome analysis of overexpression lines or higher order mutants in cold conditions could complement our current data. In addition, ChIP experiments could further dissect the direct or indirect regulation of the TFs to construct gene regulatory networks.

The TFs described here regulate different sets of COR genes, but a substantial number of COR genes are co-regulated by multiple TFs, indicating an intensive co-regulation of the COR genes shared between these TF regulatory pathways in cold acclimation (Fig. 3.5 and Table 3.2). The co-regulation of the COR genes may integrate different environmental signals and regulators to fine-tune expression of some key pathways in cold acclimation, such as carbohydrate metabolism (Table 3.3). On the other hand, subsets of COR genes are only affected by a single transcription factor, which suggests that these TFs also have different biological functions in cold acclimation.

The CBF pathway is unique among gene regulatory pathways in cold acclimation in several different ways. The correlation of the expression pattern of the CBF regulon and COR genes indicated that overexpression of CBF has a positive effect on regulatory gene expression; for example, the COR genes up-regulated in the cold are also induced by overexpressing CBF (Fig. 3.4). This could explain the increase in freezing tolerance phenotype of *CBF*-OX lines (36, 119). In addition, the CBF regulon genes are mainly involved in coping with cold, osmotic, and drought stresses in preventing the cellular damage (Table 3.3), which are critical for plant survival at freezing temperatures. The HSFC1 regulon also showed positive correlation to the COR genes at most time points. Consistent with this observation, the *HSFC1*-OX lines

increased in freezing tolerance as determined by ELA. The potential cause could be the induction of *GOLS1* and *GOLS2* resulting in the accumulation of raffinose as cryoprotectant (Figure 3.7, Fig. 3.8, and Table A1).

Most of the transgenic plants overexpressing early cold-induced TFs have dwarf phenotypes (Fig. 3.6). It is unlikely that changes in biosynthesis or degradation of GA and SA are the cause of this phenotype as reported in *CBF1*-OX. The adverse growth effects when overexpressing these TFs may cause a decrease in freezing tolerance in general (Fig. 3.7 and Fig. 3.8). The possibility that these TFs positively regulate cold acclimation cannot be totally excluded. In *ZF*-OX and *HSFC1*-OX, induction of the genes involved in starch metabolism and raffinose synthesis (Table3.3 and Table 3.4) can protect plants from damage by freezing temperatures. However, the effect of overexpressing *ZF* in mis-regulation of COR genes may in turn have a negative impact on freezing tolerance (Fig. 3.4).

Recently, Bieniawska et al. (69) demonstrated that the number of COR genes and their strength of expression during short term cold treatment are greatly dependent on the time of day, a phenomenon termed "gating". Dong et al. (65) further showed that *CCA1* and *LHY* regulate the gating response of the CBF pathway through interacting with the *CBF* promoters. Low temperature has broad effects on the circadian rhythm, which is unique among abiotic stresses (69, 71). Many central clock regulators in *Arabidopsis* and chestnuts, including *CCA1*, *LHY*, *TOC1*, *PRRs*, *GI*, and *ELFs*, are either dampened or arrhythmic in the cold under diurnal or circadian conditions (69, 70, 72). From the transcriptome analysis of *ZF* overexpressing lines, the up-regulated regulon genes mostly peak during the evening, while the down-regulated regulon genes

are enriched in the morning (Fig. 3.12). This effect was seen in several central clock regulators, including CCA1, LHY, TOC1, PRR5, PRR3, GI, and JMJD5 in the array experiment (Table 3.4). Among these genes, the transcript levels of *PRR3* and *CAB2* are considerably affected in ZF-OX lines, whereas a mild reduction of CCA1 and LHY was observed under circadian conditions (Fig. 3.13). Based on the current clock model (139, 163, 164) and our data, it is possible that ZF regulates PRR3 to modulate its expression in the cold: increased expression of PRR3 can stabilize TOC1 protein to repress the expression of CCA1 and LHY. However, this scenario does not explain the reduction of CAB2 expression, because the effects on the expression of CCA1 and LHY are minimal in the ZF-OX lines. The other explanation is that overexpression of ZF affects genes involved in chlorophyll biosynthesis, which is shown in the GO enrichment analysis (Table 3.3). In addition, overexpression of ZF induced expression of GWD1, GWD3, ISA3, DPE1, and DPE2, which encode key enzymes involved in starch degradation used as a source of sucrose in the cold (Table 3.4 and Fig.3.10). The function of ZF in modulation of the circadian clock and starch degradation pathway needs to be further elucidated using *zf* mutants.



Figure 3.1 Temporal expression of the COR genes. The 2455 cold-regulated (COR) genes were selected with criteria of more than 4-fold change and FDR of <0.01 at any time point in comparison between cold and warm samples (cold/warm). (*A*) K-mean clustering (k=6) of COR genes form 6 groups of COR genes according to their expression patterns. (*B*) The relative expression of the COR genes when the transcript levels in the cold at each time point are compared to 0h (cold/0h). (*C*) The relative expression of the COR genes when the transcript levels in warm at each time point are compared to 0h (warm/0h). The signals are shown as log2-fold change.



Figure 3.2 Cluster analysis of cold-induced transcription factors. The174 cold-induced transcription factors in G3 to G6 in Figure 3.1*A* were subjected to k-mean clustering (k=7). The signals are shown as log2-fold change.



Figure 3.3 Relative transcript levels of cold-induced transcription factors of cluster 3 in Figure 3.2 and Table 3.1. WT *Arabidopsis* was grown under LD (16h light and 8h dark) for 14d and harvested starting at 3h after dawn with or without cold treatment (4° C) for 0, 0.5, 1, 3, 6, 12, and 24h. The transcript levels were quantified by qRT-PCR, normalized with *IPP2*, and then compared to corresponding warm samples (blue solid line) or 0h samples (red dashed line). The data are presented as mean ± SEM (n=3).



Figure 3.3 (Continued)



Figure 3.4 Spearman correlation of the expression of COR genes regulated by each TF and that in WT at each time point. The y-axis represents the Spearman's correlation coefficiency (rho), and the x-axis indicates duration of cold treatments. * P-value < 0.05. ** P-value < 0.01.



Figure 3.5 The co-regulatory network of the TF-regulons. (*A*) The regulon in G1 and G2 (green dots) and G3 to G6 (red dots) of COR genes for each TF (yellow circles) are visualized with Cytoscape. The COR genes from individual circles connected to TFs are the COR genes regulated by a single TF. Other COR genes forming the two circles in the middle represent COR genes regulated by two (outer) or more than two (inner) TFs. The connectivity of the 696 COR genes in (*A*) and each TF-regulon are represented in (*B*) and (*C*). The numbers of the co-regulated genes between two TFs are shown in (*D*).



Figure 3.5 (Continued)



Figure 3.6 Phenotypes of the transgenic plants overexpressing early cold-induced TFs. Plants were grown in long day (16h light, 8h dark) for 19 days.



Figure 3.7 Freezing tolerance test of the TF-overexpressing lines under non-cold acclimated (NAc) conditions. Two independent transgenic lines and WT were grown at 22°C in 12h light/12h dark for 21d and subjected to a freezing tolerance test using electrolyte leakage assays.



Figure 3.8 Freezing tolerance test of the TF-overexpressing lines under cold acclimated (Ac) condition. Two independent transgenic lines and WT were grown at 22°C in 12h light/12h dark for 21d, and then transferred to 4°C for 7d for cold acclimation before being subjected to electrolyte leakage assays.



Figure 3.9 Expression of enzymes involved in the starch degradation pathway are affected by low temperature. (*A*) The starch degradation pathway is mediated by several key enzymes, including glucan water dikinase (GWD), phosphoglucan water dikinase (PWD), α -amylase (AMY), isoamylase3 (ISA3), β -amylase (BAM), disproportionating enzyme (DPE1), and transglucosidase (DPE2) as described in the text. (*B*) The expression profiles of *GWD1*, *GWD3*, *ISA3*, *DPE1*, and *DPE2* were measured using qRT-PCR in WT *Arabidopsis* grown in 12h light/12h dark for 12d and treated without (warm) or with cold (4[°]C) starting from 4h after dawn. The data are presented as mean ± SEM (n=3).



Figure 3.10 The expression of key enzymes in the starch degradation pathway is affected by overexpression of *ZF*. WT and two *ZF*-OX lines were grown at 22°C in 12h light/12h dark for 13d and the samples were harvest at 8h after dawn. The transcript levels were normalized with *IPP2*, and compared to the WT warm sample (set as 1). The data are presented as mean \pm SEM (n=3).



Figure 3.11 Model of plant circadian clock. The circadian clock is regulated by several central clock regulators, including CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION 1 (TOC1), PSEUDO-RESPONSE REGULATOR (PRR), GIGANTEA (GI), JUMONJI DOMAIN CONTAINING 5 (JMJD5), and ZEITLUPE (ZTL) as described in the text.



Figure 3.12 Phase enrichment of the ZF regulon. The up- and down-regulated ZF regulon (Table A1) was subjected to PHASER to compare the enrichment of its phase to the whole circadian datasets (LL12_LDHH).


Figure 3.13 The effects of overexpressing *ZF* on the expression of circadian regulators and output genes in warm conditions. WT and two *ZF*-OX lines were entrained in 12h light/12h dark at 22° C for 12d and then shifted to continuous light starting at 4h after dawn (ZT4). The samples were harvested every 4h in a 48h-period, and the transcript levels were quantified with qRT-PCR. The relative expression levels were first normalized with *IPP2*, and then compared to WT samples at ZT8, which was set as 1. The data are presented as mean ± SEM (n=3).



Figure 3.13 (Continued)

Cluster	Affy ID	Locus	Gene Name
C1	261569_at	AT1G01060	LHY
C1	259751_at	AT1G71030	MYBL2 (ARABIDOPSIS MYB-LIKE 2)
C1	260380_at	AT1G73870	zinc finger (B-box type) family protein
C1	263739_at	AT2G21320	zinc finger (B-box type) family protein
C1	263549_at	AT2G21650	MEE3 (MATERNAL EFFECT EMBRYO ARREST 3)
C1	263252_at	AT2G31380	STH
C1	265248_at	AT2G43010	PIF4 (phytochrome interacting factor 4)
C1	266719_at	AT2G46830	CCA1
C1	258497_at	AT3G02380	COL2 (constans-like 2)
C1	258723_at	AT3G09600	myb family transcription factor
C1	252917_at	AT4G38960	zinc finger (B-box type) family protein
C1	249769_at	AT5G24120	SIGE (SIGMA FACTOR E)
C1	245925_at	AT5G28770	BZO2H3
C1	248306_at	AT5G52830	WRKY27
C2	260956_at	AT1G06040	STO (SALT TOLERANCE)
C2	255937_at	AT1G12610	DDF1
C2	262028_at	AT1G35560	TCP family transcription factor, putative
C2	263735_s_at	AT1G60040	AGL49 (AGAMOUS-LIKE 49)
C2	263584_at	AT2G17040	ANAC036
C2	263253_at	AT2G31370	bZIP transcription factor (POSF21)
C2	267515_at	AT2G45680	TCP family transcription factor, putative
C2	263783_at	AT2G46400	WRKY46
C2	259293_at	AT3G11580	DNA-binding protein, putative
C2	257262_at	AT3G21890	zinc finger (B-box type) family protein
C2	251745_at	AT3G55980	SZF1 (SALT-INDUCIBLE ZINC FINGER 1)
C2	254592_at	AT4G18880	AT-HSFA4A
C2	254016_at	AT4G26150	CGA1 (CYTOKININ-RESPONSIVE GATA FACTOR 1)
C2	253405_at	AT4G32800	AP2 domain-containing transcription factor TINY, putative
C2	250099_at	AT5G17300	myb family transcription factor
C2	249415_at	AT5G39660	CDF2 (CYCLING DOF FACTOR 2)
C2	248611_at	AT5G49520	WRKY48
C2	248389_at	AT5G51990	CBF4 (C- REPEAT-BINDING FACTOR 4)
C2	247455_at	AT5G62470	MYB96 (myb domain protein 96)

 Table 3.1 List of transcription factors up-regulated at low temperature.

Cluster	Affy ID	Locus	Gene Name
C3	259364_at	AT1G13260	RAV1
C3	261263_at	AT1G26790	DOF1.3
C3	261648_at	AT1G27730	ZAT10; STZ (salt tolerance zinc finger)
C3	261470_at	AT1G28370	ERF11 (ERF DOMAIN PROTEIN 11)
C3	259834_at	AT1G69570	DOF1.10
C3	261892_at	AT1G80840	WRKY40
C3	265359_at	AT2G16720	MYB7 (MYB DOMAIN PROTEIN 7)
C3	267028_at	AT2G38470	WRKY33
C3	263379_at	AT2G40140	CZF1
C3	257022_at	AT3G19580	AZF2
C3	258139_at	AT3G24520	AT-HSFC1
C3	252278_at	AT3G49530	ANAC062
C3	252214_at	AT3G50260	DEAR1; CEJ1 (COOPERATIVELY REGULATED BY ETHYLENE AND
			JASMONATE 1)
C3	255568_at	AT4G01250	WRKY22
C3	245397_at	AT4G14560	AXR5; IAA1 (INDOLE-3-ACETIC ACID INDUCIBLE)
C3	245276_at	AT4G16780	ATHB-2
C3	245250_at	AT4G17490	ATERF6
C3	254235_at	AT4G23750	CRF2 (CYTOKININ RESPONSE FACTOR 2)
C3	254075_at	AT4G25470	CBF2
C3	254066_at	AT4G25480	CBF3
C3	254074_at	AT4G25490	CBF1
C3	253722_at	AT4G29190	zinc finger (CCCH-type) family protein
C3	246253_at	AT4G37260	MYB73 (MYB DOMAIN PROTEIN 73)
C3	245711_at	AT5G04340	CZF2; ZAT6
C3	249606_at	AT5G37260	RVE2 (REVEILLE 2); CIR2
C3	248799_at	AT5G47230	ERF5
C3	248253_at	AT5G53290	CRF3 (CYTOKININ RESPONSE FACTOR 3)
C3	247655_at	AT5G59820	ZAT12
C3	247029_at	AT5G67190	DEAR2, DREB; AP2 domain-containing transcription factor, putative
C3	246987_at	AT5G67300	MYB44; MYBR1

Table 3.1 (Continued)

Cluster	Affy ID	Locus	Gene Name				
C4	260203_at	AT1G52890	ANAC019				
C4	256149_at	AT1G55110	AtIDD7 (Arabidopsis thaliana				
			Indeterminate(ID)-Domain 7)				
C4	259992_at	AT1G67970	AT-HSFA8				
C4	263797_at	AT2G24570	WRKY17				
C4	245635_at	AT1G25250	AtIDD16 (Arabidopsis thaliana				
			Indeterminate(ID)-Domain 16)				
C4	267246_at	AT2G30250	WRKY25				
C4	263963_at	AT2G36080	DNA-binding protein, putative				
C4	266555_at	AT2G46270	GBF3 (G-BOX BINDING FACTOR 3)				
C4	259129_at	AT3G02150	PTF1 (PLASTID TRANSCRIPTION				
			FACTOR 1)				
C4	258742_at	AT3G05800	transcription factor				
C4	259244_at	AT3G07650	COL9 (CONSTANS-LIKE 9)				
C4	256430_at	AT3G11020	DREB2B				
C4	257053_at	AT3G15210	ERF4				
C4	251282_at	AT3G61630	CRF6 (CYTOKININ RESPONSE FACTOR				
.	0.450.47	474047000	6)				
C4	245247_at	AT4G17230	SCL13 (Scarecrow-like 13)				
C4	254159_at	A14G24240	WRKY7				
C4	253535_at	A14G31550	WRKY11				
C4	253485_at	AT4G31800	WRKY18				
C4	250781_at	AT5G05410	DREB2A				
C4	249746_at	AT5G24590	TIP (TCV-INTERACTING PROTEIN)				
C4	248744_at	AT5G48250	zinc finger (B-box type) family protein				
C4	247452_at	AT5G62430	CDF1 (CYCLING DOF FACTOR 1)				
C5	263656_at	AT1G04240	SHY2 (SHORT HYPOCOTYL 2)				
C5	261766_at	AT1G15580	IAA5				
C5	261663_at	AT1G18330	EPR1				
C5	259971_at	AT1G76580	transcription factor				
C5	262136_at	AT1G77850	ARF17 (AUXIN RESPONSE FACTOR 17)				
C5	245078_at	AT2G23340	AP2 domain-containing transcription factor, putative				
C5	264057 at	AT2G28550	RAP2.7 (RELATED TO AP2.7)				
C5	265842 at	AT2G35700	FRF38				
C5	260540 at	AT2G43500	RWP-RK domain-containing protein				
C5	253799 at	AT4G28140	AP2 domain-containing transcription factor				
20	at		putative				
C5	246217_at	AT4G36920	AP2 (APETALA 2)				

Figure 3.1 (Continued)

Table 3.1 ((Continued))
		,

Cluster	Affy ID	Locus	Gene Name
C5	263128_at	AT1G78600	LZF1 (LIGHT-REGULATED ZINC FINGER PROTEIN 1)
C5	250694 at	AT5G06710	HAT14
C5		AT5G11590	TINY2 (TINY2)
C5	246523_at	AT5G15850	COL1 (constans-like 1)
C5	246911_at	AT5G25810	tny (TINY)
C5	249422_at	AT5G39760	AtHB23
C5	249087_at	AT5G44210	ERF9
C5	248366_at	AT5G52510	scarecrow-like transcription factor 8 (SCL8)
C5	248328_at	AT5G52660	myb family transcription factor
C5	248160_at	AT5G54470	zinc finger (B-box type) family protein
C5	247945_at	AT5G57150	basic helix-loop-helix (bHLH) family protein
C5	247921_at	AT5G57660	zinc finger (B-box type) family protein
C5	247707_at	AT5G59450	scarecrow-like transcription factor 11 (SCL11)
C5	247601_at	AT5G60850	OBP4
C5	247519_at	AT5G61430	ANAC100
C5	266820_at	AT2G44940	AP2 domain-containing transcription factor TINY, putative
C5	266125_at	AT2G45050	zinc finger (GATA type) family protein
C5	258813_at	AT3G04060	ANAC046
C5	258325_at	AT3G22830	AT-HSFA6B
C5	257643_at	AT3G25730	AP2 domain-containing transcription factor, putative
C5	252210_at	AT3G50410	OBP1 (OBF BINDING PROTEIN 1)
C5	252175_at	AT3G50700	AtIDD2 (Arabidopsis thaliana Indeterminate(ID)-Domain 2)
C6	259417 at	AT1G02340	HFR1 (LONG HYPOCOTYL IN FAR-RED)
C6	261254_at	AT1G05805	basic helix-loop-helix (bHLH) family protein
C6	260776_at	AT1G14580	zinc finger (C2H2 type) family protein
C6	262843_at	AT1G14687	AtHB32
C6	256091_at	AT1G20693	HMGB2 (HIGH MOBILITY GROUP B 2)
C6	256092_at	AT1G20696	HMGB3 (HIGH MOBILITY GROUP B 3)
C6	259595_at	AT1G28050	zinc finger (B-box type) family protein
C6	261192_at	AT1G32870	ANAC13
C6	245807_at	AT1G46768	RAP2.1 (related to AP2 1)
C6	261610_at	AT1G49560	myb family transcription factor
C6	261613_at	AT1G49720	ABF1
C6	264190_at	AT1G54830	NF-YC3 (NUCLEAR FACTOR Y, SUBUNIT C3)

Cluster	Affy ID	Locus	Gene Name
C6	260627_at	AT1G62310	transcription factor jumonji (jmjC) domain- containing protein
C6	262166_at	AT1G74840	myb family transcription factor
C6	259711_at	AT1G77570	DNA binding / transcription factor
C6	265333_at	AT2G18350	AtHB24
C6	266695_at	AT2G19810	zinc finger (CCCH-type) family protein
C6	265662_at	AT2G24500	FZF (C2H2 zinc finger protein)
C6	267509_at	AT2G45660	AGL20 (AGAMOUS-LIKE 20)
C6	266514_at	AT2G47890	zinc finger (B-box type) family protein
C6	258603_at	AT3G02990	ATHSFA1E
C6	258809_at	AT3G04070	ANAC047
C6	258395_at	AT3G15500	ANAC055
C6	252573_at	AT3G45260	zinc finger (C2H2 type) family protein
C6	252429_at	AT3G47500	CDF3 (CYCLING DOF FACTOR 3)
C6	251623_at	AT3G57390	AGL18
C6	255625_at	AT4G01120	GBF2 (G-BOX BINDING FACTOR 2)
C6	255585_at	AT4G01550	ANAC069
C6	254778_at	AT4G12750	sequence-specific DNA binding / transcription factor
C6	254670 at	AT4G18390	TCP family transcription factor, putative
C6	253872 [_] at	AT4G27410	RD26
C6	253603_at	AT4G30935	WRKY32
C6	253423_at	AT4G32280	IAA29
C6	253245_at	AT4G34590	GBF6 (G-BOX BINDING FACTOR 6)
C6	246222_at	AT4G36900	RAP2.10 (related to AP2 10)
C6	251132_at	AT5G01200	myb family transcription factor
C6	250155_at	AT5G15160	bHLH family protein
C6	246432_at	AT5G17490	RGL3 (RGA-LIKE PROTEIN 3)
C6	249944_at	AT5G22290	ANAC089
C6	246939_at	AT5G25390	SHN2 (shine2)
C6	249065_at	AT5G44260	zinc finger (CCCH-type) family protein

Table 3.1 (Continued)

Cluster	Affy ID	Locus	Gene Name
C7	261564_at	AT1G01720	ATAF1
C7	255953_at	AT1G22070	TGA3
C7	259626_at	AT1G42990	ATBZIP60
C7	259705_at	AT1G77450	ANAC032
C7	263295_at	AT2G14210	AGL44 (AGAMOUS-LIKE 44)
C7	266839_at	AT2G25930	ELF3 (EARLY FLOWERING 3)
C7	266841_at	AT2G26150	ATHSFA2
C7	267534_at	AT2G41900	zinc finger (CCCH-type) family protein
C7	266327_at	AT2G46680	ATHB-7
C7	257610_at	AT3G13810	AtIDD11 (Arabidopsis thaliana
			Indeterminate(ID)-Domain 11)
C7	258157_at	AT3G18100	MYB4R1 (myb domain protein 4R1)
C7	257985_at	AT3G20810	transcription factor; jumonji (jmjC) domain-
			containing protein
C7	251272_at	AT3G61890	ATHB-12
C7	250858_at	AT5G04760	myb family transcription factor
C7	247795_at	AT5G58620	zinc finger (CCCH-type) family protein

Table 3.1 (Continued)

TF	Locus	DEG	CORs (% of 2455 CORs)	p-value	G1 **(512)	G2 **(579)	G3 **(298)	G4 **(276)	G5 **(586)	G6 **(204)
HSFC1	AT3G24520	632	210 (8.55)	<2.2E-16	51	54	21	22	40	22
Zinc-finger										
(ZF)	AT4G29190	460	167 (6.80)	<2.2E-16	30	46	14	21	_ 26 _	30
CBF2*	AT4G25470	238	146 (5.95)	<2.2E-16	14	13	7	8	101	3
ZAT10	AT1G27730	364	145 (5.91)	<2.2E-16	30	45	16	17	26	11
CZF1	AT2G40140	213	108 (4.4)	<2.2E-16	17	17	14	16	24	20
ZAT12*	AT5G59820	335	72 (2.97)	<2.2E-16	18	24	13	5	11	1
MYB44	AT5G67300	188	58 (2.36)	2.04E-9	13	17	7	4	9	8
DEAR1	AT3G50260	70	37 (1.51)	1.0E-14	7	8	3	9	4	7
ERF5	AT5G47230	59	24 (0.98)	2.2E-16	7	2	4	3	2	6
WRKY33	AT2G38470	18	11 (0.45)	1.09E-8	2	1	2	0	1	5
ERF6	AT4G17490	12	8 (0.33)	2.09E-7	1	1	1	2	1	2
ANAC62	AT3G49530	13	5 (0.20)	4.10E-6	1	0	2	1	1	0
CRF3	AT5G53290	33	4 (0.16)	7.14E-3	1	2	0	1	0	0
RAV1	AT1G13260	19	4 (0.16)	0.77	0	2	0	0	1	1
CRF2	AT4G23750	14	3 (0.12)	0.12	1	0	1	0	1	0
CZF2	AT5G04340	7	2 (0.08)	0.17	1	1	0	0	0	0
MYB73	AT4G37260	9	1 (0.04)	0.16	1	0	0	0	0	0
			696 (28 35)							

 Table 3.2 Summary of differentially expressed genes (DEG) in each TF-OX line.

The DEG are defined by comparing transgenic lines to WT using a criterion of more than 2-fold change (p-value< 0.01). The significance of enrichments of COR genes in DEG for each TF were tested with Fisher's exact test, and the p-values were presented in table.

The significance enrichments of COR genes regulated by each TF in each group of COR genes defined in Table A.1 and Figure 3.1 were tested with Fisher's exact test. The red block represents the p-value< 0.01, and the yellow block represents p-value< 0.05.

Table 3.2 (Continued)* The CBF2-OX and ZAT12-OX data were analyzed from GSE5536 and GSE5742 microarray experiments.** The number in parentheses represents the number of genes in each group.

							Gene C	ounts			
Description ‡	CORs	p-value	CBF (146)	ZAT12 (72)	ZAT10 (145)	ZF (167)	CZF1 (108)	HSF (210)	MYB44 (58)	ERF5 (24)	DEAR1 (37)
P response to abiotic stimulus	311	1.03E-40	31	12	24	19	16	38	10	6	8
P response to temperature stimulus	85	4.91E-27	20	4	13	6	7	14	1	0	3
P response to cold	63	5.65E-25	19	4	8	5	4	9	0	0	1
P response to water deprivation	49	1.56E-21	16	3	5	4	3	9	2	0	1
P response to osmotic stress	63	6.20E-20	14	2	7	3	2	5	3	1	0
P response to salt stress	54	1.19E-16	10	2	5	2	2	5	3	1	0
P response to heat	23	2.30E-05	0	2	2	1	4	6	0	0	2
P response to hormone stimulus	144	2.46E-27	18	7	14	5	8	18	5	4	1
P response to abscisic acid stimulus	72	4.60E-24	15	3	9	3	3	11	2	1	1
P response to salicylic acid stimulus	35	2.35E-10	3	1	3	1	3	2	2	2	3
P response to ethylene stimulus	36	7.91E-10	1	0	2	1	2	6	3	4	0
P response to wounding	64	1.71E-19	6	5	6	2	1	6	3	2	0

Table 3.3 GO analysis of COR genes and TF-regulons

						G	iene Cou	unts			
Description ‡	CORs	p-value	CBF (146)	ZAT12 (72)	ZAT10 (145)	ZF (167)	CZF1 (108)	HSF (210)	MYB44 (58)	ERF5 (24)	DEAR1 (37)
P response to biotic stimulus	163	2.40E-23	8	8	19	13	7	22	6	4	2
P response to other organism	107	7.56E-21	5	7	14	9	4	15	5	4	2
P defense response	133	4.04E-20	7	5	12	7	7	17	5	4	1
P defense response to fungi	13	3.89E-03	1	1	2	0	4	3	0	1	0
P circadian rhythm	15	3.91E-08	0	1	3	6	0	3	0	1	0
P carbohydrate metabolism	117	7.51E-11	8	4	11	15	7	20	5	0	5
P starch metabolism	10	6.51E-05	0	0	1	6	0	3	0	0	0
P glucan metabolism	20	9.34E-05	0	0	3	7	1	6	1	0	1
P polysaccharide metabolism	28	1.32E-05	1	0	4	7	2	8	2	0	2
P cell wall loosening	12	2.14E-05	3	1	3	2	2	3	1	0	0
C cell wall	41	4.25E-05	3	1	2	4	9	8	4	1	2
P cell wall modification	12	5.62E-05	3	1	3	2	2	3	1	0	0
P pigment metabolism	21	4.16E-05	0	0	5	5	0	4	0	0	1
P chlorophyll metabolism	9	3.66E-03	0	0	2	3	0	3	0	0	0
F oxidoreductase activity	21	2.92E-03	1	0	5	2	0	2	1	0	2

1. ‡: P, Biological Process; C, Celluler Component; F, Molecular Function
 2. The numbers in parentheses below the TFs represent the number of TF-regulon genes.

3. The red and yellow colors indicate a p-value less than 0.01 and 0.05 respectively with Fisher's exact test.

 Table 3.4 Genes in circadian or starch degradation pathways affected by overexpressing ZF

Locus	Gene	log2 (FC)
CIRCADIAN	RHYTHM	
AT2G46830	CIRCADIAN CLOCK ASSOCIATED (CCA1)	-1.15
AT1G01060	LATE ELONGATED HYPOCOTYL (LHY)	-1.83
AT1G29920	CAB2	-0.42
AT5G61380	TIMING OF CAB EXPRESSION 1 (TOC1)	1.52
AT5G24470	PSEUDO-RESPONSE REGULATOR 5 (PRR5)	1.71
AT5G60100	PSEUDO-RESPONSE REGULATOR 3 (PRR3)	2.84
AT1G22770	GIGANTEA (GI)	2.11
AT3G20810	JUMONJI DOMAIN CONTAINING 5 (JMJD5)	2.19

STARCH DEGRADATION

AT1G10760	GLUCAN, WATER DIKINASE; STARCH EXCESS 1 (GWD1; SEX1)	1.28
AT5G26570	PHOSPHOGLUCAN, WATER DIKINASE (GWD3)	1.54
AT3G52180	DUAL-SPECIFICITY PROTEIN PHOSPHATASE 4;	1.76
	STARCH-EXCESS 4 (DSP4, SEX4)	
AT4G09020	ISOAMYLASE 3 (ISA3)	1.5
AT5G64860	α-1,4 GLUCANOTRANSFERASE (DPE1)	1.66
AT2G40840	TRANSGLICOSIDASE (DPE2)	1.06

 Table 3.5 List of primers for qRT-PCR

Gene	Locus	Primer (Fw or Rv)	Primer sequences
WRKY33	AT2G38470	Fw	CGATGTTCCTGCAGCTCGTGG
		Rv	TGTGGTGCTCTGTTTGTGGCG
HSFC1	AT3G24520	Fw	CCGGATGGGTGGATTGTTCCTATGAC
		Rv	GTTGAATTCGAGAGCATCGACTTCGC
ERF6	AT4G17490	Fw	CTACTACTGCCACCACCAATCGATGG
		Rv	AAAATCTCCGGTTTGGGAGTGACGAG
CRF2	AT4G23750	Fw	AAAATGGGCGGCGGAGATAAGAGATC
		Rv	GAGAAATTAGTCAGAGCGTCGGGACC
ERF5	AT5G47230	Fw	GGCGACTCCTAACGAAGTATCTGCAC
		Rv	CATGGATCTGTAGCCACAGGAGAAGC
CRF3	AT5G53290	Fw	TCCGGCGTCGAGTCGTCAAC
		Rv	ATCTCCGCCGCCCATTTCCC
DEAR1	AT3G50260	Fw	GCTGACGTGGCAGTCAAAATGAAGAG
		Rv	TGAACGCTTGTTGGGTTCTCGAATCT
MYB44	AT5G67300	Fw	AATGGGGAAGTCTTTTCCCGGTAACG
		Rv	CATTGTTCCGTTGCATCTCCGTCATG
ZAT10	AT1g27730	Fw	TCGAGCACTGGACAAAGGGTAAGC
		Rv	CCTCAGTGAGGTTTTGGTGGTGGA
CZF1	AT2g40140	Fw	GCCTTGTCCCGAGTTTCGTA
		Rv	TGCGCGTACTCACACGAATC
ZF	AT4G29190	Fw	TCATTTCCTCGTAACAATCCTTTATTC
		Rv	CGGTGTTGTAGGCAGAGACTGA
RAV1	At1g13260	Fw	TAGATGCGGGTCGGGTTTT
		Rv	GTTTCTTGAACTCTCCGGTGAAA
ANAC62	AT3g49530	Fw	GCAGTTGACATGAGCAATGATGT
		Rv	TGTGCCTGGCGACTCTTAATC

Table 3.5 (Continued)

Gene	Locus	Primer (Fw or Rv)	Primer sequences
MYB73	AT4g37260	Fw	TGAGGAGTTACATGGCGGATT
		Rv	CGCCGCCAGAACTACTACCA
CZF2	AT5g04340	Fw	TCGCGACGGAGATAGAAACC
		Rv	GCAGAGGAGGTGAAGACGAAGA
AZF2	AT3G19580	Fw	TACGAAGGCAACCTCGGCGG
		Rv	CGTGCTCGACACGCTTCCAC
DEAR2	AT5G67190	Fw	GGAGGTCGGTGCTCAGGTT
		Rv	CGGTGGCGGTTATTTTGC
AXR5	AT4G14560	Fw	TGGATGGCCTCCAGTGAGATCTAACC
		Rv	GGAGCTCCGTCCATACTCACTTTCAC
WRKY22	AT4G01250	Fw	TGAGGATCATCTAGCGGTGGGAGATC
		Rv	ATCGCTAACCACCGTATCCGACAAAG
ATHB2	AT4G16780	Fw	GACGAAGACGCTGGAGTATCTTCACC
		Rv	TCCCTGTAGAGCTTGAGACTGTACTGT
ERF11	AT1G28370	Fw	ACAGAGGAGTGAGGAAGAGACCATGG
		Rv	CTTCAGGAGTGTCGAAAGTACCGAGC
WRKY40	AT1G80840	Fw	GAGGACGCATTCAGCTGCGC
		Rv	GAGCTACTCTCCGACACTCCGC
DOF1.3	AT1G26790	Fw	CCCAACAACTTCCAAGGGTTAC
		Rv	AGGCCAAGGAGGCGAAAC
RVE2	AT5G37260	Fw	CGCCTCCAAGGCCAAAG
		Rv	TTGCATCAGGAATCACAAGCTT
DOF1.10	AT1G69570	Fw	CCAACGGGTCCTAACGCTAGT
		Rv	GGGTATATCGGAACCGTGCAT
MYB7	AT2G16720	Fw	TCGCTGCGGTAAAAGCTGCC
		Rv	AGACCACTTGTTGCCTAGGAGGC

Table 3.5 (Continued)

Gene	Locus	Primer (Fw or Rv)	Primer sequences
CBF1	AT4G25490	Fw	CCGCCGTCTGTTCAATGGAATCAT
		Rv	TCCAAAGCGACACGTCACCATCTC
CBF2	AT4G25470	Fw	CGACGGATGCTCATGGTCTT
		Rv	TCTTCATCCATATAAAACGCATCTTG
CBF3	AT4G25480	Fw	TTCCGTCCGTACAGTGGAAT
		Rv	AACTCCATAACGATACGTCGTC
ZAT12	AT5G59820	Fw	GTGCGAGTCACAAGAAGCCTAACA
		Rv	GCGACGACGTTTTCACCTTCTTCA
IPP2	AT3G02780	Fw	ATTTGCCCATCGTCCTCTGT
		Rv	GAGAAAGCACGAAAATTCGGTAA
CCA1	AT2G46830	Fw	TCTGTGTCTGACGAGGGTCGAATT
		Rv	ACTTTGCGGCAATACCTCTCTGG
LHY	AT1G01060	Fw	CTGCCGCTGTGCATGACT
		Rv	GGATGAGCGGTCCACAAGAT
TOC1	AT5G61380	Fw	GCTGCACCTAGCTTCAAGCA
		Rv	TCTTCGCAGAATCCCTGTGAT
PRR3	AT5G60100	Fw	GGACAAAAAGAGCCAGTGATACTAAGA
		Rv	GGGTGCATCGGGAAATTG
PRR5	AT5G24470	Fw	CGAGAAGCCGCTTTAACCAA
		Rv	CGGCTCTCGTAACGAACCTT
PRR7	AT5G02810	Fw	AAAAGCTGTGGATGTTGATGACA
		Rv	GTGCTATCAACTCGGTCCCATAG
PRR9	AT2G46790	Fw	GCCAGAGAGAAGCTGCATTGA
		Rv	CCTGCTCTGGTACCGAACCTT
JMJD5	AT3G20810	Fw	CGGAGACCACAACAACCTCCGTC
		Rv	ATAAGCGTAACCCCCTTCAGCAGAAA

Table 3.5 (Continued)

Gene	Locus	Primer (Fw or Rv)	Primer sequences
GI	AT1G22770	Fw	GTATCTGCAACGCCAGCGA
		Rv	GCAACTCCCTTTCAGCCTGA
CAB2	AT1G29920	Fw	CCGGAAAGGCTGTGAACCT
		Rv	CACACGGCCGCTTCCA
ISA3	AT4G09020	Fw	GGTGGCCGTGACATCTATGTG
		Rv	TGGAATCAGAGCCTTGACAAAG
DPE1	AT5G64860	Fw	CAGAGGATTTGCAGGGTTTTG
		Rv	CGTCCAACCATGGCAACTTT
DPE2	AT2G40840	Fw	CCTGCAACAGAGGAGACAATCA
		Rv	CGTGTACGCGGTATCTCCAGTA
GWD1	AT1G10760	Fw	AGTACAGAGAACTTTTGCGGATGAT
		Rv	TTGCCCGACATCTCCTTCA
GWD3	AT5G26570	Fw	TTCAGATTCGGTTTGCCAAGT
		Rv	GCTCTACGGCTTAGAACAGCTCTT

Table 3.6 List of primers for cloning

Gene	Locus	Primer (Fw or Rv)	primer sequences
ZAT10	AT1g27730	Fw	ATGGCGCTCGAGGCTCTTACATCACCAA
		Rv	TTAAAGTTGAAGTTTGACCGGAAAGTCAAACCGA
CZF1	AT2g40140	Fw	ATGTGCGGTGCAAAGAGCAACCTTTGCT
		Rv	TTATGCCACAATCTGCTGCTCATGGTCTATATA
RAV1	At1g13260	Fw	ATGGAATCGAGTAGCGTTGATGAGAGT
		Rv	TTACGAGGCGTGAAAGATGCGTTGCTTCT
MYB73	AT4g37260	Fw	ATGTCAAACCCGACCCGTAAGAATATGGA
		Rv	CTACTCCATCTTCCCAATTCCGATTTGGT
CZF2	AT5g04340	Fw	ATGGCACTTGAAACTCTTACTTCTCCAAGA
		Rv	TTAGGGTTTCTCCGGGAAGTCAAACCGGA-

Materials and Methods

Plant Material and Growth Conditions

Wild type *Arabidopsis thaliana* (Col-0) and transgenic lines overexpressing TF were stratified at 4°C in the dark for 3 to 5 days prior to growth. For gene expression studies and Affymetrix GeneChip experiments, plants were grown on sterilized Gamborg's B5 medium (Caisson Laboratories) and 8% phytoagar (Caisson Laboratories) without sucrose. For freezing tolerance studies, plants were grown in soil as described (49). All plants were grown at 22°C under ~100 µmol m⁻² s⁻¹ fluorescent white light.

Abiotic Gene Expression Datasets and Cluster

The ATGenExpress Abiotic time course experiment series (113) was downloaded from Gene Expression Omnibus (GEO, <u>http://www.ncbi.nlm.nih.gov/geo/</u>). The accession numbers of the cold stress time series and its control are GSE5621and GSE5621, respectively. Only the datasets from shoot tissues were normalized and analyzed with GCRMA (166) and LIMMA (167) packages from Bioconductor (<u>http://www.bioconductor.org/</u>) in R (<u>http://www.R-project.org</u>). Differentially expressed genes were selected by more than 4-fold change and FDR <0.01 at at least one time point by comparison of cold-treated samples to control samples at the same time point. For the fold-change of cold/0h and warm (compared to 0h), GCRMA and LIMMA were applied. K-mean clustering was performed with normalized expression data using CLUSTER, and the results were visualized with TREEVIEW (168).

Phase Enrichment Analysis

The enrichment of phase (time of the peak expression of circadian genes) was analyzed with PHASER (haystack.cgrb.oregonstate.edu/) as previously described (169). The enrichment score was calculated as the ratio of genes at a specific phase within a given list divided by the ratio of the circadian regulated genes at a specific phase within GeneChip. The LL12_LDHH dataset was used for phase analysis in this experiment. Briefly, the dataset was collected from plants entrained in 12 h light/12 h dark and moved to continuous light starting at ZT0, and samples were harvested at 4 h intervals for 24 h. The graphs were plotted over a 48h period for visualization.

Determination of Gene Expression

Transcript levels were determined using real-time quantitative RT-PCR (qRT-PCR). Plant sample collection, RNA extraction, and qRT-PCR were performed as described (49), except that 100 ng of total RNA was used in a 20 µl reverse transcription reaction, and 2 µl of 10-fold diluted cDNA was used as template in a 10 µl reaction for qRT-PCR. *IPP2* (*ISOPENTENYL PYROPHOSPHATE-DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2*) was used as the reference gene. Primers are listed in Table 3.5.

Generation of Transgenic Lines Overexpressing TFs

For transgenic lines overexpressing *ZAT10*, *CZF1*, *RAV1*, *CZF2*, and *MYB73*, the coding regions were amplified from WT *Arabidopsis* cDNA and cloned into the pCR®8/GW/TOPO® vector (Invitrogen). For *CRF3*, *DEAR1*, *MYB44*, *ZF*, and *ANAC62*,

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the coding region in gateway pENTR223 entry vectors were obtained from the Arabidopsis Biological Resource Center (ABRC) (https://abrc.osu.edu). The cloned sequences were transferred to the plant gateway binary pEarleyGate100 vector (143) through recombination reactions (Invitrogen). For *ERF5*, *ERF6*, and *CRF2*, the plant binary vector pYL436 containing the coding regions of these TFs were obtained from ABRC. The floral dip method was used to transform genes into WT *Arabidopsis* (142). The expression levels of transgenes were examined using qRT-PCR and two independent T3 homozygous lines were selected for further experiments. The primer sequences are listed in Table 3.6. The clones requested from ABRC were: WRKY33, DKLAT2G38470; ERF6, DKLAT4G17490; ERF5, DKLAT5G47230; CRF2, DKLAT4G23750; HSFC1, G14335; CRF3, G68286; DEAR1, G13196; MYB44, G09033.

Affymetrix GeneChip Experiments

WT and two independent transgenic lines for each TF were grown under continuous light for 14 days on plates. Samples were collected, and RNA was extracted with the RNeasy Plant Mini Kit following the manufacture's protocol (Qiagen). Biotinylated cRNA was prepared from 200 ng of total RNA using MessageAmpTM Premier RNA Amplification Kit (Ambion), and hybridized to the Affymetrix Arabidopsis ATH1 GeneChip.

Affymetrix GeneChip Data Analysis

The CEL files from WT and transgenic lines overexpressing TFs (TF-OX) from the ATH1 GeneChip experiments were normalized and processed as described in the previous section. The differentially expressed genes (DEG) in the TF-OX lines were selected by comparison of TF-OX and WT with more than 2-fold change (p-vale< 0.01). The TF-regulon was defined by the genes' overlap between the DEG and the COR genes for each TF.

For correlation analysis of gene expression in the COR genes and the TFregulon at each time point, Spearman's correlation efficiency (rho) was calculated. The Gene Ontology (GO) analysis was performed using Classification SuperViewer Tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) with 1000 times bootstrap. Enrichment of the GO terms for the COR genes was calculated using all the genes on the Arabidopsis ATH1 GeneChips as background. For the analysis of TF-regulons, the COR genes were used as background.

Electrolyte Leakage Assays (ELA)

WT and transgenic plants were grown at 22°C under a 12 h-photoperiod (12 h light, 12 h dark) for three weeks prior to ELA as described (49). For cold acclimation, plants were moved to 4°C under a 12 h-photoperiod with ~35 μ mol m⁻² s⁻¹ fluorescent white light for 7 d.

APPENDIX

Affy ID	Locus	group	CBF2	ZAT12	HSFC1	ZF	ZAT10	CZF1	MYB44	DEAR1	ERF5
245094 at	AT2G40840	a1				1.58	-1.12				
245129 at	AT2G45350	a1									
	AT2G47440	g1									
	AT1G67830	g1									
245253_at	AT4G15440	g1									
245265_at	AT4G14400	g1			-4.16					1.69	
245266_at	AT4G17070	g1									
245296_at	AT4G16370	g1									
245325_at	AT4G14130	g1						2.27			
245326_at	AT4G14100	g1									
245334_at	AT4G15800	g1									
245341_at	AT4G16447	g1									
245362_at	AT4G17460	g1									
245386_at	AT4G14010	g1									
245422_at	AT4G17470	g1		-1.76	-2.29			-1.89			
245463_at	AT4G17030	g1			2.53	-1.96					
245592_at	AT4G14540	g1									
245642_at	AT1G25275	g1	-1.24								1.30
245672_at	AT1G56710	g1							1.67		
245692_at	AT5G04150	g1			1.71						
245760_s_at	AT1G66920;	g1									
	AT1G66910										
245776_at	AT1G30260	g1									
245800_at	AT1G46264	g1			1.77						

Table A1. List of COR genes and the regulons of each overexpressed TFThe numbers represent log2 fold-change in the transgenic lines overexpressing TF.

245881_at	AT5G09462;	g1						
	AT5G09460;	-						
	AT5G09463;							
	AT5G09461							
246004_at	AT5G20630	g1						
246011_at	AT5G08330	g1		-1.26				
246079_at	AT5G20450	g1						
246159_at	AT5G20935	g1						
246200_at	AT4G37240	g1						
246231_at	AT4G37080	g1				-1.11		
246236_at	AT4G36470	g1						
246260_at	AT1G31820	g1		2.91				
246275_at	AT4G36540	g1						
246296_at	AT3G56750	g1						
246518_at	AT5G15770	g1						
246573_at	AT1G31690	g1				1.63		
246584_at	AT5G14730	g1						
246603_at	AT1G31690	g1				1.93		
246607_at	AT5G35370	g1						
246620_at	AT5G36220	g1						
246832_at	AT5G26600	g1						
246901_at	AT5G25630	g1						
246948_at	AT5G25130	g1						
246996_at	AT5G67420	g1						
247037_at	AT5G67070	g1						
247072_at	AT5G66490	g1						
247100_at	AT5G66520	g1						
247213_at	AT5G64900	g1						
247216_at	AT5G64860	g1			1.66			

247263_at	AT5G64470	g1							
247377_at	AT5G63180	g1							
247385_at	AT5G63420	g1							
247386_at	AT5G63420	g1							
247388_s_at	AT5G63470;	g1							
	AT3G48590								
247396_at	AT5G62930	g1							
247443_at	AT5G62720	g1	-1.48						
247447_at	AT5G62730	g1		2.65					
247486_at	AT5G62140	g1				-1.28			
247524_at	AT5G61440	g1							
247529_at	AT5G61520	g1							
247540_at	AT5G61590	g1							
247549_at	AT5G61420	g1							
247600_at	AT5G60890	g1	2.24						
247628_at	AT5G60400	g1							
247638_at	AT5G60490	g1			-1.53				
247684_at	AT5G59670	g1							
247800_at	AT5G58570	g1				1.74			
247814_at	AT5G58310	g1			-2.57				
247880_at	AT5G57780	g1							
247882_at	AT5G57785	g1							
248046_at	AT5G56040	g1							
248169_at	AT5G54610	g1					-1.86	1.65	
248186_at	AT5G53880	g1							
248199_at	AT5G54170	g1							
248230_at	AT5G53830	g1							
248270_at	AT5G53450	g1			1.19				
248291_at	AT5G53020	g1							

248329_at	AT5G52780	g1						
248330_at	AT5G52810	g1						
248336_at	AT5G52420	g1						
248348_at	AT5G52190	g1						
248353_at	AT5G52320	g1				-1.90		
248385_at	AT5G51910	g1						
248606_at	AT5G49450;	g1			1.23			
	AT5G49448							
248685_at	AT5G48500	g1						
248764_at	AT5G47640	g1					-1.84	
248807_at	AT5G47500	g1						
248839_at	AT5G46690	g1						
248865_at	AT5G46790	g1						
248867_at	AT5G46830	g1						
248868_at	AT5G46780	g1						
248873_at	AT5G46450	g1						
248969_at	AT5G45310	g1						
248994_at	AT5G45250	g1						
249006_at	AT5G44660	g1						
249008_at	AT5G44680	g1						
249047_at	AT5G44410	g1						
249052_at	AT5G44420	g1	-3.85					5.87
249072_at	AT5G44060	g1						
249140_at	AT5G43190	g1						
249144_at	AT5G43270	g1						
249190_at	AT5G42750	g1						
249209_at	AT5G42620	g1						
249211_at	AT5G42680	g1						
249279_at	AT5G41920	g1						

249320_at	AT5G40910	g1							
249325_at	AT5G40850	g1			-1.34				
249408_at	AT5G40330	g1							
249410_at	AT5G40380	g1		2.12					
249480_s_at	AT5G38990;	g1							
	AT5G39000								
249482_at	AT5G38980	g1							
249486_at	AT5G39030	g1							
249493_at	AT5G39080	g1							
249515_at	AT5G38530	g1							
249542_at	AT5G38140	g1							
249639_at	AT5G36930	g1							
249728_at	AT5G24390	g1							
249800_at	AT5G23660	g1							
249810_at	AT5G23920	g1							
249869_at	AT5G23050	g1							
249872_at	AT5G23130	g1							
249904_at	AT5G22700	g1							
249923_at	AT5G19120	g1							
249941_at	AT5G22270	g1	-1.46					1.27	
250002_at	AT5G18690	g1							
250063_at	AT5G17880	g1							
250132_at	AT5G16560	g1							
250160_at	AT5G15210	g1							
250167_at	AT5G15310	g1							
250216_at	AT5G14090	g1					1.77		
250255_at	AT5G13730	g1							
250265_at	AT5G12900	g1							
250424_at	AT5G10550	g1							

250487_at	AT5G09690	g1							
250503_at	AT5G09820	g1							
250550_at	AT5G07870	g1		1.61					
250582_at	AT5G07580	g1							
250689_at	AT5G06610	g1							
250704_at	AT5G06265	g1							
250777_at	AT5G05440	g1				1.45			
250828_at	AT5G05250	g1							
250844_at	AT5G04470	g1							
250942_at	AT5G03350	g1			-2.23		-1.62	1.13	
250975_at	AT5G03050	g1			1.45				
250981_at	AT5G03140	g1							
251017_at	AT5G02760	g1		-1.62					
251155_at	AT3G63160	g1			1.17				
251169_at	AT3G63210	g1							
251199_at	AT3G62980	g1							
251218_at	AT3G62410	g1							
251230_at	no_match	g1							
251299_at	AT3G61950	g1							
251342_at	AT3G60690	g1							
251427_at	AT3G60130	g1							
251509_at	AT3G59010	g1		4.79					
251575_at	AT3G58120	g1							
251586_at	AT3G58070	g1							
251661_at	AT3G56950	g1							
251677_at	AT3G56980	g1		1.87	3.70	2.38			
251704_at	AT3G56360	g1							
251705_at	AT3G56400	g1							
251746_at	AT3G56060	g1	-1.54		-1.85				

251919 at	AT3G53800	n1							
251931 at	AT3G53950	01							
252040 at	AT3G52060	 							
252040_at	AT3G52550	g1 							
252117 ot	AT3G52330	g1							
252117_at	AT3G51430	91 a1							
252168_al	AT3G50440	g1		0.04		-			
252170_at	AT3G50480	gi		2.34					
252173_at	A13G50650	g1							
252365_at	AT3G48350	g1		-1.65					
252367_at	AT3G48360	g1							
252427_at	AT3G47640	g1							
252478_at	AT3G46540	g1	-1.13						
252485_at	AT3G46530	g1							
252534_at	AT3G46130	g1							
252549_at	AT3G45860	g1							
252615_at	AT3G45230	g1			-1.42			-1.42	
252618_at	AT3G45140	g1							
252692_at	AT3G43960	g1	-1.13	-1.15					
252698_at	AT3G43670	g1							
252701_at	AT3G43700	g1							
252712_at	AT3G43800	g1							
252736_at	AT3G43210	g1							
252958_at	AT4G38620	g1		1.20					
252965_at	AT4G38860	g1							
252970_at	AT4G38850	g1							
252972_at	AT4G38840	g1							
252992_at	AT4G38520	g1							
253022_at	AT4G38060	g1							
253043_at	AT4G37540	g1							

253050_at	AT4G37450	g1		-2.00	-1.76					
253061_at	AT4G37610	g1						1.29		
253220_s_at	AT4G34930;	g1								
	AT4G34920									
253228_at	AT4G34630	g1								
253255_at	AT4G34760	g1		-1.77	-1.47					
253268_s_at	AT4G34131;	g1			2.81	1.33	1.31			
	AT4G34135									
253326_at	AT4G33440	g1			1.74					
253411_at	AT4G32980	g1								
253553_at	AT4G31050	g1							1.18	
253580_at	AT4G30400	g1								
253597_at	AT4G30690	g1								
253636_at	AT4G30500	g1								
253697_at	AT4G29700	g1			-1.30				-1.33	
253708_at	AT4G29210	g1								
253709_at	AT4G29220	g1								
253729_at	AT4G29360	g1			-1.27					
253779_at	AT4G28490	g1								
254024_at	AT4G25780	g1								
254032_at	AT4G25940	g1								
254133_at	AT4G24810	g1								
254163_s_at	AT4G24340;	g1	-1.27							
	AT4G24350									
254251_at	AT4G23300	g1								
254424_at	AT4G21510	g1								
254512_at	AT4G20230	g1	-1.34							
254532_at	AT4G19660	g1								
254533_at	AT4G19670	g1								

254547_at	AT4G19860	g1								
254697_at	AT4G17970	g1		2.16						
254794_at	AT4G12970	g1							1.18	
254803_at	AT4G13100	g1					-1.34			
254851_at	AT4G12010	g1								
254870_at	no_match	g1								
254878_at	AT4G11660	g1								
254931_at	AT4G11460	g1	-1.74			1.95		2.83	3.14	
254982_at	AT4G10470	g1								
255064_at	AT4G08950	g1							-1.45	
255294_at	AT4G04750	g1								
255306_at	AT4G04740	g1								
255437_at	AT4G03060	g1								
255438_at	AT4G03070	g1								
255484_at	AT4G02540	g1								
255579_at	AT4G01460	g1							1.56	
255740_at	AT1G25390	g1								
255786_at	AT1G19670	g1			-1.90					
255794_at	AT2G33480	g1								1.62
255857_at	AT1G67080	g1								
255908_s_at	AT1G18010;	g1								
	AT1G18000									
255926_at	AT1G22190	g1								
255943_at	AT1G22370	g1								
255962_at	AT1G22330	g1			-2.79	-1.32				
255969_at	AT1G22330	g1								
256222_at	AT1G56210	g1								
256237_at	AT3G12610	g1								
256281_at	AT3G12560	g1								

256332_at	AT1G76890	g1		-1.44					
256383_at	AT1G66820	g1							1.24
256400_at	AT3G06140	g1							
256427_at	AT3G11090	g1							
256446_at	AT3G11110	g1							
256489_at	AT1G31550	g1							
256518_at	AT1G66080	g1							
256598_at	AT3G30180	g1							
256599_at	AT3G14760	g1				1.44			
256617_at	AT3G22240	g1		-1.85	-1.70				
256622_at	AT3G28920	g1							
256664_at	AT3G12040	g1							
256698_at	AT3G20680	g1							
256721_at	AT2G34150	g1							
256746_at	AT3G29320	g1						1.17	
256766_at	AT3G22231	g1			-3.44	-1.96			
256796_at	AT3G22210	g1							
256828_at	AT3G22970;	g1							
	AT3G22968								
256869_at	AT3G26420;	g1							
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256914_at	A13G23880	g1					1.39		
256948_at	AT3G18930	g1							
256980_at	AT3G26932	g1							
257051_at	AT3G15270	g1							
257175_s_at	AT3G23480;	g1							
	AT3G23470								
257203_at	AT3G23730	g1		-1.43					
257272_at	AT3G28130	g1							

257474_at	AT1G80850	g1							
257502_at	AT1G78110	g1							
257504_at	AT1G52250	g1							
257507_at	AT1G29600	g1							
257615_at	AT3G26510	g1							
257625_at	AT3G26230	g1							
257634_s_at	AT3G26170;	g1							
	AT3G26180								
257709_at	AT3G27325	g1							
257748_at	AT3G18710	g1							
257750_at	AT3G18800	g1							
257763_s_at	AT3G23120;	g1							
	AT3G23110								
257815_at	AT3G25130	g1							
257832_at	AT3G26740	g1			1.32		1.72	1.44	
257900_at	AT3G28420	g1							
257923_at	AT3G23160	g1							
257964_at	AT3G19850	g1							
258063_at	AT3G14620	g1		1.33					
258133_at	AT3G24500	g1		2.82					
258156_at	AT3G18050	g1							
258225_at	AT3G15630	g1				1.56			
258334_at	AT3G16010	g1							
258432_at	AT3G16570	g1							
258528_at	AT3G06770	g1							
258530_at	AT3G06840	g1							
258537_at	AT3G04210	g1		-1.76					
258609_at	AT3G02910	g1							
258787_at	AT3G11840	g1							

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258919_at	AT3G10525	g1							
258932_at	AT3G10150	g1				-1.14			
258956_at	AT3G01440	g1							
259073_at	AT3G02290	g1							
259104_at	AT3G02170	g1							
259115_at	AT3G01360	g1							
259398_at	AT1G17700	g1							
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259507_at	AT1G43910	g1							
259528_at	AT1G12330	g1							
259529_at	AT1G12400	g1							
259531_at	AT1G12460	g1							
259535_at	AT1G12280	g1							
259545_at	AT1G20560	g1							
259560_at	AT1G21270	g1	-1.15						
259561_at	AT1G21250	g1	-2.36				-1.43	1.70	
259602_at	AT1G56520	g1							
259637_at	AT1G52260	g1							
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259664_at	AT1G55330	g1							
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	AT5G27780								
259830_at	AT1G80630	g1							
259839_at	AT1G52190	g1			-1.52				
259909_at	AT1G60870	g1							
259954_at	AT1G75130	g1							
259994_at	AT1G68130	g1							

260141_at	AT1G66350	g1								
260146_at	AT1G52770	g1								
260169_at	AT1G71990	g1								
260327_at	AT1G63840	g1								
260414_at	AT1G69850	g1		-1.38						
260427_at	AT1G72430	g1	-1.34		-1.37					
260453_s_at	AT1G72510; AT2G09970	g1								
260541_at	AT2G43530	g1			1.65	1.37				
260592_at	AT1G55850	g1			1.35					
260602_at	AT1G55920	g1								
260635_at	AT1G62422	g1								
260640_at	AT1G53350	g1								
260734_at	AT1G17600	g1								
260735_at	AT1G17610	g1								
260759_at	AT1G49180	g1								
260769_at	AT1G49010	g1								
260770_at	AT1G49200	g1		1.44					1.73	
260884_at	AT1G29240	g1								
260887_at	AT1G29160	g1								
260976_at	AT1G53650	g1								
260999_at	AT1G26580	g1								
261013_at	AT1G26440	g1								
261023_at	AT1G12200	g1			1.58					
261032_at	AT1G17430	g1								
261081_at	AT1G07350	g1								
261084_at	AT1G07440	g1					-1.49			
261144_s_at	AT1G19660; AT1G75380	g1								
261177_at	AT1G04770	g1								
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261221_at	AT1G19960	g1				-1.50	-2.79	-1.30		
261266_at	AT1G26770	g1								
261280_at	AT1G05860	g1								
261294_at	AT1G48430	g1								
261339_at	AT1G35710	g1						-2.25		
261402_at	AT1G79670	g1								
261407_at	AT1G18810	g1								
261409_at	AT1G07640	g1								
261426_at	AT1G18680	g1								
261460_at	AT1G07880	g1			-1.49					
261485_at	AT1G14360	g1								
261487_at	AT1G14340	g1								
261500_at	AT1G28400	g1								
261558_at	AT1G01770	g1								
261570_at	AT1G01120	g1	-1.23	-1.42	-1.15	-1.55				
261684_at	AT1G47400	g1								
261711_at	AT1G32700	g1								
261715_at	AT1G18485	g1								
261758_at	AT1G08250	g1								
261893_at	AT1G80690	g1								
261905_at	AT1G65070	g1								
261949_at	AT1G64670	g1			-1.70					
261982_at	AT1G33780	g1			1.20					
262118_at	AT1G02850	g1		1.77	2.28					
262126_at	AT1G59620	g1								
262133_at	AT1G78000	g1								
262137_at	AT1G77920	g1								
262162_at	AT1G78020	g1								

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262196_at	AT1G77870	g1						
262232_at	AT1G68600	g1			-1.38			
262236_at	AT1G48330	g1				1.29		
262286_at	AT1G68585	g1	-2.34	1.76	-2.75			
262411_at	AT1G34640	g1					1.57	
262533_at	AT1G17090	g1						
262536_at	AT1G17100	g1	-4.99					
262543_at	AT1G34245	g1					1.18	
262552_at	AT1G31350	g1						
262598_at	AT1G15260	g1						
262656_at	AT1G14200	g1						
262661_s_at	AT1G14230;	g1						
	AT1G14250							
262698_at	AT1G75960	g1				2.36		
262705_at	AT1G16260	g1						
262736_at	AT1G28570	g1	1.22		-1.80			
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262780_at	AT1G13090	g1						
262811_at	AT1G11700	g1						
262850_at	AT1G14920	g1		-1.48				
262854_at	AT1G20870	g1						
262891_at	AT1G79460	g1						
262935_at	AT1G79410	g1						
262986_at	AT1G23390	g1						
263014_at	AT1G23400	g1						
263106_at	AT2G05160	g1						
263111_s_at	AT1G65190;	g1						
	AT1G65250							
263150_at	AT1G54050	g1						

263184_at	AT1G05560	g1							
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263369_at	AT2G20480	g1							
263597_at	AT2G01870	g1							
263647_at	AT2G04690	g1							
263737_at	AT1G60010	g1							
263776_s_at	AT2G46430;	g1				-1.19		1.69	
	AT2G46440								
263947_at	AT2G35820	g1			1.35	-1.42			
263953_at	AT2G36050	g1							
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264037_at	AT2G03750	g1		-1.60					
264091_at	AT1G79110	g1				1.27			
264229_at	AT1G67480	g1							
264240_at	AT1G54820	g1		-2.24					
264244_at	AT1G60440	g1							
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264310_at	AT1G62030	g1							
264340_at	AT1G70280	g1				-1.45			
264379_at	AT2G25200	g1							
264408_at	AT1G10240	g1							
264435_at	AT1G10360	g1							
264463_at	AT1G10150	g1							
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264517_at	AT1G10120	g1							
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264769_at	AT1G61350	g1							

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264826_at	AT1G03410	g1								
264874_at	AT1G24240	g1				1.54				
264898_at	AT1G23205	g1								
264931_at	AT1G60590	g1								
264947_at	AT1G77020	g1								
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265451_at	AT2G46490	g1								
265452_at	AT2G46510	g1								
265547_at	AT2G28305	g1								
265699_at	AT2G03550	g1								
265724_at	AT2G32100	g1				-1.18				
265767_at	AT2G48110	g1								
265817_at	AT2G18050	g1	2.62							
265837_at	AT2G14560	g1			-3.54					
265869_at	AT2G01760	g1		1.63			-1.43			
265871_at	AT2G01680	g1								
265894_at	AT2G15050	g1								1.14
265902_at	AT2G25590	g1			1.14	1.22				
265929_s_at	AT2G18560;	g1								
	AT2G18570									
265943_at	AT2G19570	g1								ļ
265985_at	AT2G24220	g1								
265993_at	AT2G24160	g1						-1.91		

266007_at	AT2G37380	g1						
266187_at	AT2G38970	g1				-1.34		
266204_at	AT2G02410	g1						
266257_at	AT2G27820	g1						
266300_at	AT2G01420	g1						
266372_at	AT2G41310	g1						
266426_x_at	AT2G07140; AT3G44120	g1						
266472_at	no_match	g1						
266483_at	AT2G47910	g1						
266552_at	AT2G46330	g1						
266635_at	AT2G35470	g1						
266663_at	AT2G25790	g1		-1.85				
266707_at	AT2G03310	g1						
266869_at	AT2G44660	g1						
266873_at	AT2G44740	g1						
267034_at	AT2G38310	g1	-1.48					
267076_at	AT2G41090	g1						
267135_at	AT2G23430	g1						
267192_at	AT2G30890	g1						
267219_at	AT2G02590	g1						
267238_at	AT2G44130	g1			2.70			
267265_at	AT2G22980	g1						
267289_at	AT2G23770	g1						
267336_at	AT2G19310	g1						
267425_at	AT2G34810	g1						
267462_at	AT2G33735	g1						
267495_at	no_match	g1						

267500_s_at	AT2G45510;	g1				1.74				
007505	AT2G44890									
267535_at	AT2G41940	g1								
267569_at	AT2G30790	g1						-1.80		
245130_at	AT2G45340	g2								
245141_at	AT2G45400	g2								
245196_at	AT1G67750	g2								
245277_at	AT4G15550	g2		-1.76						
245304_at	AT4G15630	g2								
245318_at	AT4G16980	g2								
245321_at	AT4G15545	g2								
245479_at	AT4G16140	g2		-1.47		-1.12	-2.38			
245524_at	AT4G15920	g2								
245574_at	AT4G14750	g2								
245583_at	AT4G14920	g2								
245626_at	AT1G56700	g2								
245657_at	AT1G56720	g2					-1.66			
245690_at	AT5G04230	g2	-1.34		-1.22					
245696_at	AT5G04190	g2								
245759_at	AT1G66900	g2								
245783_s_at	AT1G35180; AT1G35170	g2								
245845_at	AT1G26150	g2								
245877_at	AT1G26220	g2								
245906_at	AT5G11070	g2								
245984_at	AT5G13090	g2								
246021_at	AT5G21100	g2		8.78	-2.19					
246028_at	AT5G21170	g2								
246034_at	AT5G08350	g2					1.69			

246212 of	AT1021020	~)							
240313_al	ATTG31920	gz							
246427_at	AT5G17400	g2							
246462_at	AT5G16940	g2							
246487_at	AT5G16030	g2							
246520_at	AT5G15790	g2							
246522_at	AT5G15830	g2							
246540_at	AT5G15600	g2		-1.39	-1.96				
246576_at	AT1G31650	g2							
246591_at	AT5G14880	g2							
246633_at	AT1G29720	g2				-1.12			
246681_at	AT5G33280	g2							
246701_at	AT5G28020	g2							
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246781_at	AT5G27350	g2		1.63					
246783_at	AT5G27360	g2							
246909_at	AT5G25770	g2							
246932_at	AT5G25190	g2							-1.17
246968_at	AT5G24870	g2							
246997_at	AT5G67390	g2							
247040_at	AT5G67150	g2							
247162_at	AT5G65730	g2		-1.37	-1.52	-2.75		-1.35	
247193_at	AT5G65380	g2							
247266_at	AT5G64570	g2	-1.64				1.43		
247278_at	AT5G64380	g2							
247284_at	AT5G64410	g2				-1.39			
247304_at	AT5G63850	g2		-1.55	-1.50		-1.18	-1.52	
247413_at	no_match	g2							

247522 at	AT5G61340	q2							
	AT5G59780	g2							
247780_at	AT5G58770	g2							
247819_at	AT5G58350	g2							
247826_at	AT5G58480	g2							
247848_at	AT5G58120	g2							
247884_at	AT5G57800	g2	-1.50						
247946_at	AT5G57180	g2							
247954_at	AT5G56870	g2							
247977_at	AT5G56850	g2							
248028_at	AT5G55620	g2							
248064_at	no_match	g2							
248091_at	AT5G55120	g2							
248153_at	AT5G54250	g2							
248179_at	AT5G54380	g2							
248246_at	AT5G53200	g2							
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248395_at	AT5G52120	g2					1.13		
248460_at	AT5G50915	g2							
248566_s_at	AT5G49740;	g2				-1.79			
	AT5G49730								
248600_at	AT5G49390	g2							
248622_at	AT5G49360	g2		1.38					
248683_at	AT5G48490	g2			-1.12			-1.16	
248719_at	AT5G47910	g2							
248759_at	AT5G47610	g2		3.52					
248776_at	AT5G47900	g2							
248890_at	AT5G46270	g2							
248912_at	AT5G45670	g2							

248924_at	AT5G45960	g2							
248961_at	AT5G45650	g2							
249122_at	AT5G43850	g2							
249215_at	AT5G42800	g2							
249355_at	AT5G40500	g2							
249383_at	AT5G39860	g2				2.59			
249546_at	AT5G38150	g2							
249693_at	AT5G35750	g2							
249694_at	AT5G35790	g2							
249727_at	AT5G35490	g2							
249732_at	AT5G24420	g2							
249774_at	AT5G24150	g2			-1.44	-1.22		-1.62	
249775_at	AT5G24160	g2							
249777_at	AT5G24210	g2	-1.49						
249818_at	AT5G23860	g2							
249860_at	AT5G22860	g2							
249862_at	AT5G22920	g2							
250007_at	AT5G18670	g2						-1.33	
250008_at	AT5G18630	g2							
250017_at	AT5G18140	g2							
250079_at	AT5G16650	g2							
250102_at	AT5G16590	g2							
250110_at	AT5G15350	g2			-1.31				
250180_at	AT5G14450	g2					-1.62		
250217_at	AT5G14120	g2		-1.40					
250249_at	AT5G13760	g2							
250261_at	AT5G13400	g2							
250286_at	AT5G13320	g2							
250304_at	AT5G12110	g2		1.52		-2.31			

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250478_at	AT5G10250	g2								
250482_at	AT5G10320	g2								
250485_at	AT5G09990	g2						-1.24		
250549_at	AT5G07860	g2								
250598_at	AT5G07690	g2								
250613_at	AT5G07240	g2					-1.54			
250633_at	AT5G07460	g2								
250642_at	AT5G07180	g2								
250669_at	AT5G06870	g2								
250696_at	AT5G06790	g2					-1.11			
250720_at	AT5G06180	g2								
250742_at	AT5G05800	g2								
250880_at	AT5G04070	g2								
250968_at	AT5G02890	g2							1.17	
251010_at	AT5G02550	g2								
251011_at	AT5G02560	g2							-1.88	
251028_at	AT5G02230	g2								
251068_at	AT5G01920	g2								
251108_at	AT5G01620	g2								
251142_at	AT5G01015	g2	-1.48		-1.61	-1.18	-1.72			
251160_at	AT3G63240	g2								
251195_at	AT3G62930	g2				1.14				
251219_at	AT3G62390	g2								
251324_at	AT3G61430	g2				-1.18				
251360_at	AT3G61210	g2								
251391_at	AT3G60910	g2								
251497_at	AT3G59060	g2						-1.52		
251503_at	AT3G59140	g2								
251519_at	AT3G59400	g2			-1.96	-1.25				

251524_at	AT3G58990	g2							
251601_at	AT3G57800	g2							
251759_at	AT3G55630	g2							
251771_at	AT3G56000	g2							
251773_at	AT3G55960	g2							
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251890_at	AT3G54220	g2							
251968_at	AT3G53100	g2							
251982_at	AT3G53190	g2	-1.86						
252011_at	AT3G52720	g2		-2.50	-1.64		-1.33		
252033_at	AT3G51950	g2							
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252167_at	AT3G50560	g2	-1.44						
252178_at	AT3G50750	g2		-2.35					
252189_at	AT3G50070	g2							
252317_at	AT3G48720	g2	-1.86						
252353_at	AT3G48200	g2							
252381_s_at	AT3G47760;	g2							
	AT3G47750								
252411_at	AT3G47430	g2							
252421_at	AT3G47540	g2							
252433_at	AT3G47560	g2							
252594_at	AT3G45680	g2							
252607_at	AT3G44990	g2							
252629_at	AT3G44970	g2			1.28	1.18	1.74		
252648_at	AT3G44630	g2							
252652_at	AT3G44720	g2							
252716_at	AT3G43920	g2							

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252870_at	AT4G39940	g2			-1.14				
252911_at	AT4G39510	g2							
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252949_at	AT4G38670	g2							
253042_at	AT4G37550	g2							
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	AT4G37520								
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253270_at	AT4G34160	g2							
253278_at	AT4G34220	g2							
253302_at	AT4G33660	g2							
253305_at	AT4G33666	g2	-1.65		-1.84			-1.18	
253332_at	AT4G33420	g2							
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253394_at	AT4G32770	g2				-1.76			
253397_at	AT4G32710	g2							
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253440_at	AT4G32570	g2							
253534_at	AT4G31500	g2	-1.27						
253548_at	AT4G30993	g2							
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253806_at	AT4G28270	g2							
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253815_at	AT4G28250	g2	1.67		-1.13	-2.31			
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253946_at	AT4G26790	g2	-3.13		-1.29		-1.21		
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253966_at	AT4G26520	g2							
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254280_at	AT4G22756	g2				-1.34			
254301_at	AT4G22790	g2							
254305_at	AT4G22200	g2							
254328_at	AT4G22570	g2		-1.12	-1.67	-1.83			
254332_at	AT4G22730	g2							
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254564_at	AT4G19170	g2							
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254649_at	AT4G18570	g2							
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254705_at	AT4G17870	g2							
254746_at	AT4G12980	g2			-1.98				
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255008_at	AT4G10060	g2	-1.25						
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	AT4G04540							
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255511_at	AT4G02075	g2						
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255583_at	AT4G01510	g2						
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255617_at	AT4G01330	g2						
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255626_at	AT4G00780	g2						
255698_at	AT4G00150	g2						
255753_at	AT1G18570	g2						
255773_at	AT1G18590	g2		-1.62	-1.45			
255774_at	AT1G18620	g2						
255779_at	AT1G18650	g2	1.15					
255793_at	AT2G33250	g2						
255802_s_at	AT4G10150;	g2						
	AT4G10160							
255817_at	AT2G33330	g2						
255822_at	AT2G40610	g2						
255881_at	AT1G67070	g2						
255895_at	AT1G18020;	g2						
050000	AT1G17990	-						
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256015 of	AT1C10150	a)						
250015_at	ATTG19150	g∠						

256096_at	AT1G13650	g2		-1.21		-2.96			
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256262_at	AT3G12150	g2							
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256299_at	AT1G69530	g2	-2.52				1.70		
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256603_at	AT3G28270	g2			3.56				
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256671_at	AT3G52290	g2							
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256894_at	AT3G21870	g2					-1.64		
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257793_at	AT3G26960	g2								
257801_at	AT3G18750	g2						-1.11		
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258676_at	AT3G08600	g2			-1.16				
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258962_at	AT3G10570	g2							
258983_at	AT3G08860	g2							
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259042_at	AT3G03450	g2					-1.40		
259072_at	AT3G11700	g2							
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	AT1G77580	g2							
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260309_at	AT1G70580	g2							
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260693_at	AT1G32450	g2							
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261016_at	AT1G26560	g2				-1.96			
261031_at	AT1G17360	g2							
261053_at	AT1G01320	g2							
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261346_at	AT1G79720	g2	-2.57						
261375_at	AT1G53160	g2							
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261576_at	AT1G01070	g2				1.37			
261594_at	AT1G33240	g2							
261609_at	AT1G49740	g2							
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261751_at	AT1G76080	g2							

261765_at	AT1G15570	g2							
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261913_at	AT1G65860	g2			-1.73				
261914_at	AT1G65870	g2							
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262039_at	AT1G80050	g2						1.25	
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262290_at	AT1G70985	g2				-1.88			
262354_at	AT1G64200	g2							
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	AT1G72930								-
262376_at	AT1G72970	g2		-1.74					
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262577_at	AT1G15290	g2	-1.12						
262582_at	AT1G15410	g2							
262587_at	AT1G15490	g2							
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262733_s_at	AT1G28660;	g2							
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262830_at	AT1G14700	g2			-1.34				
262847_at	AT1G14840	g2							
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263591_at	AT2G01910	g2							
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263777_at	AT2G46450	g2		-1.22		-1.37			
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263946_at	AT2G36000	g2	-3.39						

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264692_at	AT1G70000	g2							
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265175_at	AT1G23480	g2						
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	AT2G36790							
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265387_at	AT2G20670	g2				1.28		
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265444_s_at	AT2G37180; AT2G37170	g2						
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265680_at	AT2G32150	g2						
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265990_at	AT2G24280	g2						
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266104_at	AT2G45150	g2						
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266682_at	AT2G19780	g2						
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267063_at	AT2G41120	g2						
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	AT2G32530							
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267260_at	AT2G23130	g2						
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	AT3G11470							
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267516_at	AT2G30520	g2		-2.84	-1.49		-1.55	
267517_at	AT2G30520	g2			-1.31			
267520_at	AT2G30460	g2						
267544_at	AT2G32720	g2						
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245119_at	AT2G41640	g3						
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245329_at	AT4G14365	g3						
245346_at	AT4G17090	g3		-1.67				
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245504_at	AT4G15660	g3						
245528_at	AT4G15530	g3		1.22				
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245635_at	AT1G25250	g3						
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245731_at	AT1G73500	g3						
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246125_at	AT5G19875	g3		2.51						
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246293_at	AT3G56710	g3					-1.76			
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247177_at	AT5G65300	g3								
247208_at	AT5G64870	g3								

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247455_at	AT5G62470	g3								
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247706_at	AT5G59480	g3								
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250335_at	AT5G11650	g3					
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252374_at	AT3G48100	g3					1.15			
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252474_at	AT3G46620	g3								
252679_at	AT3G44260	g3								
253066_at	AT4G37770	g3								
253104_at	AT4G36010	g3	3.30	-5.97						
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253284_at	AT4G34150	g3								

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253535_at	AT4G31550	g3							
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253628_at	AT4G30280	g3							
253643_at	AT4G29780	g3							
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253830_at	AT4G27652	g3			1.25		1.16		
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254066_at	AT4G25480	g3							
254074_at	AT4G25490	g3							
254075_at	AT4G25470	g3	8.25						
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254293_at	AT4G23060	g3							
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256129_at	AT1G18210	g3							

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	AT1G66500							
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256548_at	AT3G14770	g3			-1.72			
256627_at	AT3G19970	g3						
256633_at	AT3G28340	g3	-1.59					
256715_at	AT2G34090	g3						
256755_at	AT3G25600	g3				1.29		
256763_at	AT3G16860	g3						
256799_at	AT3G18560	g3						
256891_at	AT3G19030	g3						
256999_at	AT3G14200	g3						
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257057_at	AT3G15310	g3		-2.34				
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257375_at	AT2G38640	g3						
257654_at	AT3G13310	g3						
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258167_at	AT3G21560	g3						
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258436_at	AT3G16720	g3						

258497_at	AT3G02380	g3				-2.47	-1.83			
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258792_at	AT3G04640	g3								
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259129_at	AT3G02150	g3								
259244_at	AT3G07650	g3							1.42	1.30
259293_at	AT3G11580	g3				1.68				
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259364_at	AT1G13260	g3								
259428_at	AT1G01560	g3								
259445_at	AT1G02400	g3			1.46					
259466_at	AT1G19050	g3					1.47			
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259734_at	AT1G77500	g3								
259792_at	AT1G29690	g3								
259834_at	AT1G69570	g3								
259879_at	AT1G76650	g3								
259925_at	AT1G75040	g3				1.22				
260203_at	AT1G52890	g3								
260205_at	AT1G70700	g3	-1.34							
260227_at	AT1G74450	g3								
260399_at	AT1G72520	g3		-2.92						
260429_at	AT1G72450	g3						-1.54		
260656_at	AT1G19380	g3		-3.49	1.67		1.55	1.86		
260744_at	AT1G15010	g3								
260753_at	AT1G49230	g3								

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261033_at	AT1G17380	g3							
261037_at	AT1G17420	g3							
261150_at	AT1G19640	g3		-1.58	-1.77				
261263_at	AT1G26790	g3							
261405_at	AT1G18740	g3							
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261456 at	AT1G21050	α3							
261470 at	AT1G28370	ge 							
261526 at	AT1G14370	ge 				1.45			
261564 at	AT1G01720	go 							
261648 at	AT1G27730	g2				9.68	1.23		
	AT1G76130	g3							
261892_at	AT1G80840	g3							
261958_at	AT1G64500	g3							
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262028_at	AT1G35560	g3							
262212_at	AT1G74890	g3						1.31	
262383_at	AT1G72940	g3							
262384_at	AT1G72950	g3							
262526_at	AT1G17050	g3							
262801_at	AT1G21010	g3			1.33				
262803_at	AT1G21000	g3							
262883_at	AT1G64780	g3		-1.84	-1.57	-1.35			
262940_at	AT1G79520	g3		1.13					
263122_at	AT1G78510	g3							
263253_at	AT2G31370	g3							

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263379_at	AT2G40140	g3		1.15	1.18	5.72			
263584_at	AT2G17040	g3							
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263735_s_at	AT1G60040;	g3							
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263783_at	AT2G46400	g3							
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263845_at	AT2G37040	g3							
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264217_at	AT1G60190	g3							
264232_at	AT1G67470	g3							
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264512_at	AT1G09575	g3							
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264636_at	AT1G65490	g3	2.16						
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264758_at	AT1G61340	g3		1.65					
264852_at	AT2G17480	g3							
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265184_at	AT1G23710	g3							
265327_at	AT2G18210	g3							
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265999_at	AT2G24100	g3								
266071_at	AT2G18680;	g3								
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266545_at	AT2G35290	g3								
266908_at	AT2G34650	g3								
267028_at	AT2G38470	g3								
267069_at	AT2G41010	g3								
267083_at	AT2G41100	g3	-3.65				1.38			
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267337_at	AT2G39980	g3								
267515_at	AT2G45680	g3								
267595_at	AT2G32990	g3								
245152_at	AT2G47490	g4								
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245434_at	AT4G17140	g4								
245563_at	AT4G14580	g4								
245619_at	AT4G13990	g4								
245982_at	AT5G13170	g4			6.23					
246071_at	AT5G20150	g4								
246272_at	AT4G37150	g4				1.17				
246310_at	AT3G51895	g4			-1.81		-1.15	-2.86		
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246523_at	AT5G15850	g4								
246911_at	AT5G25810	g4								
247025_at	AT5G67030	g4								
247029_at	AT5G67190	g4								
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247438_at	AT5G62460	g4								
247477_at	AT5G62340	g4								
247519_at	AT5G61430	g4		2.57						
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253125_at	AT4G36040	g4				1.46			
253129_at	AT4G36020	g4							
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	AT4G13800	g4			1.57						
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258189_at	AT3G17860	g4								
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247937_at	AT5G57110	g5								
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247989_at	AT5G56350	g5			-1.69					
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255585_at	AT4G01550	g5						
255625_at	AT4G01120	g5						
255723_at	AT3G29575	g5						
255795_at	AT2G33380	g5				-1.53		
255931_at	AT1G12710	g5			1.13			
256014_at	AT1G19200	g5						
256017_at	AT1G19180	g5						
256069_at	AT1G13740	g5						
256091_at	AT1G20693	g5						
256114_at	AT1G16850	g5	8.45				-1.17	
256116_at	AT1G16858;	g5						
	AT1G16860							
256149_at	AT1G55110	g5						
256235_at	AT3G12490	g5			1.53			
256285_at	AT3G12510	g5						
256288_at	AT3G12270	g5						
256296_at	AT1G69480	g5						
256310_at	AT1G30360	g5	1.79					
256340_at	AT1G72070	g5						
256576_at	AT3G28210	g5						

256595_x_at	AT3G28530; AT1G36770	g5					
256797_at	AT3G18600	g5					
256833_at	AT3G22910	g5					
257022_at	AT3G19580	g5	1.31				
257035_at	AT3G19270	g5		5.19			
257154_at	AT3G27210	g5	1.56				
257188_at	AT3G13150	g5					
257226_at	AT3G27880	g5					
257237_at	AT3G14890	g5					
257444_at	AT2G12550	g5					
257484_at	AT1G01650	g5					
257487_at	AT1G71850	g5					
257519_at	AT3G01210	g5					
257593_at	AT3G24840	g5					
257610_at	AT3G13810	g5					
257650_at	AT3G16800	g5			1.76		
257652_at	AT3G16810	g5					
257653_at	AT3G13225	g5					
257708_at	AT3G13330	g5					
257719_at	AT3G18440	g5					
257876_at	AT3G17130	g5	5.45				
258078_at	AT3G25870	g5	3.59				
258092_at	AT3G14595	g5					
258104_at	AT3G23620	g5					
258137_at	AT3G24515	g5					
258166_at	AT3G21540	g5					
258180_at	no_match	g5					
258202_at	AT3G13940	g5					

258201 at	AT3G13060	a5						
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200209_al	AT3014000	yo «F		-1.56		4 4 4	-1.00	
258252_at	AT3G15720	g5				1.44		
258316_at	A13G22660	g5						
258395_at	AT3G15500	g5						
258505_at	AT3G06530	g5						
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258562_at	AT3G05980	g5						
258603_at	AT3G02990	g5						
258664_at	AT3G08700	g5						
258665_at	AT3G08710	g5						
258683_at	AT3G08760	g5						
258719_at	AT3G09540	g5	2.68					
258777_at	AT3G11850	g5						
258805_at	AT3G04010	g5						
258809_at	AT3G04070	g5						
258871_at	AT3G03060	g5						
258878_at	AT3G03170	g5						
258893_at	AT3G05660	g5	5.66					
258965_at	AT3G10530	g5						
258979_at	AT3G09440	g5						
258981_at	AT3G08880	g5						
259037_at	AT3G09350	g5						
259077_s_at	AT5G15650;	g5						
	AT3G02230							
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259109_at	AT3G05580	g5						

259132 at	AT3G02250	d2								
259137 at	AT3G10300	90 5								
259173 at	AT3G03640	90 05				1 1 1		1 92		
259175_at	AT3G01560	90 05						1.52		
250231 at	AT3G11/10	90 05								
259231_at	AT3G11410	95 05								
259/17 at	AT1G02340	95 05								
259/26 at	AT1G02340	95 05	1 57	1 10						
259420_at	AT1G01470	95 95	4.57	1.13						
259430_at	AT1G01500	95 95								
259442_at	AT1G02310	yo aF								
259444_al	ATIG02370	yo aF	2.66	2.02						
259516_at	AT1G20450	go ar E	3.00	-2.92						
259568_at	AT1G20490	g5								-
259570_at	AT1G20440	g5	3.30							
259582_at	AT1G28060	g5								
259588_at	AT1G27930	g5								
259595_at	AT1G28050	g5								
259605_at	AT1G27910	g5	2.34							
259606_at	AT1G27920	g5								
259611_at	AT1G52280	g5								
259626_at	AT1G42990	g5								
259704_at	AT1G77680	g5								
259711_at	AT1G77570	g5			1.66		1.24			
259789_at	AT1G29395	g5	5.29		-2.56	-2.28	-1.18		-1.55	
259841_at	AT1G52200	g5			1.74	-1.26				
259876_at	AT1G76700	g5	1.15	-1.76						
259878_at	AT1G76790	g5								
259971_at	AT1G76580	g5	2.42							
259977_at	AT1G76590	g5	2.18		1.19					

259992_at	AT1G67970	g5				1.41	1.2	2		
260075_at	AT1G73700	g5								
260176_at	AT1G71950	g5	1.35							
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260262_at	AT1G68470	g5			-1.31					
260264_at	AT1G68500	g5	5.36				1.2	8		
260276_at	AT1G80450	g5								
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260338_at	AT1G69250	g5								
260345_at	AT1G69270	g5								
260352_at	AT1G69295	g5								
260410_at	AT1G69870	g5	2.22							
260489_at	AT1G51610	g5								
260556_at	AT2G43620	g5	7.38	1.39			1.8	2		
260628_at	AT1G62320	g5								
260674_at	AT1G19370	g5								
260727_at	AT1G48100	g5	5.26			-1.66			-1.17	
260776_at	AT1G14580	g5	2.22							
260804_at	AT1G78410	g5								
260832_at	AT1G06780	g5	1.15							
260870_at	AT1G43890	g5								
260876_at	AT1G21460	g5	2.46				1.4	6		
260921_at	AT1G21540	g5					1.4	3		
261004_at	AT1G26450	g5								
261048_at	AT1G01420	g5	3.49							
261076_at	AT1G07420	g5								
261077_at	AT1G07430	g5								
261109_at	AT1G75450	g5								

261168 at	AT1G04945	d5						
261187 at	AT1G32860	ge 05	3 72					
261192 at	AT1G32870	ge a5	0.1.2					
261225 at	AT1G20100	gs q5						
261248 at	AT1G20030	g= q5						
261254 at	AT1G05805	a5						
	AT1G53035	q5			1.35	1.12		
	AT1G79660	g5	1.97					
261366 at	AT1G53100	g5						
261377_at	AT1G18850	g5						
261436_at	AT1G07870	g5						
261453_at	AT1G21130	g5						
261482_at	AT1G14530	g5						
261506_at	AT1G71697	g5						
261522_at	AT1G71710	g5	1.19					
261566_at	AT1G33230	g5	2.12					
261610_at	AT1G49560	g5						
261613_at	AT1G49720	g5						
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261655_at	AT1G01940	g5		1.35				
261664_s_at	AT1G18320;	g5						
	AT3G10110							
261718_at	AT1G18390	g5						
261726_at	AT1G76270	g5						
261728_at	AT1G76160	g5						
261745_at	AT1G08500	g5						
261749_at	AT1G76180	g5						
261818_at	AT1G11390	g5						
261899_at	AT1G80820	g5						

262050_at	AT1G80130	g5	3.63	1.23				
262061_at	AT1G80110	g5						
262099_s_at	AT4G37390;	g5						
	AT1G59500							
262119_s_at	AT1G02930;	g5						
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262164_at	AT1G78070	g5	2.95					
262166_at	AT1G74840	g5						
262219_at	AT1G74750	g5						
262307_at	AT1G71000	g5		2.42				
262313_at	AT1G70900	g5						
262440_at	AT1G47710	g5	2.19		1.11			
262452_at	AT1G11210	g5	2.64					
262478_at	AT1G11170	g5						
262496_at	AT1G21790	g5	3.60					
262503_at	AT1G21670	g5						
262584_at	AT1G15440	g5						
262644_at	AT1G62710	g5						
262706_at	AT1G16280	g5						
262760_at	AT1G10770	g5	2.17					
262843_at	AT1G14687	g5						
262881_at	AT1G64890	g5	2.16					
262953_at	AT1G75670	g5						
263004_at	AT1G54510	g5						
263082_at	AT2G27200	g5				1.17		
263221_at	AT1G30620	g5						
263222_at	AT1G30640	g5						
263249_at	AT2G31360	g5						

263259 at	AT1G10560	q5						
263330_at	AT2G15320	g5	1.12			-1.19		1
263352_at	AT2G22080	g5	2.22					
263495_at	AT2G42530	g5	7.29					
263497_at	AT2G42540	g5	9.79					
263517_at	AT2G21620	g5						
263574_at	AT2G16990	g5	5.45					
263789_at	AT2G24560	g5	6.85					
263797_at	AT2G24570	g5						
263823_s_at	AT2G40340; AT2G40350	g5						
263963_at	AT2G36080	g5						
263981_at	AT2G42870	g5						
264000_at	AT2G22500	g5						
264019_at	AT2G21130	g5			1.94			
264024_at	AT2G21180	g5						
264042_at	AT2G03760	g5						
264118_at	AT1G79150	g5						
264123_at	AT1G02270	g5						
264131_at	AT1G79150	g5						
264190_at	AT1G54830	g5						
264211_at	AT1G22770	g5		1.46	2.11	1.85		
264246_at	AT1G60140	g5						
264261_at	AT1G09240	g5						
264289_at	AT1G61890	g5						
264398_at	AT1G61730	g5						
264452_at	AT1G10270	g5						
264458_at	AT1G10410	g5	2.34	1.69				
264511_at	AT1G09350	g5	9.15					

			1					
264515_at	AT1G10090	g5						
264516_at	AT1G10090	g5	2.34					
264529_at	AT1G30820	g5						
264560_at	AT1G55820	g5						
264580_at	AT1G05340	g5			1.74			
264624_at	AT1G08930	g5						
264652_at	AT1G08920	g5		1.63				
264654_s_at	AT1G08890; AT1G08900	g5	1.72					
264767_at	AT1G61380	g5						
264787_at	AT2G17840	g5	2.40					
264818_at	AT1G03530	g5						
264841_at	AT1G03740	g5						
264893_at	AT1G23140	g5						
264907_at	AT2G17280	g5						
264948_at	AT1G77050	g5						
264953_at	AT1G77120	g5						
264968_at	AT1G67360	g5		2.22				
264989_at	AT1G27200	g5	2.87					
264999_at	AT1G67310	g5	1.31					
265025_at	AT1G24575	g5			1.37			
265061_at	AT1G61640	g5						
265093_at	AT1G03905	g5						
265119_at	AT1G62570	g5	4.29				-1.36	
265147_at	AT1G51380	g5						
265154_at	AT1G30960	g5						
265197_at	AT2G36750	g5						
265214_at	AT1G05000	g5				1.17		

265244_at	AT2G43018;	g5						
	AT2G43020							
265276_at	AT2G28400	g5						
265283_at	AT2G20370	g5						
265290_at	AT2G22590	g5			1.75			
265333_at	AT2G18350	g5				1.57		
265354_at	AT2G16700	g5	1.66					
265480_at	AT2G15970	g5	2.41					
265634_at	AT2G25530	g5						
265662_at	AT2G24500	g5						
265728_at	AT2G31990	g5						
265886_at	AT2G25620	g5						
265913_at	AT2G25625	g5						
265931_at	AT2G18520	g5						
265935_at	AT2G19580	g5						
265941_s_at	AT2G19490;	g5						
	AT3G32920							
266049_at	AT2G40780	g5						
266100_at	AT2G37980	g5						
266119_at	AT2G02100	g5						
266141_at	AT2G02120	g5		1.33	3.49	2.49		
266184_s_at	AT3G54700;	g5						
	AT2G38940							
266225_at	AT2G28900	g5	3.45					
266229_at	AT2G28840	g5		-2.64				
266259_at	AT2G27830	g5	2.45					
266327_at	AT2G46680	g5						
266358_at	AT2G32280	g5						
266510_at	AT2G47990	g5						

266514_at	AT2G47890	g5	2.20						
266532_at	AT2G16890	g5							
266555_at	AT2G46270	g5							
266695_at	AT2G19810	g5							
266702_at	AT2G19860	g5							
266799_at	AT2G22860	g5	1.21						
266831_at	AT2G22830	g5					1.52		
266861_at	AT2G26830	g5							
266934_at	AT2G18900	g5							
266946_at	AT2G18890	g5							
267019_at	AT2G39130	g5							
267036_at	AT2G38465	g5			2.21				
267081_at	AT2G41210	g5							
267163_at	AT2G37520	g5							
267201_at	AT2G31010	g5							
267261_at	AT2G23120	g5	3.68						
267266_at	AT2G23150	g5			1.40	1.34			
267280_at	AT2G19450	g5							
267315_at	no_match	g5							
267335_s_at	AT2G19440;	g5							
	AT1G64760								
267361_at	AT2G39920	g5			2.34	1.73			
267364_at	AT2G40080	g5		1.15	1.26				
267429_at	AT2G34850	g5							
267509_at	AT2G45660	g5					-1.67		
267534_at	AT2G41900	g5			1.88				
267576_at	AT2G30640	g5							
267631_at	AT2G42150	g5							
245188_at	AT1G67660	g6						1.15	

245319_at	AT4G16146	g6					1.66	
245405_at	AT4G17150	g6						
245432_at	AT4G17100	g6						
245433_at	AT4G17140	g6						
245437_at	no_match	g6						
245505_at	AT4G15690	g6						
245600_at	AT4G14230	g6						
245602_at	AT4G14270	g6		1.38	1.85	1.32		
245668_at	AT1G28330	g6			1.94	2.72		
245694_at	AT5G04170	g6						
246288_at	AT1G31850	g6						
246550_at	AT5G14920	g6			1.12	1.86		
246791_at	AT5G27280	g6						
246829_at	AT5G26570	g6			1.54			
246881_at	AT5G26040	g6						
247013_at	AT5G67480	g6			1.52	1.38		
247302_at	AT5G63880	g6						
247348_at	AT5G63810	g6				1.16		
247488_at	AT5G61820	g6		2.56	1.24			
247541_at	AT5G61660	g6						
247668_at	AT5G60100	g6			2.84			
247723_at	AT5G59220	g6		1.53				
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248138_at	AT5G54960	g6						
248410_at	AT5G51570	g6						
248587_at	AT5G49550	g6						
248698_at	AT5G48380	g6						
248793_at	AT5G47240	g6						

249015_at	AT5G44730	g6								
249128_at	AT5G43440	g6								
249220_at	AT5G42420	g6								
249346_at	AT5G40780	g6								
249456_at	AT5G39410	g6								
249619_at	AT5G37500	g6	3.98	2.59						
249719_at	AT5G35735	g6		1.15						1.76
249850_at	AT5G23240	g6		1.28	1.96	1.94			2.25	1.67
249917_at	AT5G22460	g6		5.38				1.46		
249989_at	AT5G18525	g6								
249990_at	AT5G18540	g6								
250028_at	AT5G18130	g6								
250096_at	AT5G17190	g6								
250127_at	AT5G16380	g6								
250252_at	AT5G13750	g6								
250290_at	AT5G13310	g6								
250412_at	AT5G11150	g6								
250648_at	AT5G06760	g6								
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250826_at	AT5G05220	g6								
250858_at	AT5G04760	g6								
250935_at	AT5G03240	g6			1.13					
250987_at	AT5G02860	g6			1.16			1.41	1.65	
251039_at	AT5G02020	g6								
251221_at	AT3G62550	g6					1.19			
251248_at	AT3G62150	g6	-2.59			1.94	1.46			
251400_at	AT3G60420	g6			-1.42					
251529_at	AT3G58570	g6								
251725_at	AT3G56260	g6								

	· · /		1		1					
251789_at	AT3G55450	g6								
251791_at	AT3G55500	g6								
251975_at	AT3G53230	g6		1.85						
251977_at	AT3G53250	g6								
252166_at	AT3G50500	g6		1.20	1.62					
252355_at	AT3G48250	g6								
252391_at	AT3G47860	g6								
252464_at	AT3G47160	g6						1.11		
252468_at	AT3G46970	g6		1.58	1.55			1.47		
252475_s_at	AT5G59570; AT3G46640	g6								
252880_at	AT4G39730	g6								
252882_at	AT4G39675	g6								
252885_at	AT4G39260	g6								
252908_at	AT4G39670	g6								
252927_at	AT4G39090	g6								
252940_at	AT4G39270	g6								
252976_s_at	AT4G38550	g6								
253215_at	AT4G34950	g6					1.32			
253245_at	AT4G34590;	g6								
	AT4G34588									
253293_at	AT4G33905	g6		2.46						
253331_at	AT4G33490	g6				1.75				
253581_at	AT4G30660	g6								
253592_at	AT4G30840	g6								
253622_at	AT4G30560	g6								
253627_at	AT4G30650	g6	4.38							
253871_at	AT4G27440	g6				1.12				
253981_at	AT4G26670	g6					1.37	1.34	1.11	

254232_at	AT4G23600	g6				1.21			
254408_at	AT4G21390	g6							
254490_at	AT4G20320	g6							
254580_at	AT4G19390	g6							
254634_at	AT4G18650	g6							
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254646_at	AT4G18530	g6							
254659_at	AT4G18240	g6							
254778_at	AT4G12750	g6							
254833_s_at	AT4G12290; AT4G12280	g6	1.24				1.57		1.24
255070_at	AT4G09020	g6		1.72	1.50				
255221_at	AT4G05150	g6							
255232_at	AT4G05330	g6							
255331_at	AT4G04330	g6			1.62		1.78		
255430_at	AT4G03320	g6		1.68		1.82			
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255923_at	AT1G22180	g6							
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255980_at	AT1G33970	g6			1.11				
256060_at	AT1G07050	g6			1.98			3.13	1.39
256062_at	AT1G07090	g6							
256092_at	AT1G20696	g6							
256221_at	AT1G56300	g6		1.86	1.26	1.98			
256426_at	AT1G33420	g6							
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256601_s_at	AT3G28290; AT3G28300	g6							

256623_at	AT3G19960	g6							
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256825_at	AT3G22120	g6							
256839_at	AT3G22930	g6							
257144_at	AT3G27300	g6			1.16				
257147_at	AT3G27270	g6							
257271_at	AT3G28007	g6					-1.38		
257985_at	AT3G20810	g6			2.19				
258157_at	AT3G18100	g6							
258347_at	AT3G17520	g6							
258397_at	AT3G15357	g6							
258435_at	AT3G16740	g6							
258498_at	AT3G02480	g6		3.50					
258735_at	AT3G05880	g6							
258751_at	AT3G05890	g6							
258827_at	AT3G07150	g6							
258833_at	AT3G07274	g6							
258887_at	AT3G05630	g6							
258901_at	AT3G05640	g6							
258939_at	AT3G10020	g6					1.47		
258970_at	AT3G10410	g6		1.89	1.48				
259080_at	AT3G04910	g6				1.28			
259118_at	AT3G01310	g6			1.59				
259178_at	AT3G01650	g6							
259302_at	AT3G05120	g6							
259705_at	AT1G77450	g6							
260005_at	AT1G67920	g6							
260357_at	AT1G69260	g6							
260412_at	AT1G69830	g6							

260627_at	AT1G62310	g6								
260688_at	AT1G17665	g6					1.45			
260974_at	AT1G53440	g6								
261125_at	AT1G04990	g6								
261203_at	AT1G12845	g6						1.59		
261272_at	AT1G26665	g6			1.54					
261428_at	AT1G18870	g6								
262128_at	AT1G52690	g6				-1.13		-1.81		
262248_at	AT1G48370	g6								
262296_at	AT1G27630	g6								
262324_at	AT1G64170	g6					1.18			
262477_at	AT1G11220	g6								
262548_at	AT1G31280	g6								
262607_at	AT1G13990	g6								
262609_at	AT1G13930	g6								
262680_at	AT1G75880	g6								
262690_at	AT1G62720	g6								
262703_at	AT1G16510	g6								
262722_at	AT1G43620	g6								
262775_at	AT1G13000	g6								
262784_at	AT1G10760	g6			1.29					
262892_at	AT1G79440	g6		1.16					1.51	
263065_at	AT2G18170	g6		1.60	1.13					
263295_at	AT2G14210	g6								
263472_at	AT2G31955	g6								
263493_at	AT2G42520	g6								
263548_at	AT2G21660	g6								
263653_at	AT1G04310	g6		1.12						
263708_at	AT1G09320	g6								
Table A.1 (Continued)

263881_at	AT2G21820	g6							
263912_at	AT2G36390	g6							
264045_at	AT2G22450	g6			1.23				
264048_at	AT2G22400	g6							
264209_at	AT1G22740	g6							
264525_at	AT1G10060	g6							
264888_at	AT1G23070	g6							
264957_at	AT1G77000	g6		1.22					
264972_at	AT1G67370	g6							
264992_at	AT1G67300	g6							
265082_at	AT1G03830	g6							
265216_at	AT1G05100	g6							
265271_at	AT2G28360	g6							
265358_at	AT2G16710	g6							
265478_at	AT2G15890	g6					1.44		
265481_at	AT2G15960	g6				1.52			
265900_at	AT2G25730	g6			1.15				
266399_at	AT2G38670	g6							
266462_at	AT2G47770	g6							
266500_at	AT2G06925	g6							
266503_at	AT2G47780	g6							
266578_at	AT2G23910	g6				-1.63			
266839_at	AT2G25930	g6							
266841_at	AT2G26150	g6							
266952_at	AT2G34555	g6							
267080_at	AT2G41190	g6							
267084_at	AT2G41180	g6							
267254_at	AT2G23030	g6					1.55		
267378_at	AT2G26200	g6							

Table A.1 (Continued)

267461_at	AT2G33830	g6	1.14		2.87		1.34

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