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CONSERVATION OF THE LOW TEMPERATURE TRANSCRIPTOMES AND CBF REGULONS BETWEEN *SOLANUM* SPECIES AND ARABIDOPSIS

By

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ABSTRACT

CONSERVATION OF THE LOW TEMPERATURE TRANSCRIPTOMES AND CBF REGULONS IN *SOLANUM* SPECIES AND ARABIDOPSIS

By

Marcela A. Carvallo-Pinto

Plants from tropical regions have no freezing tolerance whereas plants from temperate regions can survive freezing after a period of cold acclimation (exposure to low nonfreezing temperature). In *Arabidopsis* the AP2 transcriptional activators CBF1, CBF2 and CBF3 have an important role in cold acclimation. They are quickly induced in response to low temperature followed by expression of the CBF regulon, which results in an increase in freezing tolerance. Little is known about the conservation of low temperature transcriptomes and CBF regulons in different plant species. *Solanum tuberosum* (common potato) (St) and its wild close relative *S. commersonii* (Sc) are two closely related species with different levels in freezing tolerance, therefore they constitute an excellent model to study conservation of the cold transcriptomes and CBF regulons.

The work in this dissertation focused on the identification and comparison of the low temperature transcriptomes of Sc and St, and also their CBF regulons. Using the St 10K cDNA array, the cold- and CBF-transcriptomes of these species were analyzed, and by identification of putative orthologous groups between St and Arabidopsis, the transcriptomes of Sc and St were compared to that of Arabidopsis. With the criteria used (2FC, $p<0.05$) there was more than 50% overlap between cold transcriptomes of the two *Solanum*

species, suggesting that there are species specific cold regulated genes. However, no obvious differences could be identified between Sc and St cold-transcriptomes that explain their differences in freezing tolerance. Only around 10% of the cold regulated genes in *Solanum* species, that have Arabidopsis orthologs, were also identified as cold regulated in Arabidopsis. This indicates significant differences between the two *Solanum* species and Arabidopsis cold transcriptomes.

The Sc and St CBF regulons were identified, as genes that are differentially expressed by cold treatment and by CBF overexpressionfracture. About 48% of the Sc CBF regulon is also part of the St CBF regulon, suggesting that the genes that are members of the CBF regulon in each of these two *Solanum* species have evolved different cis-acting DNA regulatory elements. When compared to Arabidopsis, only 14% of the Sc and St CBF regulons identified in this study are also part of the Arabidopsis CBF regulon, indicating that there are important differences between these CBF regulons.

The identification of low temperature transcriptomes of the two *Solanum* species provides a start point to the study of these two closely related species with different levels in freezing tolerance. Future analysis of the sequenced potato genome will provide the bases for novel strategies to expand our knowledge of these plants freezing stress mechanism.

PREFACE

In chapter 2, the *S. tuberosum* and *S. commersonii* plant growth and treatments were conducted by Jeff Skinner and Zoran Jeknic from Tony Chen's laboratory at Oregon State University. *35S::AtCBF3 S. tuberosum* and *S. commersonii* transgenic plants were obtained from Maria Teresa Pino at Tony Chen's laboratory, Oregon State University. Expression profiles generated by microarray hybridizations and real time PCR, as well as data analysis was conducted by the author of this thesis. The groups of putative orthologs between Arabidopsis and *S. tuberosum* were generated by Cheng Zou from Shinhan Shiu's laboratory at Michigan State University. The list of cold regulated genes in Arabidopsis was generated by Colleen Doherty. Cold data analysis and comparison across species was performed by the author of this thesis.

In chapter 3, the *S. tuberosum* and *S. commersonii* plant growth and treatments were conducted by Zoran Jeknic from Tony Chen laboratory at Oregon State University. Real time PCR and data analysis was conducted by the author of this thesis.

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KEY TO ABREVIATIONS

CAC = Cold Acclimation Capacity

CHX = Cycloheximide

DE = Differentially expressed

EST = Expressed Sequence Tags

FC = Fold Change

GO = Gene Ontology

NAFT = Non acclimated Freezing Tolerance

PUT = Putative Unique Transcripts

Sc = *Solanum commersonii*

St = *Solanum tuberosum* (common potato)

TIGR = The Institute for Genomic Research

ZT = Zeitgeiber time

CHAPTER ONE

Literature review

Due to the sedentary nature of plants, they have evolved strategies to adapt to different environmental changes. Plants that grow in different climates exhibit differences in cold tolerance. Many plants from temperate regions, such as Arabidopsis, wheat, rye, barley and canola survive freezing temperatures and are able to cold acclimate, process whereby plants increase in freezing tolerance after exposure to low non-freezing temperatures (1,2). For instance, non-acclimated wheat plants are killed at freezing temperatures of about -5°C, but cold acclimated wheat can increase its freezing tolerance and survive to about -20°C (3).

In contrast, plants that grow in tropical or subtropical regions, including crop species such as rice, maize, tomato and potato are freezing sensitive and generally do not cold acclimate (4-7).

Improving the tolerance of crop species to lower temperatures would increase the land where the crops could be grown and would also lengthen the growing season, improving the food supply for a growing world population.

Freezing Damage and Cold Acclimation

When plants are exposed to freezing temperatures, ice formation occurs in the extracellular space due to the extracellular fluid having a higher freezing point than the intracellular fluid. Freezing of the extracellular fluid increases the solute concentration outside the cell. This high osmotic potential draws out water from the cell causing dehydration (1,8). Freezing-induced dehydration can cause a series of cellular injuries including protein denaturation and precipitation of molecules, and membrane damage. Freezing-induced dehydration can cause different types of membrane lesions. At freezing temperatures between -2°C and -4°C the freezing-thaw cycles can cause expansion-induced cell lysis; at lower temperatures, between -4°C and -10°C, the most common form of membrane injury is the phase transition of bilayer lipids from lamellar to hexagonal II (an interbilayer event that involves fusion of cellular membranes); at temperatures below -10°C severe dehydration occurs and causes fracture jump lesions, an alteration in membrane ultrastructure that is manifested as localized deviations of the plasma membrane fracture plane to subtending lamellae (2,9,10).

The gradual exposure to low non freezing temperatures during fall allows plants to increase their freezing tolerance during the winter. This cold acclimation process involves adjustment of metabolism and cellular functions to the constraints imposed by low temperature and the induction of freezing tolerance (1). Cold acclimation induces changes in membrane lipid composition,

increasing levels of fatty acid desaturation in the membrane phospholipids (11). It also prevents expansion-induced lysis and the formation of hexagonal II phase lipids in the plasma membrane (9). Additionally, there is accumulation of small cryoprotective molecules such as soluble sugars and proline during cold acclimation (12,13), and it has been suggested that through interaction with proteins and membranes by hydrogen bonding these could prevent protein denaturation and stabilize membranes (14).

Another event that occurs during cold acclimation is the accumulation of certain hydrophilic polypeptides that help to stabilize membranes against freeze-induced damage. Among these polypeptides are the *COR* (cold-regulated) proteins such as *COR6.6*, *COR15a*, *COR47* and *COR78* (10,15,16). *COR47* is a member of the group II late embryogenesis abundant (LEA) type proteins, also known as dehydrins (10,17). The role of these hydrophilic polypeptides has been elusive for many years but they are thought to be □□□□□□□□□□□□□□□n□□'□□□□□□□□□□□□□□□□'□□□□□□□□□□□□ been shown to increase freezing tolerance of isolated protoplast due to a decrease in incidence of lamellar to hexagonal II phase transitions. These occurred in regions where the plasma membrane comes into close proximity with the chloroplast envelope upon freeze-induced dehydration (18).

Microarray studies have revealed induction of many genes by low temperature. Among these, numerous genes encode proteins that share the *COR* proteins property of being highly hydrophilic but their functions are still unknown (19,20).

Cold responsive CBF pathway in *Arabidopsis thaliana*

Discovery of CBF

The process of cold acclimation involves changes in gene expression that accounts for specific biochemical changes that are thought to contribute to the increase in freezing tolerance. The *COR* gene transcripts accumulated after 4h of cold treatment, and it has been observed that they can stay induced for 2 weeks in the cold. *COR* gene transcripts come back to their warm levels as soon as 4h after transfer to warm (deacclimation) (21). A cis-acting DNA regulatory element present in the *COR* gene promoters was identified to be responsible for their cold-induction, the C-repeat/Dehydration Responsive Element (CRT/DRE) (core sequence = CCGAC) (22). The CBF1 (CRT/DRE Binding Factor 1) transcription factor was found to bind to this CRT/DRE element and activate transcription of CRT/DRE reporter gene fusions in yeast (23).

CBF proteins are members of the AP2/ERBP family of transcription factors (24). In the model plant Arabidopsis, there are 6 members of the CBF family, three of which are cold-induced: CBF1, CBF2 and CBF3, also known as DREB 1B, 1C, and 1A respectively (25,26). CBF1-3 are the major regulators of cold acclimation in Arabidopsis. *CBF* transcripts accumulate soon after exposure to 4°C. They are detectable by Northern hybridization within 15 minutes of exposure to low temperature and they peak at around 2h (27). Constitutive expression of any of the three cold-inducible CBFs in Arabidopsis

leads to induction of the CBF target genes at warm temperatures and results in the ability of these plants to be freezing tolerant without the requirement of a period of cold acclimation (26,28,29).

CBF regulon and its predominant role in configuring the low temperature responses in Arabidopsis

Microarray technology has allowed the identification of hundred of genes that are responsive to low temperature in Arabidopsis. The *COR* gene transcripts accumulate in the cold soon after CBF transcript accumulation (27). Other genes that accumulate by low temperature include enzymes involved in synthesis of protective sugars such as sucrose synthase and galactinol synthase. By overexpression of each of the three CBFs in Arabidopsis and comparison to the cold regulated genes, about 100 genes have been identified as members of the CBF regulon in Arabidopsis, but beside those, several hundred cold-induced genes fall outside CBF regulation, which implies that additional transcription factors play a role in the process of cold acclimation (19,20).

Despite the presence of additional cold-responsive pathways, the CBF pathway plays a predominant role in cold acclimation. Among the genes that are cold responsive, the most highly induced ones are members of the CBF regulon (20). It is known that overexpression of any of the CBF proteins in

Arabidopsis leads to constitutive expression of the CBF regulon and enhanced freezing tolerance without cold acclimation (26,28-30).

Besides the large changes in gene expression caused by CBF overexpression, changes in metabolite profiles have also been studied. Metabolite profiling has demonstrated that 79% of the metabolites that increase in response to low temperature in Arabidopsis Wassilewskija-2 (Ws-2), also increase in non-acclimated plants by *AtCBF3* overexpression. Moreover, the Arabidopsis Cape Verde Islands-1 (Cvi), which is less tolerant to freezing, expressed less *CBF*1-3 and *CBF* target genes in response to the cold, and the low temperature metabolome of Cvi-1 plants was depleted in metabolites affected by *CBF3* overexpression (31).

CBF regulation in response to low temperature

Given the predominant role of CBF in freezing tolerance, much effort has been put into identifying regulators of its induction.

Inducer of CBF expression 1 (*ICE1*) is a MYC-like bHLH transcriptional activator that has been identified as a positive regulator of *CBF3* in Arabidopsis. ICE1 binds specifically to the Myc recognition site in the *CBF3* promoter. A point mutation in ICE1 (*ice1* mutant) almost completely abolished expression of the endogenous *CBF3* gene, but CBF1 and CBF2 expression are only reduced at 1h of cold treatment and reach similar levels to wild type after 6h of cold. Many CBF target genes have decreased expression in the *ice1* mutant after

cold treatment, which leads to a reduction in plant chilling and freezing tolerance. Overexpression of ICE1 enhances the expression of CBF2, CBF3, and the CBF regulon in the cold and improves freezing tolerance (32). However, ICE1 overexpression is not able to induce any of the three CBF transcripts at warm temperatures, indicating that ICE1 alone is not sufficient to induce CBF expression (32). Maybe ICE1 needs to have a modification that only occurs in the cold, or alternatively there are other factors needed to activate CBF.

ICE1 is expressed constitutively, and cold induces the degradation of ICE1 through the E3 ligase, HOS1, a negative regulator of cold acclimation, that targets ICE1 for ubiquitination (33). SIZ1, a SUMO E3 ligase, mediates SUMO (small ubiquitin-related modifier) conjugation of ICE1 during cold acclimation, reducing its polyubiquitination and leading to an enhanced cold induction of CBF and COR genes and increased freezing tolerance (34). Given that there is little effect on CBF1 and CBF2 expression in the *ice1* mutant, it is thought that the regulation of these three CBF genes may be independent.

Another transcription factor has been recently identified as a positive regulator of *CBF2* expression, the CAMTA3. This protein belongs to the CAMTA family of calmodulin-binding transcription factors that has six members in Arabidopsis. A *camta3* single knock out mutant had 50% reduction of *CBF2* transcript and 40% reduction of *CBF1* transcript under low temperature compared to WT. CAMTA3 binds to the conserved motif 2 (CM2) present in the *CBF2* promoter. The double *camta1/camta3* mutant is impaired in freezing

tolerance after cold acclimation, indicating that CAMTA1 and CAMTA3 are both needed to attain full levels of freezing tolerance (35).

Negative regulation of *CBF*1-3 gene expression has also been identified. The null *cbf2* mutant has more CBF1 and 3 transcripts in warm and cold conditions, suggesting that CBF2 could be a negative regulator of CBF1 and CBF3 (36). Two other transcription factors repress CBF1-3 accumulation. The overexpression of Myb15 (a R2R3 type Myb transcription factor) and ZAT12 (a zinc finger transcription factor) reduce CBF1-3 cold accumulation (20,37). Myb15 and ZAT12 transcripts are cold-induced. The Myb15 protein binds to the Myb recognition sequences in the promoters of *CBF1-3* genes. A knock out mutation of Myb15 causes increased expression of CBF genes under low temperature. However, overexpression or knock out of Myb15 does not change the transcripts of CBF regulon genes such as COR15 or RD29a genes. All these studies suggest that the regulation of CBFs is very complex.

CBF regulation in response to other environmental cues

CBF genes are not only responsive to low temperature signals but also to other environmental changes. It has previously been shown that Arabidopsis CBFs are induced in response to mechanical agitation and inhibition of protein synthesis (cycloheximide treatment) (20,27,38). The CBF2 promoter has two sequences, ICEr1 and ICEr2 (Induction of CBF expression region 1 and 2) that impart cold-regulated gene expression and also stimulate transcription in

response to mechanical agitation and the protein synthesis inhibitor, cycloheximide. It is possible that there is a regulatory link between these different responses that it is yet to be discovered.

In addition to these responses Arabidopsis CBF3 has also been shown to be regulated by the circadian clock (39). Harmer et al. (2000) showed that at warm temperature, *CBF3* transcripts undergo circadian cycling, with a peak at ZT4 (Zeitgeiber Time, hours after dawn) and a trough at ZT16 (39). Given this circadian regulation of *CBF3* at warm temperatures, the question was raised whether the circadian clock also gated the expression of *CBF1-3* in response to low temperature. Fowler et al. (2005) showed that indeed it did. The circadian clock has a gating effect on the low temperature induction of *CBF1-3* genes. When plants are shifted to low temperature at ZT4 (4h after dawn), which coincides with the peak of CBF3 circadian expression in the warm, the cold induction of *CBF1-3* is higher than when transferred to cold at the trough of *CBF3* expression (ZT16) (40). Furthermore, disruption of the circadian clock by overexpression of Circadian Clock Associated 1 (*CCA1*), a Myb-related transcription factor member of the Arabidopsis clock (41,42), also disrupted the cycling of *CBF1-3* (40).

Circadian gating of CBFs has also been suggested in tomato. When *Solanum lycopersicum* (tomato) and its wild relative *Solanum pimpinellifolium* were entrained under a 16:8 L:D photoperiod, *CBF1* expression cycled, reaching higher responsiveness during the light period in both species. When plants were shifted to constant dark or constant light CBF transcripts continued

to cycle, however the peaks and troughs did not correspond to those observed in plants grown under a normal 16:8 L:D photoperiod (43).

Another environmental factor that has been observed to be involved in CBF regulation is light quality. More *CBF1-3* transcripts accumulate at 16°C under a low R/FR ratio compared to a high R/FR, and this increase is dependent on the circadian clock. Plants grown at 16°C in low R/FR light are more freezing tolerant than plants treated with high R/FR light, indicating that this light quality-dependent increase in CBF expression is sufficient to confer freezing tolerance at higher temperatures than those required for cold acclimation (4°C). In nature, a decrease in R/FR light occurs during twilight periods. It is reasonable to think that low temperature, shorter day length and longer twilight periods during fall will trigger CBF expression to confer freezing tolerance before the winter comes (44).

Recently, the transcription factor PIF7 (Phytochrome Interacting Factor 7) was demonstrated to bind specifically the G-box of *CBF1* and *CBF2* promoters. PIF7 is a basic helix-loop-helix (bHLH) transcription factor that interacts with the far red light-absorbing Pfr form of phytochrome B (phyB) (45). Transactivation experiments showed that PIF7 acts as a transcriptional repressor for *CBF2* expression and this activity is mediated by two PIF7-interacting factors, TOC1 and PhyB (components of the circadian clock and the red light photoreceptor respectively). PIF7 is localized to the nucleus and it is expressed under warm conditions in rosette leaves. After entrainment by the clock in a 12:12h L:D photoperiod, the *pif7* mutant showed no repression of

CBF1 and *CBF2* under continuous light, which translated in no cycling of these genes, indicating that PIF7 functions as a transcriptional repressor of *CBF1* and *CBF2* under circadian control (46).

The integration of knowledge about regulation of CBF under different environmental signals will enable a better understanding of CBF regulation under low temperature stress.

CBF independent pathways in cold acclimation

Some recent evidence suggests that, besides the CBF pathway, there are CBF-independent pathways that contribute to freezing tolerance. Microarray studies have revealed that there are hundreds of genes that are cold-regulated but are not affected by *CBF* overexpression in warm grown plants, even though constitutive expression of *CBF* is sufficient to increase freezing tolerance in warm conditions (19,20). These genes may be part of a CBF independent pathway in the cold or may require one or more additional factor(s) that are only present in the cold. Moreover, when *CBF1*, 2 or 3 is overexpressed, cold acclimated plants have an increase in freezing tolerance compared to warm non acclimated plants (26,28). This additional freezing tolerance may be due to CBF independent pathways, additional components that act in concert with CBF to confer freezing tolerance or may just be a quantitative effect due to more CBF transcript present in the cold as a result of endogenous CBF accumulation at low temperature.

Mutational analysis has supported the idea of CBF independent pathways involved in freezing tolerance. For instance, the *hos10* mutation in the R2R3-type Myb transcription factor, produced a mutant that is extremely sensitive to freezing and is unable to cold acclimate; however the cold-induction of *CBF*1-3 transcripts and *CBF* target genes (*COR15a*, *COR78*) is not altered (47). Similarly, Gigantea (GI), a protein involved in developmental regulation of flowering in response to day length and circadian clock, may be involved in CBF-independent cold acclimation. *GI* transcript has been shown to be cold-induced. The *gi-3* mutation shows increased sensitivity to freezing stress with no changes in the transcript accumulation of *CBF1-3* or CBF target genes (*COR15a*, *COR78*, *KIN1*) (48).

Cold responsive CBF pathway in other plant species

Considerable evidence suggests that the CBF cold responsive pathway is present in a wide variety of plant species and that it functions in the development of freezing tolerance in many of them. CBF proteins are highly conserved and are not limited only to cold acclimating plants. Cold-inducible CBF genes have been identified in *B. napus*, wheat, rye, barley, rice, maize, *Populus*, tomato, and potato among others (3,43,49-52). The region of highest amino acid sequence identity among the CBF proteins is within the AP2/EREBP (Apetala2/Ethylene Responsive Element Binding Protein) DNA binding domain (3). This domain is common to several transcription factors in plants known as

the AP2/EREBP proteins (53). CBF proteins form a subset of this group and are characterized by having two regions flanking the AP2/EREBP DNA binding domain. These two regions (PKK/RPAGRxKFxETRHP upstream and DSAWR downstream of the DNA binding domain) are called the "signature sequences" and are very well conserved in CBF-like proteins from *B. napus*, wheat, rye, tomato, and barley; and less conserved in pepper, rice, and maize.

Several studies have shown that CBFs from species other than Arabidopsis also bind to the CRT/DRE DNA binding motif. In *B. napus*, the CRT/DRE element is critical to the low temperature response of the *Bn115* gene (54). BnCBF5 and BnCBF17 (*B. napus* homologs of the Arabidopsis CBF proteins) are able to bind to this element in vitro and are able to trans activate promoter regions containing CRT/DRE elements fused to a *lacZ* reporter gene in yeast (55). The cold-inducible *HvCBF1* from barley is able to bind to the GCCGAC motif and is involved in the regulation of cold-responsive genes from barley (56). The rice cold-inducible *OsDREB1A* (ortholog of AtCBF) has been shown to bind efficiently to the GCCGAC CRT/DRE elements (51). Another example is the CBF maize ortholog *ZmDREB1A* that is also cold-inducible and able to bind the DRE motif (52).

Interestingly, it has been shown that CBFs confer freezing tolerance in other plant species. Overexpression of any of the three Arabidopsis *CBF* genes increase freezing tolerance of *B. napus* in non-acclimated conditions (3). When maize *ZmDREB1A* (an ortholog of CBF) is overexpressed in Arabidopsis there is constitutive expression of *AtCOR15a* and other cold-induced genes and this

results in an increase in freezing and drought tolerance (52). Overexpression of *AtCBF1* increases freezing tolerance of Populus (50).

CBFs have also been shown to have an effect in freezing sensitive species. Overexpression of wheat *CBF2* in transgenic tobacco increase freezing tolerance (57). Overexpression of *AtCBF3* increased chilling tolerance in tobacco and resulted in a small increase in freezing tolerance in potato (58,59), while *AtCBF1* overexpression has been demonstrated to increase freezing tolerance in transgenic potato plants (50,60).

In tomato, a freezing and chilling sensitive non acclimating plant, there are three orthologs of the CBF genes, *LeCBF 1, 2* and *3*, which, as in Arabidopsis, are present in tandem array in the genome. However, only *LeCBF1* is induced by low temperature (61). Overexpression of *LeCBF1* or *AtCBF3* in Arabidopsis leads to induction of the CBF target genes and an increase in freezing tolerance. This indicates that *LeCBF1* encodes a functional CBF protein. However, overexpression of the *LeCBF1* or *AtCBF3* genes in tomato plants do not increase freezing tolerance and they regulate very few genes (61), indicating that *AtCBF3* overexpression is not sufficient to induce cold acclimation in tomato. This suggests that tomato and Arabidopsis have critical differences, resulting in an absence of response to *AtCBF* in tomato. It is possible that there is some co-activator, a protein that works in concert with CBF that is not present in tomato. Alternatively the CBF target genes do not have CRT/DRE elements in their promoters, which would explain why CBF

does not induce many genes in tomato. To date, it is unknown why tomato is freezing sensitive.

Another level of conservation in the CBF pathway may lay upstream of CBF. Recently, two wheat *ICE* (Inducer of CBF expression) genes have been identified: *TaICE41* and *TaICE87*. Both genes are expressed constitutively as is the Arabidopsis *ICE1* (62). *TaICE41* and *TaICE87* bind to different MYC elements in the wheat *TaCBFIVd-B9* promoter, and both *TaICE* proteins can activate *TaCBFIVd-B9* transcription when transiently transformed in *N. benthamiana* plants. As observed with the *AtICE1* in Arabidopsis (32), overexpression of either *TaICE41* or *TaICE87* genes in Arabidopsis increased freezing tolerance only after cold acclimation, suggesting that other factors induced by low temperature are required for ICE activity.

All these studies suggest that the CBF cold-responsive pathway is conserved in diverse plant species. However, little is known about what the differences are between freezing tolerant and freezing sensitive species. Microarray technology has provided the opportunity to study gene expression changes under cold stress at a whole genome level. For instance, wheat, one of the most freezing tolerant crop plants, has been used to compare cold transcriptomes among cultivars with different levels of freezing tolerance. The highly cold tolerant winter wheat cultivar CDC Clair was compared to the less tolerant spring cultivar, Quantum (63). It was found that a large number of genes had altered levels of expression in each cultivar and there were significant differences in expression between the two cultivars. After 6 hours of

cold, the number of up regulated genes was higher in the spring cultivar; however throughout the time course (up to 14 days of cold acclimation) the number of up regulated genes was higher in the winter wheat.

In the future, it would be very interesting to find what the differences are between freezing sensitive and freezing tolerant species. Is there one gene or many genes? Are there any differences in the cis elements that drive cold expression? Are these factors transferable from freezing tolerant to freezing sensitive species? Answers to these questions would not only be important for our basic knowledge of plants responses to environmental changes, but also to improve crop production.

CHAPTER TWO

TRANSCRIPTOME PROFILES OF *SOLANUM* SPECIES WITH DIFFERENT LEVELS OF FREEZING TOLERANCE

INTRODUCTION

Cold acclimation is the process whereby plants increase their level of freezing tolerance by exposure to low non-freezing temperatures. The CBF (CRT/DRE Binding Factor) family of transcription factors (CBF1, CBF2 and CBF3, also known as DREB 1B, 1C, and 1A respectively) is a major regulator of cold acclimation in Arabidopsis. The CBF genes are induced soon after exposure to 4°C and CBF transcripts are detectable by Northern hybridization within 15 minutes (27).

Arabidopsis CBF proteins bind the C-repeat/Dehydration Responsive Element (CRT/DRE) (core sequence = CCGAC) present in the promoters of many cold responsive (*COR*) genes to induce their expression (20,23). Transcripts for the *COR* genes accumulate soon after *CBF* transcript accumulation in response to cold (27). About 100 genes have been identified as CBF target genes in Arabidopsis, but beside these, several hundred fall outside CBF regulation, which implies that additional transcription factors play a role in the process of cold acclimation (19,20).

Considerable evidence suggests that the CBF cold response pathway is present in a wide variety of plant species and that it functions in the

development of freezing tolerance in many of them. Genes that may encode CBF orthologs have been found in many species; they are highly conserved and not limited to cold acclimating plants. For instance, orthologs of CBF genes from *B. napus*, wheat, rye, tomato, barley, pepper, grape, rice, and maize are induced in response to low temperature (3,49,51,52,64,65). Furthermore, CBF proteins are highly conserved. The region of highest amino acid sequence identity among the CBF proteins is within the AP2/EREBP (Apetala2/Ethylene Responsive Element Binding Protein) DNA binding domain (3,52,66).

Overexpression of any of the CBF genes in Arabidopsis leads to constitutive expression of the CBF regulon and enhanced freezing tolerance (26,28-30). Additionally, it has been shown that CBF overexpression increases freezing tolerance in other plant species. Overexpression of any of the three Arabidopsis CBF genes increases freezing tolerance of *B. napus*, in non-acclimating conditions (3). Overexpression of *AtCBF1* has also been shown to increase freezing tolerance under non-acclimating conditions in populus and potato, and overexpression of *AtCBF3* has a small increase in freezing tolerance in potato (50,51,59,60).

Despite the increasing evidence for conservation of the CBF pathway, little is known about the differences and similarities between plants with different levels of freezing tolerance. It has been previously shown that tomato, a freezing and chilling sensitive non acclimating plant, encodes three orthologs of the CBF genes, *LeCBF 1, 2* and *3*, which, as in Arabidopsis, are present in

tandem array in the genome. However, only *LeCBF1* is induced by low temperature (61).

LeCBF proteins are 70-84% identical to each other and 51-59% identical to those of Arabidopsis CBF proteins. The 3 tomato CBF proteins have the two conserved "signature sequences" that distinguishes the CBF proteins from other AP2/ERBP proteins (PKKPAGR and DSAWR). Overexpression of *LeCBF1* or *AtCBF3* in Arabidopsis leads to induction of the CBF target genes and an increase in freezing tolerance. This indicates that *LeCBF1* encodes a functional CBF protein. However, overexpression of the *LeCBF1* or *AtCBF3* genes in tomato plants does not increase freezing tolerance (61), indicating that *AtCBF3* overexpression is not sufficient to induce cold acclimation in tomato. This suggests that tomato and Arabidopsis have critical differences, resulting in an absence of response to *AtCBF* in tomato.

Recently, it was found that *Solanum tuberosum* (common potato) (St) and its wild relative *Solanum commersonii* (Sc) may have conserved parts of the CBF cold responsive pathway. Sc is able to cold acclimate and has a moderate level of freezing tolerance; it is killed at -4.5°C and after cold acclimation is able to survive down to – 11.5°C. On the other hand, St does not cold acclimate and is freezing sensitive; it is killed at -3°C before and after cold acclimation (7). Sc has four *CBFs* genes and St has five. Both these species also have *CBF1-3* genes in tandem array in their genomes; and each of these species has two cold-induced CBFs (*CBF1* and *CBF4*) (43). The Sc and St CBFs are highly similar to those of Arabidopsis (54-64% identity), and they also

contain the signature sequences. It has also been demonstrated that overexpression of *AtCBF1* or *AtCBF3* increases freezing tolerance of St by 2°C, but *AtCBF2* does not increase freezing tolerance; and *AtCBF1* increases freezing tolerance of Sc by 4°C (59,60).

The low temperature transcriptomes of Sc and St are as yet unknown. It is not clear if they are similar to each other or what portion of them is regulated by CBF. Because these are very closely related species with different levels of freezing tolerance, they constitute an excellent model to study conservation of the cold transcriptomes and CBF regulons.

Variation in gene expression can result in phenotypic differences. Studies of comparative transcriptomes are still not very prevalent. Some studies have focused on expression variation, for instance, between species of primates and between yeast species (67,68). In plants, a few recent studies have attempted to compare differences in gene expression between species challenged by stress (63,69-71).

The main goal of the experiments described in this chapter was to identify and compare the low temperature transcriptomes of Sc and St, and also their CBF regulons. Using the St 10K cDNA array, the cold- and CBF-transcriptomes of these species were analyzed, and by identification of putative orthologous groups between St and Arabidopsis the transcriptomes of Sc and St were compared to that of Arabidopsis. Hierarchical clustering analysis of the low temperature transcriptomes of Sc and St indicates that, in general, they are very similar. With the criteria used (2FC, $p<0.05$) there was more than 50%

overlap between cold transcriptomes of the two *Solanum* species. In general, there are no obvious differences between the Sc and St cold transcriptomes that could account for their differences in freezing tolerance. Only around 10% of the cold regulated ESTs in *Solanum* species, that have Arabidopsis orthologs, were also identified as cold regulated in Arabidopsis. This indicates significant differences between the two *Solanum* species and Arabidopsis cold transcriptomes.

The Sc and St CBF regulons were identified. There are significant differences between the genes that are regulated by *AtCBF3* overexpression in Sc and St. About 48% of the Sc CBF regulon is also part of the St CBF regulon, suggesting that these CBF regulons are not very well conserved. When compared to Arabidopsis, only 14% of the Sc and St CBF regulons identified in this study are also part of the Arabidopsis CBF regulon, indicating that there are important differences among these CBF regulons.

RESULTS

The transcriptomes of both *S. commersonii* and *S. tuberosum* are significantly altered in response to low temperature

S. commersonii (Sc) and *S. tuberosum* (St) have very different tolerances to freezing. Moreover, Sc is able to cold acclimate but St is not. It was hypothesized that differences in their low temperature gene expression could result ultimately in their differences in freezing tolerance. Therefore, the low temperature transcriptomes of these two *Solanum* species were identified and compared. The TIGR potato cDNA array (10K, version 4) was used to compare the low temperature transcriptomes of Sc and St. The array represents about 10,000 of the ~70,000 Putative Unique Transcripts (PUT) available at PlantGDB. Plants were grown for three weeks at 25°C and then transferred to 2°C for 2h, 24h and 168h. RNA was isolated from plants at the various time points and their transcriptomes determined.

First, the log ratios of all the Expressed Sequence Tags (ESTs) in the array were hierarchical clustered to compare the general patterns of cold regulated kinetics between Sc and St low temperature transcriptomes. This hierarchical cluster was done including all flagged spots and is prior to statistical selection. Fig 2.1 shows this hierarchical cluster done with average log ratios of three biological replicates per time point. A large part of the ESTs spotted on the array showed cold regulation. The highest up regulated cluster (A)

correspond to ESTs cold-induced in Sc and St at 2h and St at 24h of cold. Only some of the ESTs in this cluster show up regulation in Sc at 24h of cold. This indicates that the ESTs in this cluster are only transiently induced by cold in Sc, but they stay at least until 24h of cold in St. This different kinetic between Sc and St in cluster A could be a reason why these species are different in freezing tolerance, but there is no enough evidence at this stage to support that.

Another major cluster is B; this corresponds to a group of ESTs that are cold-induced only at 168h in both Sc and St. A small cluster (C) corresponds to ESTs that are induced in response to low temperature at all time points tested in both species. A fourth cluster is D; the pattern of cold induction in this cluster indicates up regulated ESTs at 24h and 168h in both Sc and St. The highest cold down regulated cluster (E) has ESTs from Sc 168h and St 168h.

The hierarchical cluster analysis of all the data revealed that the Sc and St transcriptomes are largely changed by low temperature exposure, with very similar responses in Sc and St at the different cold time points tested and possibly with some kinetic differences. However, the analysis described here serves the purpose of a general overview of all EST clones present in the array, lacking statistical significance.

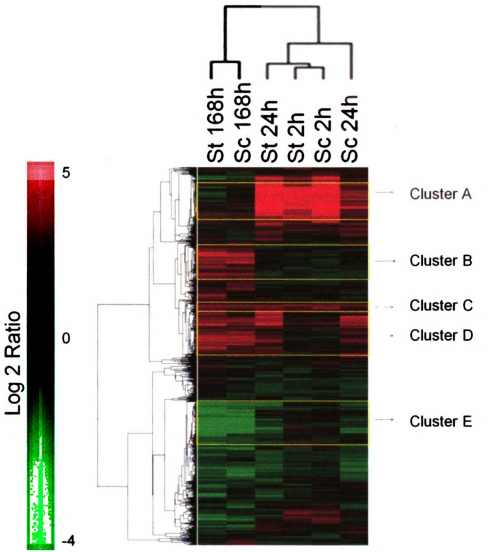

Fig 2.1: Hierarchical clustering and expression profiles of Sc and St EST clones at 2, 24 and 168h of cold treatment (2°C). Sc: *S. commersonii*, St: *S. tuberosum*. Data showed as average log ratio from 3 biological replicates. The figure shows all spots on the array (including bad flagged spots) prior to statistical selection.

After the previous general analysis was done, the idea was to identify a core set of cold responsive genes in each of the two *Solanum* species. To do that, a list of ESTs that were reproducibly cold regulated were obtained, employing linear models (Limma package, (72)) as a statistical tool to rank the ESTs in order of evidence (p value) of differential expression (DE), thus addressing any variability between biological replicates. After ranking the ESTs based on p value, a $p<0.05$ and 2 fold change (FC) was used as the cut off. A list of cold-regulated ESTs for Sc and St can be accessed at http://www.prl.msu.edu/Facultypages/NSF_MFT_Site/data.html. About 13% of the ESTs on the array were cold-induced and 5% cold-repressed at one or more of the time points in Sc. Similarly, about 10% of the ESTs were cold-induced and 6% cold-repressed in St.

Given that without applying selection criteria, a large part of the ESTs in the array showed cold regulation, but after applying the criteria (2FC, $p<0.05$) only small percentage of ESTs were cold regulated, this indicates that many ESTs were lowly expressed and there was large technical or biological variability.

Similarities and differences in the cold-induced gene sets of Sc and St

A total of 1532 and 1084 ESTs were cold-induced in Sc and St, respectively (totals were determined by combining the results from all three cold-treated time points). Around 50% of the cold-induced ESTs in Sc were also

induced in St (Fig 2.2a). Early in the cold (2h and 24h) there was a large overlap between cold-induced ESTs in Sc and St, and there were more ESTs induced apparently only in Sc (Fig 2.2b). However, late in the cold (168h) (Fig 2.2c) there was a large group of ESTs that were cold-induced apparently only in St. These results suggest that the cold-regulation of these genes could be different between Sc and St.

The results presented above indicated that even when statistics are applied to the data, the overlap between the cold-induced transcriptomes of Sc and St is still considerable. The differences could be real or apparent due to the arbitrary criteria used to define cold-induced genes. Changing the criterion to make it more stringent or more relaxed increased the overlap between cold-induced ESTs in Sc and St. Moreover, with the criteria of two-fold change and $p<0.05$, there were no cold-induced ESTs for the 2h St RNA, but by relaxing the criteria to two-fold and $p<0.07$, the number of ESTs that were cold-induced at 2h was 675. These findings indicate that the overlaps detected between the Sc and St transcriptomes were minimal estimates of conservation.

The functions encoded by the genes that were significantly cold-induced at early and late time points in both Sc and St were compared to determine whether the functional categories of the genes changed with time of exposure to low temperature (Fig 2.2d). The results indicated that the categories did not change much between the early and late samples, but that there were some differences. Earlier in the cold there were more cold-induced ESTs annotated as transcription factors than later in the cold. Later in the cold, there were more

ESTs annotated as structural molecules (like ribosomal proteins) and translation factors being induced. These results suggest protein synthesis is activated later in the cold.

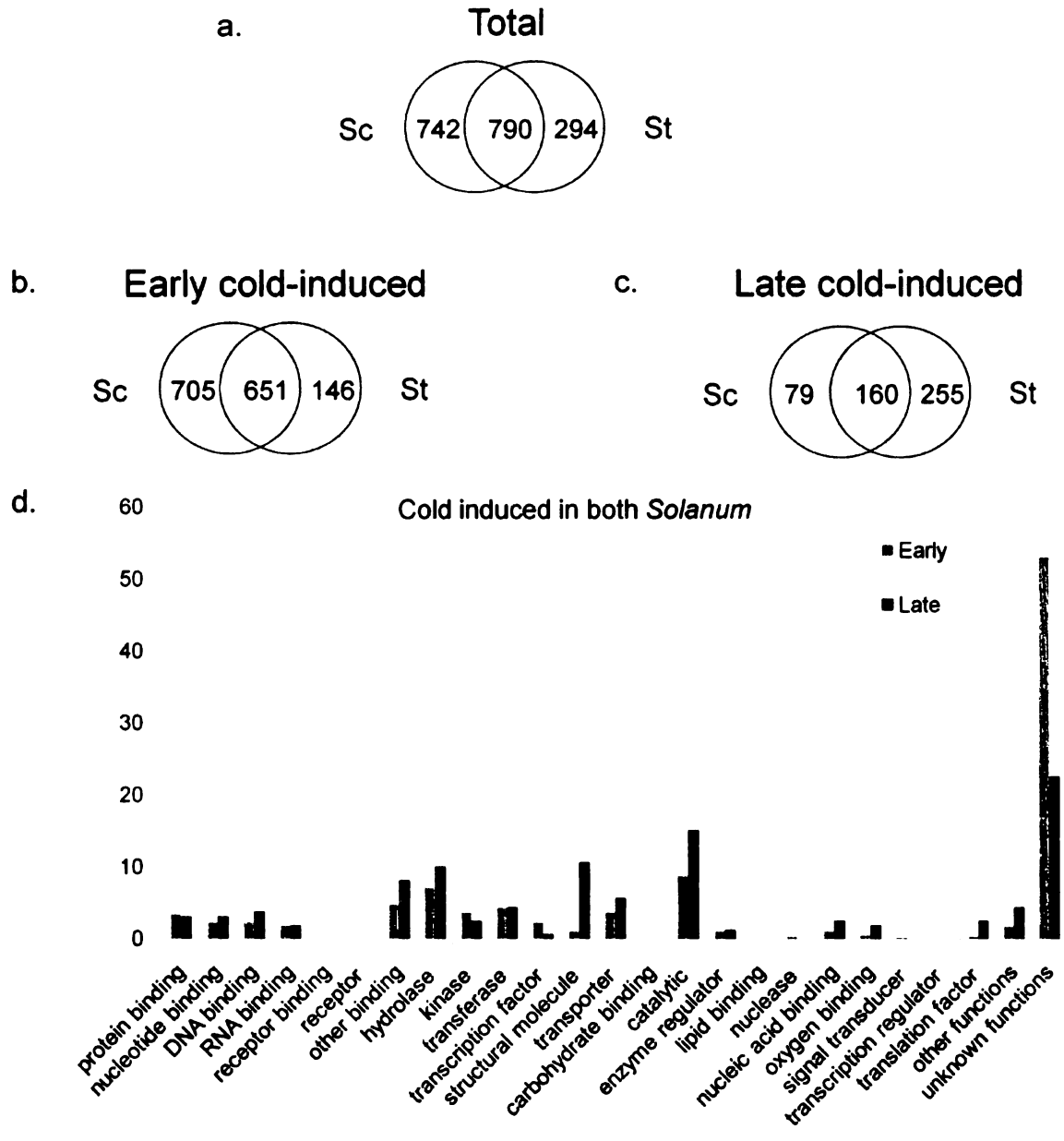

Fig 2.2: Comparison of early and late cold-induced ESTs (2FC, $p<0.05$) in both *Solanum* species (a) Number of total ESTs cold-induced (determined by combining the results from all three cold treated time points). (b) Number of ESTs cold-induced early (2h and 24h at 2°C) and (c) late (7 days at 2°C). (d) The ESTs that were induced either early or late in both *Solanum* species were classified according to their functional categories. Each category is shown as a percentage of the total 651 early cold-induced or total 160 late cold-induced ESTs.

Similarities and differences in the cold-repressed gene sets of Sc and St

A total of 530 and 688 ESTs were cold-repressed in Sc and St, respectively (totals include results from the three cold-treated time points) (Fig 2.3a). Around 70% of the cold-repressed ESTs in Sc were also down regulated in St. The percentage of overlap between cold-repressed ESTs was similar between early and late cold treatments (Fig 2.3b, and c). Functional analysis of the ESTs cold-repressed in both Sc and St indicated that early in the cold there were more down regulated genes annotated as transferases and oxygen binding proteins than later in the cold (Fig 2.3d).

Together, these results indicate that the transcriptomes of Sc and St are significantly altered in response to low temperature. Even though some small differences can be identified between Sc and St cold transcriptomes, there were not dramatic differences at a global level that could account for their differences in freezing tolerance, but rather similarities in the patterns of gene expression were identified.

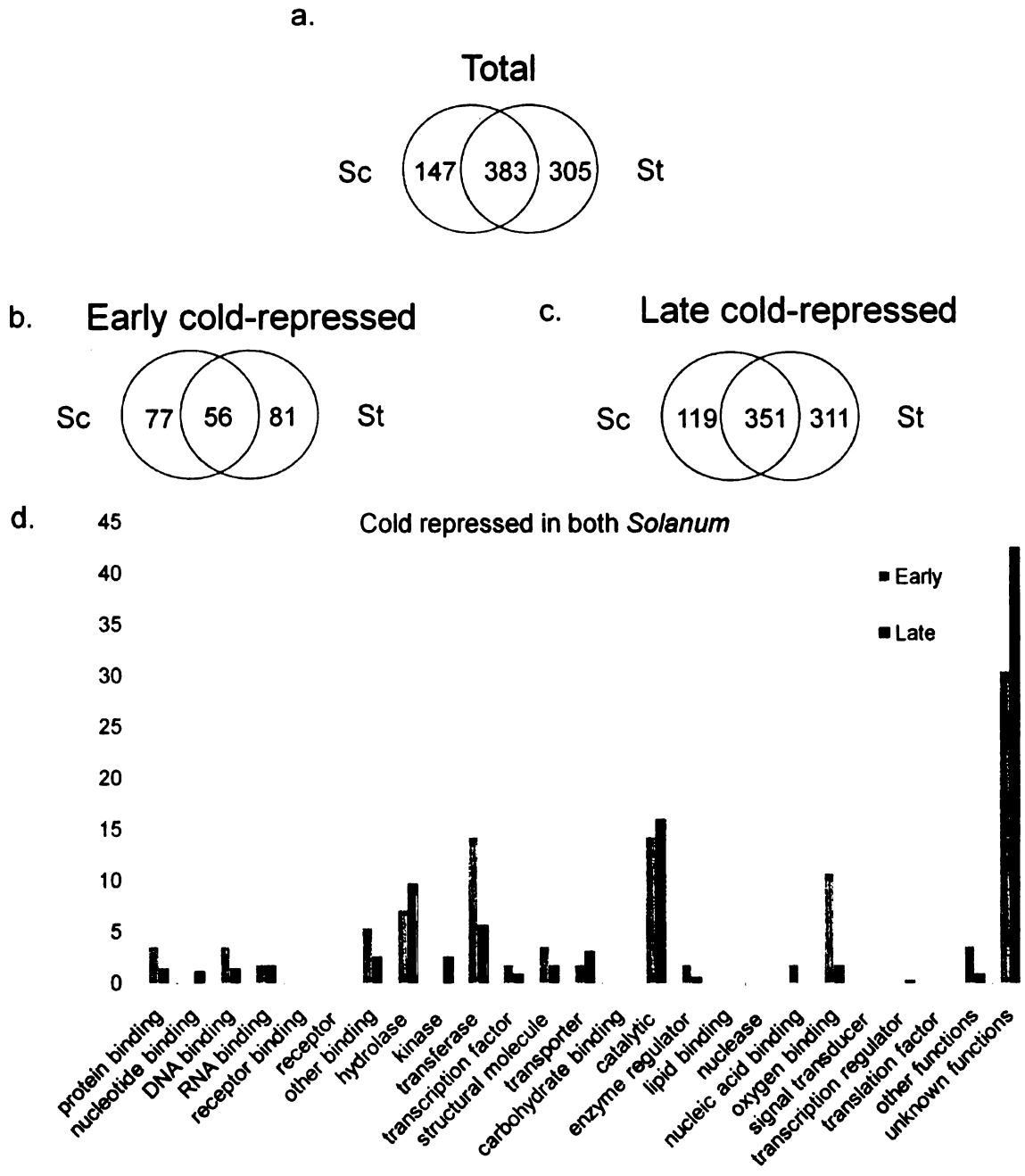

Fig 2.3: Comparison of early and late cold-repressed ESTs (2FC, $p<0.05$) in both *Solanum* species (a) Number of total ESTs cold-repressed (determined by combining the results from all three cold treated time points). (b) Number of ESTs cold-repressed early (2h and 24h at 2°C) and (c) late (7 days at 2°C). (d) The ESTs that were repressed either early or late in both *Solanum* species were classified according to their functional categories. Each category is shown as a percentage of the total 56 early cold-repressed or total 351 late cold-repressed ESTs.

Comparison of the low temperature transcriptomes between the two *Solanum* species and Arabidopsis

The major goal is to understand to which extent cold responsive pathways are conserved in plant species. To explore conserved aspects of the cold response pathways, experiments were conducted to determine the degree to which the cold-regulated genes in the two *Solanum* species were also cold-regulated in Arabidopsis. To conduct this comparison, a list of putative orthologous gene groups (pOG) between *S. tuberosum* (St) and Arabidopsis was obtained from Shinhan Shiu's laboratory. This list was generated using all the Arabidopsis protein sequences (TAIR) and all the potato protein sequences predicted from the ~70,000 PUT (Putative Unique Transcripts) available at PlantGDB (see methods). This resulted in the identification of 8,714 pOGs between Arabidopsis genes and potato PUTs (Fig 2.4). A pOG may have more than one Arabidopsis gene and/or more than one potato PUT.

The potato genome is not known therefore the ESTs that are spotted in the potato array are only small portions of potato genes. There are many ESTs that cannot be assembled into transcripts (PUTs), because there are not more ESTs known for those genes, and a single EST could be just a small portion of a transcript therefore not enough sequence to identify its Arabidopsis ortholog gene. That is why, only the ESTs that could be assigned to transcripts (PUTs) were used to identify their Arabidopsis ortholog. From the 11,366 ESTs represented in the potato array, most of them (9,900 ESTs) can be assigned to

a PUT, and only those were considered in the pOGs identification. From these 9,900 ESTs only 3,934 ESTs belong to pOGs with Arabidopsis members (within the 8,714 pOGs). The rest of pOGs between At and St include PUTs that are not represented in the potato array (Fig 2.4).

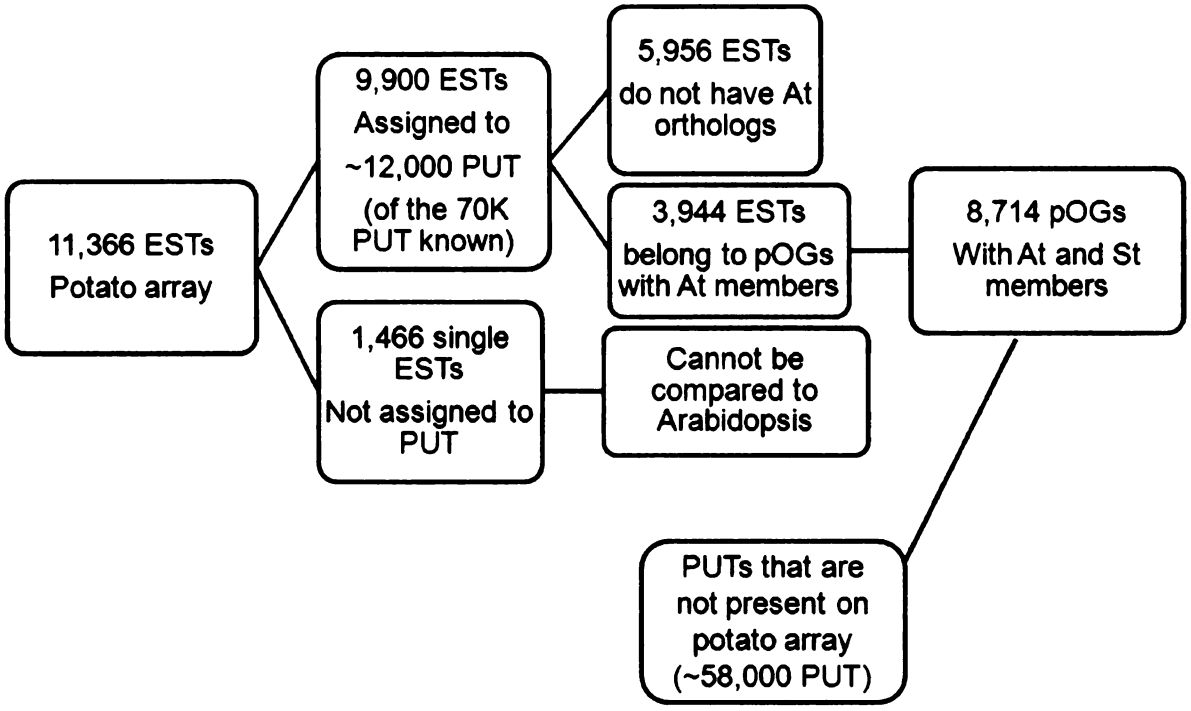

Fig 2.4: Putative orthologs groups (pOGs) between Arabidopsis and *S. tuberosum*. 8,714 pOGs were identified between Arabidopsis proteins and the 70,000 PUT (potato unique transcripts). From the 11,366 ESTs present in the potato array, 9,900 can be assigned to potato PUTs, and only those were considered for the pOGs identification. Only 3,944 ESTs present in the potato array belong to pOGs that have Arabidopsis members. The rest of pOGs between Arabidopsis and potato includes PUTs that are not present in the potato array.

The cold-regulated ESTs that were conserved between Sc and St were first analyzed to identify how many of them had Arabidopsis putative orthologs. From the 790 ESTs that were identified as cold-induced in both Sc and St above, 278 ESTs (35%) were represented in the list of orthologous groups and correspond to 244 pOGs (Fig 2.5a). From the 383 ESTs that were identified as cold-repressed in both Sc and St above, 174 ESTs (45%) were represented in the list of orthologous groups and correspond to 129 pOGs (Fig 2.5a). These results indicate that a big percentage of the cold-regulated ESTs in the two *Solanum* species do not have Arabidopsis orthologous genes under the criteria used.

Of the 8,714 pOGs identified between St and Arabidopsis genes, only 2,944 pOGs were determined to have at least one Arabidopsis gene present on the ATH1 Affymetrix chip, and at least one potato EST present on the potato array. These pOGs represented in both arrays were considered for the following analysis (Fig 2.5b).

To study the conservation of cold responsive pathways, the low temperature transcriptomes of Sc and St were compared to a list of Arabidopsis cold-regulated genes generated in our laboratory (unpublished data). This list was obtained from the AtGeneexpress website, from experiments done in 16:8h L:D photoperiod with cold treatments of 4°C for different times. The criteria of DE genes selection was 2FC and $p<0.05$, and the lists consisted of 1,151 cold-induced and 1,095 cold-repressed Arabidopsis genes.

Based on the 2,944 pOGs represented in both arrays, the overlap between the cold-induced transcriptomes of the three species was determined (Fig2.5c). Numbers of pOGs with at least one cold induced gene from each species were identified. As the potato array does not include all potato genes—likely well less than half—these values are minimum estimates of pOGs that include cold-regulated genes; that is, it is possible that a given pOG includes multiple genes, one or more of which is cold-induced, but the EST on the array is one that is not cold-induced.

Forty pOGs with at least one cold-induced gene in each of the three species were identified (Fig 2.5c). Thus, only 9% and 13% of the pOGs with cold-induced genes in Sc and St, respectively, are also cold-induced in Arabidopsis. Given that the Arabidopsis cold-induced list of genes comes from a microarray that represents almost its entire genome, this result likely indicates a real difference between cold-induced transcriptomes between the two *Solanum* species and Arabidopsis. Forty four percent of the 197 pOGs that are only induced in both *Solanum* species but are not induced in Arabidopsis are pOGs with genes of unknown molecular function; many are pOGs with genes encoding proteins with catalytic activity (10%), hydrolases (8%), transporters (6%) and kinases (5%).

The 40 pOGs common to all three species (Table A.1) include genes that are thought to have protective roles against freezing and drought such as LEA14 (late embryogenesis abundant) (73), ERD10 (early responsive to desiccation) and ERD14 (73,74), as well as ELIP (early light inducible protein),

which is thought to have a protective role against photooxidative damage (75). There were also conserved cold-induced transcription factors in these three species: Agamous-like 20, also called Suppressor of overexpression of CO (*SOC1*); *NAC019*; *RD26* (responsive to dessication 26); *ADOF1*; and Heat shock factor 8 (*HSFA8*).

Thirteen pOGs with at least one cold-repressed gene in each of the three species were identified (Fig 2.5d). Thus, only 8% and 6% of the pOGs with cold-repressed genes in Sc and St, respectively, also have Arabidopsis cold repressed genes. The 13 pOGs that were cold-repressed in the three species (Table A.2) include chloroplast metabolic genes such as carbonic anhydrase 1, glucose-6-phosphate dehydrogenase, and a cell wall metabolic gene, xyloglucan endotransglycosylase.

In addition to the conservation in cold-regulated genes identified by pOGs present in both microarrays, pOGs with Arabidopsis and potato members that were represented in only one of the two arrays were also identified. Among the around five thousand present only in the Arabidopsis array, many were cold-regulated in Arabidopsis (Fig 2.5b). Therefore, from the total 1,145 pOGs cold-regulated in At that have putative potato orthologs, 65% (478 pOGs up and 268 pOGs down) cannot be compared to *Solanum* species, because they are not present on the potato array. Given this, the number of genes that are cold-regulated in the two *Solanum* species could be larger and therefore the conservation with Arabidopsis could be underestimated in these experiments.

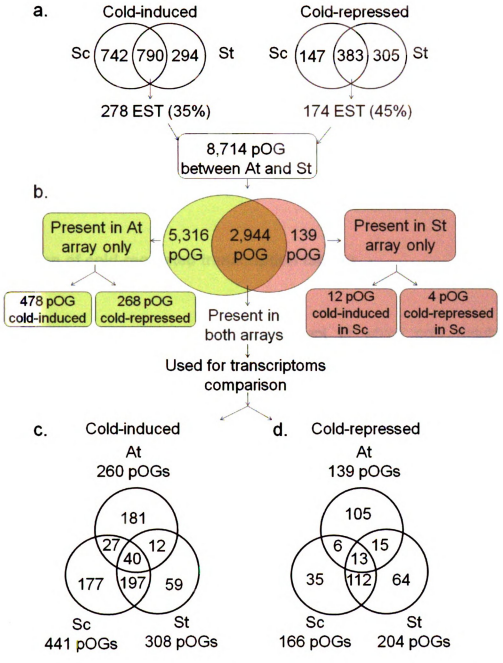

Fig 2.5: Comparison of cold transcriptomes of Arabidopsis (At), *S. commersonii* (Sc) and *S. tuberosum* (St). (a) Cold-induced and cold-repressed ESTs in Sc and St, showing the percentage that have Arabidopsis putative orthologous genes. (b) Putative orthologous groups (pOG) distribution on the Arabidopsis ATH1 Chip and potato cDNA array. Venn diagram shows pOGs with at least one Arabidopsis gene on the ATH1 chip (green), at least one potato clone on the potato cDNA array (brown) or at least one gene from each species in both arrays. Among the pOGs that are present only in one of the two arrays, the number of cold-induced or cold-repressed pOGs in the corresponding species is shown. From the pOGs present in both arrays, overlaps of (c) cold-induced or (d) cold-repressed pOGs are shown.

Together these results suggest that a large percentage (around 60%) of the cold-regulated ESTs in the two *Solanum* species do not have Arabidopsis orthologs. From the ones that do have, a large percentage (around 90%) is not cold regulated in Arabidopsis. This suggests differences in the evolution of low temperature transcriptomes between the two *Solanum* species and Arabidopsis.

Conservation of cold-regulated transcription factors

A major goal is to determine the extent to which cold regulatory pathways are conserved in plants. It was hypothesized that differences in freezing tolerance between Sc and St could be explained by differences in the cold-induction of transcription factors. In the previous section, 27 conserved orthologous groups (pOGs) were identified as cold-induced in the two freezing tolerant species Arabidopsis and Sc, but not in the freezing sensitive St (Table A3). Among them, only two were classified as transcription factors: cycling DOF factor 1 and 3 (*CDF1*, *CDF3*, both in the same pOG); and Short Hypocotyl 2 (*SHY2*) also called IAA3. Additionally, two genes were classified as regulators of transcription: BTB AND TAZ domain protein 4 (*BT4*), and a gene involved in RNA modification, CCR4 Associated Factor 1a (*CAF1a*). The array results showed that these genes were not cold-induced in St. In order to confirm this result, the expression of two of them was tested by quantitative real time PCR (qRT-PCR) (Fig 2.6). The *CDF3*-like and *CAF1a*-like genes are cold-induced in

both St and Sc, with similar kinetics of expression in both species. Therefore these genes are false negatives in the microarray for St. These results expand the list of conserved cold-induced TFs in the three species.

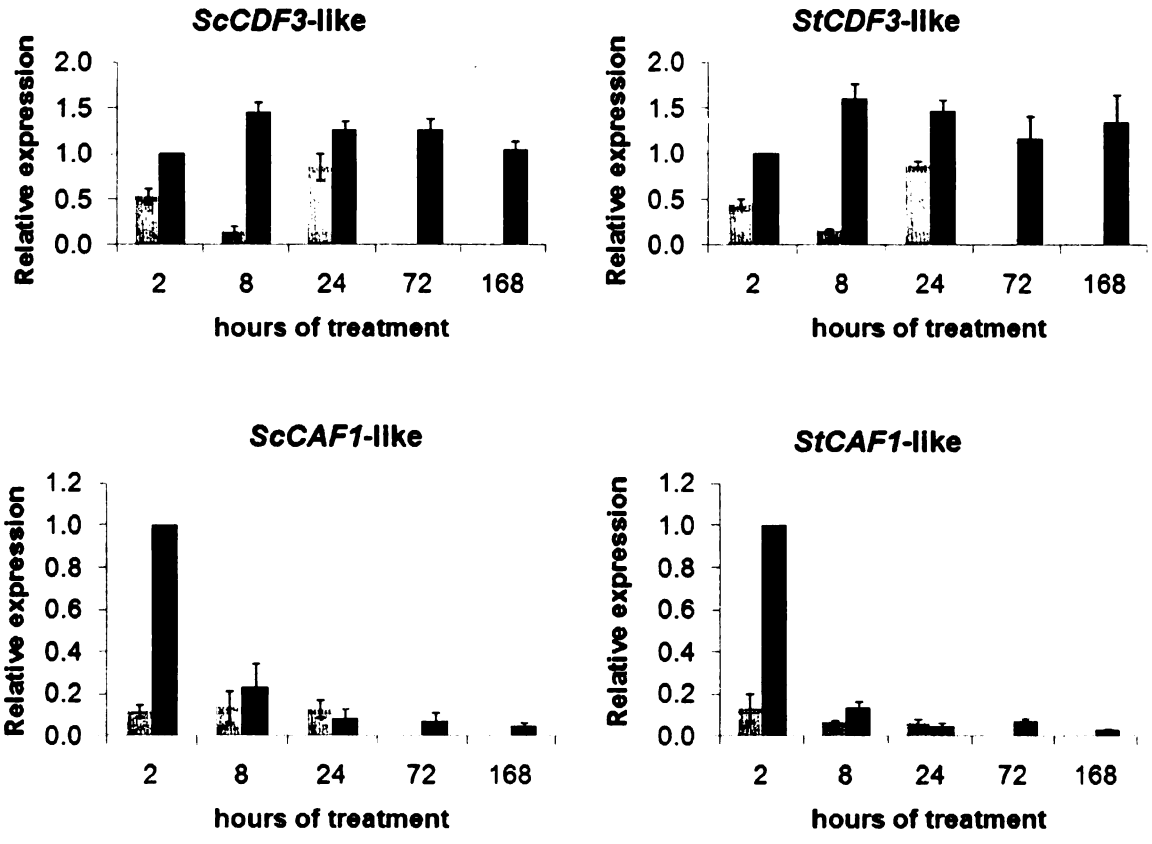

Fig 2.6: Transcript accumulation of *CAF1a-like* and *CDF3-like* in *S. commersonii* (Sc) and *S. tuberosum* (St). Sc and St wild type plants were grown for 3 weeks under a 16:8h L:D photoperiod. Eight hours after dawn, plants were either transferred to 2°C (black) or kept at 25°C (grey). Tissue was collected at the different times shown. qRT-PCR analysis was used to determine the transcript levels of Sc and St genes. Average values of three different experiments are shown. Relative expression levels of each transcript were normalized using the potato 60S gene (clone STMCK67) as an internal reference. Relative expression of the 2h cold sample was set to 1. Error bars indicate SE. Cold samples were significantly different from warm samples (ANOVA, $p<0.0001$, $n=3$).

In Arabidopsis, there are a number of transcription factors that are quickly induced in response to low temperature in addition to CBF1-3. The potato array does not represent the whole genome, nor the whole set of PUTs known to date, and does not include orthologs of known rapidly cold-induced transcription factors including Myb73, CZF1, ZAT10, ZAT12, and RAV1 (20). Based on the pOG list, PUT sequences of the putative potato orthologous genes were selected, and primers were designed for qRT-PCR based on those St sequences. The results are shown in Figure 2.7. The expression levels for all gene transcripts were compared between Sc and St, given that the same primers were used for both species. If some differences in hybridization efficiency of the primers were to happen, the primers could have had less homology to the Sc genes (the Sc genes are unknown) and that could result in less hybridization efficiency, but in almost all the cases the Sc gene transcript was higher than the St, suggesting that primers hybridized to Sc transcripts as well as to St transcripts. In the pOG list, only one potato PUT was identified as a putative ortholog of *AtMyb73* (PUT_69025). The *ScMyb73*-like transcript accumulates in the cold; the cold samples are significantly different from the warm sample (ANOVA, $p=0.005$, $n = 3$). However, the *StMyb73*-like transcript accumulation by cold was not statistically significant (ANOVA, $p = 0.2$, $n = 3$) (Fig 2.7a). Additionally, the Sc transcript accumulates to a higher level than the St transcript. The *ScMyb73*-like transcript accumulation kinetics is similar to the one previously observed in Arabidopsis (20).

Three *CZF1* putative potato orthologs were identified (PUT12601, PUT25814, and PUT25815). Given the high identity between PUT25814 and 25815, primers that target both sequences were tested by qRT-PCR (Fig 2.7c). The transcript accumulation of these genes in response to cold was significant in both Sc and St (ANOVA, $p<0.0001$, $n = 3$), and the transcript levels were very similar in both species. The Sc and St transcripts had kinetics very similar to those observed for the Arabidopsis *CZF1* gene (20). The other *CZF1*-like gene was PUT12601. The transcript accumulation of PUT12601 by cold was significant in St (ANOVA, $p = 0.02$, $n = 3$), but not in Sc (ANOVA, $p = 0.3$, $n = 3$) (Fig 2.7d). The St transcript accumulates to a higher level than the Sc transcript. The kinetics of PUT12601, however, are different to those previously observed for *At CZF1*.

Four potato EST contigs were identified as putative orthologs of *AtZAT10*: PUT22120, PUT22122, PUT32825, and PUT45213. Two of them (PUT32825 and PUT45213) were highly identical, so primers that target both sequences were used in qRT-PCR (Fig 2.7e). Both Sc and St transcript were highly cold induced at 2h reaching similar levels in both species. Cold samples were significantly different from the warm sample in both Sc and St (ANOVA, $p< 0.0001$, $n = 3$). Their kinetics of transcript accumulation were similar to those observed for Arabidopsis *ZAT10* (20). The other two PUTs (PUT22122 and PUT 22120) were highly identical, so primers that target both sequences were used (Fig 2.7f). These genes are significantly induced by cold in Sc (ANOVA,

p= 0.005, n = 3) but not in St (ANOVA, p = 0.3, n = 3). These transcripts have different kinetics to the *AtZAT10*.

Only one EST contig was identified as a putative ortholog of *AtZAT12*: PUT68089. The expression of this gene, even though very low, was detected in St (Fig 2.7b), but it could not be detected in Sc. The gene was significantly induced by cold (ANOVA, p=0.02, n = 3). The expression kinetics of St transcript was similar to the one observed for *AtZAT12*, but in the latter case the expression goes down at 24h (Vogel et al, 2005).

Two EST contigs were identified as putative potato orthologs of *AtRAV1*: PUT3404 and PUT3405. Given their high identity, primers that target both sequences were designed (Fig 2.7h). Transcripts for these genes accumulate in response to cold in both Sc and St; in both cases cold samples were significantly different than the warm sample (ANOVA, p<0.0001 for Sc and p = 0.05 for St, n = 3). However, the Sc transcript reached higher levels compared to the St transcript. Their kinetic pattern is similar to that of *AtRAV1* (Vogel et al., 2005).

The transcript accumulation of *ScCBF1* and *StCBF1* is also shown (Fig 2.7g). The *CBF* genes (5 in St and 4 in Sc) are highly similar, therefore primers that primarily, but not exclusively, amplify *ScCBF1 and StCBF1* were used. Both genes are highly cold-induced at 2h, however *ScCBF1* reached higher levels than *StCBF1*.

It can be concluded that most of the putative potato orthologs to the Arabidopsis early cold-induced transcription factors, are cold-induced too in

both *Solanum* species. Only *Myb73*-like transcript was significantly cold induced in Sc but not in St. However, it appears that some of these gene transcripts reach higher levels of accumulation in Sc than in St, suggesting a quantitative rather than qualitative difference in their cold response between Sc and St. Together these results expand the list of conserved cold-induced transcription factors in Sc, St and Arabidopsis.

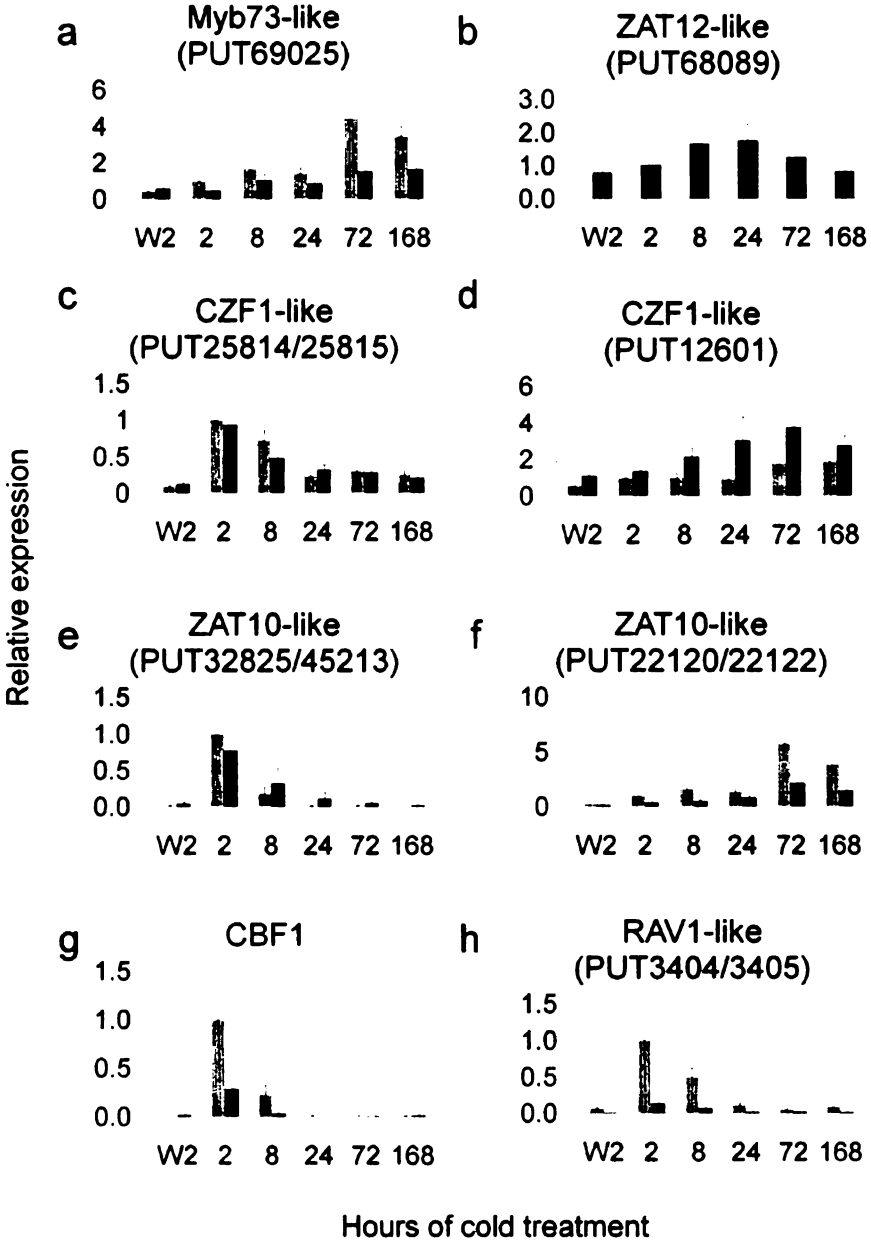

Fig 2.7: Transcript accumulation in response to low temperature of *Myb73*-like, *ZAT10*-like, *CZF1*-like, *ZAT12*-like, *RAV1*-like, and *CBF1* *Solanum* genes. Sc and St wild type plants were grown for 3 weeks under 16:8h L:D photoperiod. Eight hours after dawn, plants were either transferred to 2°C for 2, 8, 24, 72 and 168h or kept at 25°C for the same periods of time. Since, for each gene, all warm samples reached same levels, only samples kept at 25°C for 2h are shown as control (W2). qRT-PCR analysis was performed to determine the transcript levels of Sc and St genes. Average values of three different experiments are shown. Relative expression level of each transcript was normalized using the potato 60S gene (clone STMCK67) as an internal reference. Relative expression of the Sc 2h cold sample was set to 1. Error bars indicate SE.

S. commersonii and potato CBF regulons

CBF overexpression is sufficient to increase freezing tolerance in Arabidopsis (26,28-30). In order to explore if differences in the CBF regulon of Sc and St could account for their differences in freezing tolerance, the next objective was to determine how much of the cold transcriptomes are regulated by CBF in these species, and how do they compare to each other.

To identify the CBF regulons of these two *Solanum* species microarray hybridizations were conducted, using Sc and St transgenic lines expressing *AtCBF3* under the constitutive CaMV35S promoter (obtained from Tony Chen's laboratory). These lines were first tested for *AtCBF3* transgene expression (Fig 2.8). The Sc transgenic lines expressed higher levels of *AtCBF3* transgene than the St lines did. RNA from *35S::AtCBF3* Sc and St transgenic lines was hybridized to the potato cDNA array (see methods). Sc and St WT plants were used as reference samples in each case.

Figure 2.9a shows expression profiles as average log ratio of the three *35S::AtCBF3* Sc lines (Sc ox 3) and two *35S::AtCBF3* St lines (St ox 2). The data indicated that *AtCBF3* overexpression produced many changes in gene expression in both Sc and St, and only a small number of those changes (highly up or down regulated) are similar in both species. There were some clusters where there was induction by CBF overexpression in both Sc and St and also some clusters where there was repression by CBF overexpression in both Sc and St. However there were some clusters where there was either induction or

repression by CBF overexpression only in one of the two species. Interestingly, there were clusters where there was induction in one species and repression in the other one.

The next step was to determine which ESTs were not only regulated by *AtCBF3* overexpression but also by cold. For that, the expression profiles from Sc and St WT cold treated plants at different time points were compared to that of *35S::AtCBF3* Sc and St lines (Fig 2.9b). Many of the up or down regulated ESTs in transgenic lines were also up or down regulated by cold at 168h to similar levels. This result suggests that CBF is responsible mainly for the induction or repression of ESTs late in the cold.

There was a cluster (F) where the majority of the genes were induced by CBF overexpression in both Sc and St (Sc and St lines) and also induced by cold at 24h and 168h in both Sc and St. Another cluster of ESTs (H) from Figure 2.9b was identified as having the majority of its ESTs repressed in Sc and St lines and also repressed by cold at 24h and 168h.

There were a few clusters where there was high activation by CBF overexpression in both Sc and St, but not in the cold treated WT samples (Fig 2.9b). This could be explained by the fact that CBF overexpression produces a stunted phenotype in Arabidopsis (28); therefore it is possible that CBF is altering expression of some developmentally regulated genes that are not necessarily affected by cold.

The cluster with the highest number of ESTs up regulated by cold at 2h in Sc and St and 24h in St (Cluster G) was not up regulated by CBF

overexpression (or just induced to a low level) either in Sc or in St. It is reasonable to assume that if CBF itself reaches its peak of expression at around 2h of low temperature (3,38), the ESTs being induced by cold at 2h will not be regulated by CBF and, more likely, CBF target ESTs will be in the second wave of induction. These data suggest that other TFs are responsible for the early cold-induction of ESTs in Sc and St.

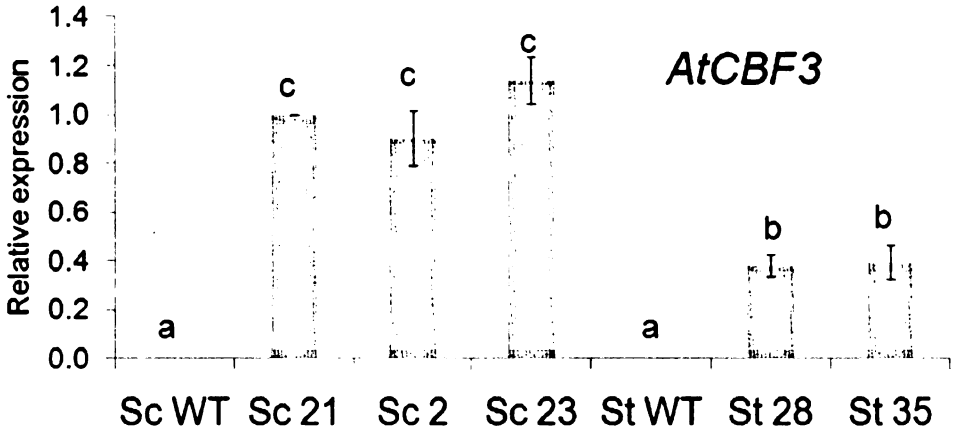

Fig 2.8: *AtCBF3* transgene accumulation in St and Sc 35S::*AtCBF3* transgenic lines. The *AtCBF3* transgene accumulation was tested in St and Sc 35S::*AtCBF3* transgenic lines in warm conditions. qRT-PCR was performed using 100ng of RNA for each sample. Relative expression was calculated using the potato 60S gene (clone STMCK67) as an internal reference. ScWT, *S. commersonii* wild type; StWT, *S. tuberosum* wild type. Relative expression of Sc 21 was set to 1. The letters a, b and c indicate statistically significant differences (ANOVA, $p<0.05$, n=2).

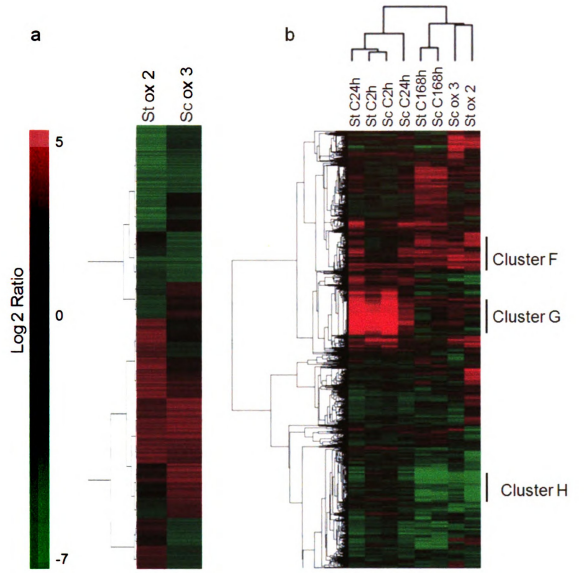

Fig 2.9: Hierarchical clustering and expression profiles of Sc and St EST clones at 2, 24 and 168h of cold treatment (2°C), in combination with *35S::AtCBF3* Sc lines and *35S::AtCBF3* St lines. (a) *35S::AtCBF3* Sc lines (Sc ox 3) and *35S::AtCBF3* St lines (St ox 2). (b) Same as (a) plus WT cold treated. Data shown as average log ratio (lines/wild type) of 3 Sc lines and 2 St lines, and 3 biological replicates for each time of cold treatment. The figure shows all spots on the array (including bad flagged spots) prior to statistical selection. Sc C2h, Sc C24h, and Sc C168h correspond to Sc WT cold treated for 2h, 24h and 168h respectively. St C2h, St C24h, and St C168h correspond to St WT cold treated for 2h, 24h and 168h respectively.

To identify ESTs that were likely to be part of the CBF regulon in Sc and St the data were selected using the following criteria. The Sc CBF regulon was defined by ESTs being DE in the three *35S::AtCBF3* Sc transgenic lines compared to WT and also being DE by cold in the three biological replicates at any time point by 2FC and $p<0.05$ (Limma package, (72)). Similarly, the St CBF regulon was defined by ESTs being DE in the two *35S::AtCBF3* St transgenic lines compared to WT and also being DE by cold in the three biological replicates at any time point. The criteria used were 2FC and $p<0.05$; however, since we had only two St transgenic lines, these p-values were generated by Limma using the average of these two transgenic lines as a third replicate.

Fifteen and thirty percent of the cold regulated ESTs in Sc and St, respectively, can be assigned to their CBF regulons under the criteria used ($p<0.05$, 2FC). The CBF regulon lists can be accessed at http://www.prl.msu.edu/Facultypages/NSF_MFT_Site/data.html. A total of 160 ESTs in Sc and 170 ESTs in St were members of the CBF regulon of induced ESTs (Fig 2.10). The overlap between the Sc and St CBF regulons of induced EST is not very large (around 30%). A total of 137 ESTs in Sc and 364 ESTs in St were members of the CBF regulon of down regulated ESTs (Fig 2.10). There is a large number of ESTs that are members of the St CBF regulon of repressed ESTs that apparently are not members of the Sc CBF regulon.

Together these data indicate that among the most likely CBF regulon members defined by the 2FC and $p<0.05$ criterion, the overlap between Sc and

St ESTs is not very big. These differences between Sc and St CBF regulons could account for the differences in their freezing tolerance.

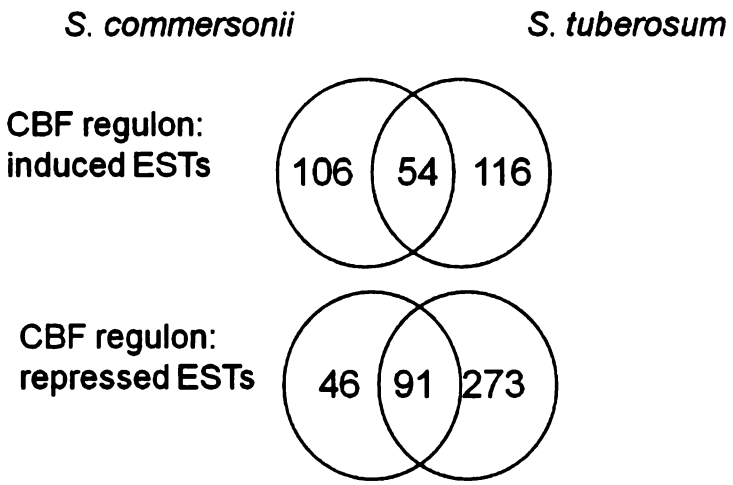

Fig 2.10: ***S. commersonii* and *S. tuberosum* CBF regulons.** CBF regulated genes were selected as being DE in *35S::AtCBF3* transgenic lines compared to WT and also DE in 3 WT cold treated biological replicates (all times cold considered) with $p<0.05$ and 2FC cut off.

Comparison between Sc, St and Arabidopsis CBF regulons

Given that CBF pathway plays a predominant role in freezing tolerance in Arabidopsis, it is important to know to which extent the CBF regulons are conserved in different plant species. To address this issue, the Sc and St CBF regulons were compared to that of Arabidopsis. For this analysis, the Arabidopsis-potato pOG list was used.

From the 54 ESTs that were identified as CBF regulon ESTs that were up regulated in both Sc and St, 28 ESTs (52%) were represented in the list of 8,714 putative orthologous groups (pOGs) and correspond to 22 pOGs (Fig 2.11a). From the 91 ESTs that were identified as down regulated CBF regulon ESTs in both Sc and St, 38 ESTs (42%) were represented in the list of pOGs between Arabidopsis and St and correspond to 31 pOGs (Fig 2.11a). These results indicate that a large percentage of the CBF regulon ESTs that are conserved in Sc and St do not have Arabidopsis orthologs based on the criteria used in this study. The CBF regulons of Sc and St were compared to that of Arabidopsis. The Arabidopsis CBF regulon used in this comparative study corresponds to the group of genes that were DE by 2FC cut off ($p<0.05$) in *35S::CBF2* Arabidopsis transgenic lines (20) and also DE in the Arabidopsis cold data sets described above.

Figure 2.11c shows the overlap of CBF regulon up regulated genes between the three species. Seven pOGs were identified that have at least one CBF regulon up regulated member from each species. The Arabidopsis

members of these seven pOGs are: early light-induced protein 2 (*ELIP2*); *LEA14*; *ERD10*; *COR47*; *ADOF1*; responsive to desiccation 26 (*RD26*); sucrose synthase 1 (*SUS1*); and invertase/pectin methylesterase inhibitor family protein. Among the CBF regulon activated pOGs, only 15% of the pOGs with CBF regulon genes in Sc and 14% of the pOGs with St CBF regulon genes also have Arabidopsis CBF regulon members. Given that the Arabidopsis array represents almost its entire genome, these *Solanum* CBF regulon members that are not members of Arabidopsis CBF regulon represent a real difference between these CBF regulons, suggesting differences in the evolution of CBF upregulated genes.

Figure 2.11d shows the overlap of CBF regulon down regulated genes between the three species. Two pOGs were identified that have at least one CBF regulon down regulated member from each species. The Arabidopsis members of these 2 pOGs are a phosphoethanolamine N-methyltransferase; and two beta galactosidases (*BGAL1* and *BGAL4*). Among the CBF regulon repressed pOGs, only 4% and 2% of the pOGs with CBF regulon members from Sc and St respectively also have Arabidopsis CBF regulon members.

With these results it was concluded that there is more conservation between the CBF regulons of the two *Solanum* species than between the two freezing tolerant species (Sc and Arabidopsis).

Despite these differences, the results reported here are minimal estimates of conservation, given that the potato array used does not represent the complete potato genome; therefore the number of CBF regulon genes

conserved between these species could be an underestimate. As was determined for the cold transcriptomes, CBF-regulon pOGs that are present in only one of the two arrays (either Arabidopsis or potato) were also identified, these therefore escape this conservation analysis (Fig 2.11b). Of the approximately 5,316 pOGs present on the Arabidopsis array, but not in the potato array, many had members of the Arabidopsis CBF regulon. There were 69 pOGs with CBF regulon up regulated members and 15 pOGs with CBF regulon down regulated members (58% of the total pOGs with Arabidopsis CBF regulon members). These pOGs cannot be compared to those of the *Solanum* species because the putative St orthologs are not represented in the potato array. Given this, the number of CBF regulon genes that are conserved between the two *Solanum* species and Arabidopsis could be largely underestimated in these experiments.

From these results it was concluded that the overlap identified between CBF regulons from Arabidopsis and the two *Solanum* species is small. This suggests that CBF is regulating different genes in Arabidopsis and in the two *Solanum* species, suggesting a significant difference in the evolution of these CBF regulons.

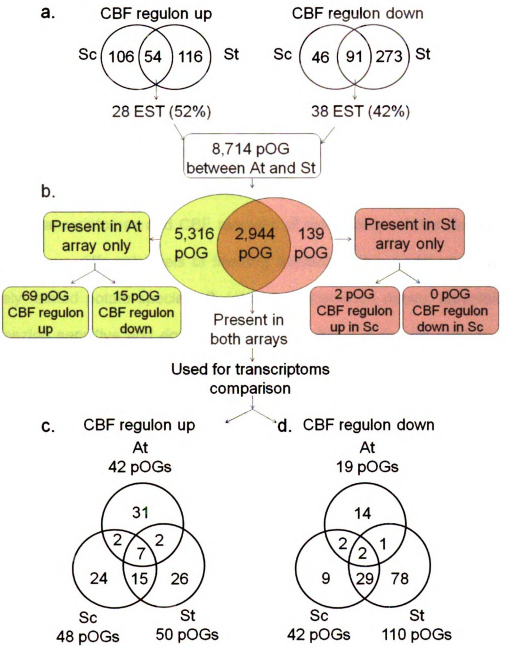

Fig 2.11: Comparison of CBF regulons among *S. commersonii* (Sc), *S. tuberosum* (St) and Arabidopsis (At) based on putative orthologous groups (pOGs). (a) CBF regulon up and CBF regulon down ESTs in Sc and St, showing the percentage that have Arabidopsis putative orthologous genes. (b) Putative orthologous groups (pOGs) distribution on the Arabidopsis ATH1 Chip and potato cDNA array. The venn diagram shows pOGs with at least one Arabidopsis gene on the ATH1 chip (green), at least one potato clone on the potato cDNA array (brown) or at least one gene from each species in both arrays. Among the pOGs that are present only in one of the two arrays, the number of CBF regulon induced or CBF regulon repressed pOGs in the corresponding species is shown. (c) Overlaps of CBF regulon induced or (d) CBF regulon repressed pOGs from pOGs present in both arrays.

DISCUSSION

To date, there is no explanation for the differences in freezing tolerance between Sc and St. It is known that the CBF pathway is the only known pathway to play a predominant role in freezing tolerance in Arabidopsis. Therefore, the goal of this study was to identify and compare the low temperature transcriptomes and CBF regulons of two closely related *Solanum* species that have different levels of freezing tolerance. The model used was two closely related potato species, *S. tuberosum* (common potato, tetraploid) that is freezing sensitive and does not cold acclimate, and its wild relative *S. commersonii* (diploid) that is freezing tolerant and can cold acclimate. The low temperature transcriptomes of these two species were identified. In both, hundreds of ESTs were cold-induced and cold-repressed. A global view of the cold regulated ESTs in the two *Solanum* species showed that the changes in gene expression are very similar between Sc and St (Fig 2.1). The results suggest that these two *Solanum* species share between 50 to 70% of their cold-regulated ESTs. The ESTs regulated in both species might be involved in tolerance to chilling temperatures or may be part of the general response to stress damage.

From the transcriptome analysis done in this study, there is nothing immediately obvious to conclude why Sc and St have differences in their freezing tolerance. Presumably the specific differences in the kinetics of gene cold induction or quantitative differences in the low temperature transcriptomes

of Sc and St could account for their differences in freezing tolerance, but there is not enough evidence at this stage. There is a group of transcription factors that are candidates to configure the low temperature transcriptome in Arabidopsis. The potato orthologs of these genes were not represented in the potato array, therefore they were tested for cold induction in Sc and St by qRT-PCR. Results revealed that most of these genes were cold induced in both Sc and St, moreover with similar kinetics than the Arabidopsis orthologs (Fig 2.7). However, in some cases the Sc genes were induced at a higher level compared to the St genes. The role of these transcription factors in freezing tolerance it is not known, then whether these quantitative differences are responsible for the differences in freezing tolerance between Sc and St is yet to be discovered. In the future it would be interesting to study the role of these conserved transcription factors in freezing tolerance.

It is also possible that the freezing tolerance difference between Sc and St comes from differences at a post transcriptional level, possibly due to differences in protein levels, protein modifications, or metabolite levels.

Another group (76) has previously studied other potato species under cold stress: *S. phureja* CHS (diploid), *S. tuberosum* cv. Desiree (tetraploid) and *S. tuberosum* PS3 (dihaploid). Electrolyte leakage experiments demonstrated that *S. phureja* CHS was the most cold tolerant at a constitutive level (LT50 of -9.6 under non acclimating conditions and -11 after 3 weeks of cold acclimation). Transcriptome analysis at one and three days of cold treatment revealed significant changes in expression of genes related to amino acid metabolism

and carbohydrate metabolism in the three potato species. There are no immediately obvious differences between these three species transcriptomes. However, they found that *S. phureja* CHS had higher constitutive levels and higher accumulation upon cold exposure of protective sugars such as sucrose, galactose, trehalose, galactinol, raffinose, and glucose.

Another study (77) also looked at *S. tuberosum* transcriptomes under cold stress. This study found that cold stress resulted in a large number of DE ESTs (2,318, $p<0.01$) (77). Using the same cut off criteria they used, 1,128 ESTs (50%) can be identified as cold regulated in St in this study. The differences observed between Rensink et al and this study could be due to differences in the growth and experimental conditions. Rensink et al grew plantlets on magenta boxes, and their cold treatments were done by transferring the magenta boxes to ice just after dawn. In our study we transferred the plants to cold 8h after dawn. It is suspected that some potato genes might be under circadian regulation, as is the case with Arabidopsis CBFs (40) and tomato CBF1 (43); therefore the results could be different depending on the time in the photoperiod when the plants were transferred to cold. They collected leaf and root samples at different time points, and they also mentioned that their cold treated plants collapsed after 3h of cold treatment, and later they recover. This could have had an effect on gene expression.

Given that whole genome duplication and gene duplication and retention following duplication has been very extensive in plants (78), orthologous genes are not only in a one-to-one relationship, but rather organized in groups of

orthology. In this study a list of groups of putative orthologous genes between Arabidopsis and potato were used to compare their low temperature transcriptomes. First, many of the ESTs that were cold-regulated in both Sc and St did not have Arabidopsis orthologous genes according to the criteria used (Fig 2.5a). This could indicate an important difference between the cold responses of the two *Solanum* species and Arabidopsis. Thirteen percent (1,466) of the ESTs present in the potato array have not been assigned to a PUT, and therefore were not compared to Arabidopsis proteins (Fig 2.4). Given that this is a small percentage, it is not believed that having more sequence information for these genes would affect largely the results.

Second, the cold regulated list of genes in the two *Solanum* species could be underestimated, given that many Arabidopsis genes that are cold regulated, have potato orthologs that are not present in the potato array (Fig 2.5b). Therefore, the overlap between the two *Solanum* species and Arabidopsis could be larger (Fig2.5c and d). This is supported by the qRT-PCR results of known early cold induced transcription factors in Arabidopsis whose potato orthologs were not represented in the potato array. The expression of these potato orthologs was also induced in both Sc and St (Fig 2.7). However, given that the number of cold regulated genes in Arabidopsis likely reflects reality (the array represent almost its entire genome), then the number of pOGs that are only cold-regulated in the two *Solanum* species, but not in Arabidopsis (Fig 2.5c and d) could be the same or even larger. Therefore this result indicates another level of difference between *Solanum* and Arabidopsis low

temperature transcriptomes. This suggests significant differences in the evolution of these low temperature responses, a result that is not surprising considering the evolutionary distance between Arabidopsis and potato (around 125 Mya) (79).

To date, there is no large scale comparison of low temperature transcriptomes between distantly related species. Only one study has been published comparing stress regulated genes (including cold stress) of rice and Arabidopsis (80). By a combination of rice cDNA microarray (1,700 rice cDNAs) and northern analysis, they identified 73 genes as stress inducible in rice, 36 of which were induced by cold. Fifty percent of these stress inducible rice genes were identified as having similar functions or gene names with already reported Arabidopsis stress inducible genes. Therefore this study is the first large scale comparison of low temperature transcriptomes between distant related species such as potato and Arabidopsis.

The CBF regulons of Sc and St were identified in this study. With the criteria used (2FC and $p<0.05$), the overlap between the Sc and St CBF regulons is not very big (Fig 2.10). This suggests that CBF turns on different genes in Sc and St. When these CBF regulons are compared to that of Arabidopsis, there is more conservation between the two *Solanum* CBF regulons than to that of Arabidopsis. Many genes that are members of CBF regulon in the two *Solanum* species are not in Arabidopsis. Considering that the Arabidopsis array represents almost its entire genome, these genes likely represent a real difference between these CBF regulons. This result suggests

that there has been divergence in the evolution of CBF regulons. It could be that CBF is binding to different sites in these different species or there could have been loss of cis-acting DNA regulatory sequences in some genes of one species, therefore CBF no longer binds to the same targets.

Examples of cis-acting regulatory mutations that cause evolutionary changes have been observed in different species (81). In yeast for example, it has been shown that transcription factor binding sites are evolving at a fast rate and could be the major cause of divergence between related species. The transcription factor binding sites for the two pseudohyphal regulators Ste12 and Tec1 were studied in three species of *Saccharomyces* and it was found that these binding sites have diverged across species. Most target genes were bound in only one or two of the three *Saccharomyces* species studied. This group identified many examples where a species-specific loss of binding and/or loss of cis-acting sequence had occurred (82).

A previous study has shown conservation between the *Populus* and Arabidopsis CBF regulon (50). They searched the Poplar cDNA array (POP1 13K) for homologs of previously identified CBF responsive genes in Arabidopsis. They found that 12 (32%) of the 38 CBF3 regulon up-regulated members in Arabidopsis (83) had orthologous in the poplar array, and of those 12, 7 were up regulated in their *AtCBF1* overexpressing poplar lines. They do not mention if those 7 genes were also cold regulated. They identified 22 Populus genes as being up regulated by cold and by *AtCBF1* overexpression. Given that only 7 genes that had Arabidopsis orthologs were also regulated by

AtCBF1 overexpression in Populus, the majority of these 22 Populus CBF regulon genes either did not have Arabidopsis orthologs or were not members of the Arabidopsis CBF3 regulon, indicating important differences between Arabidopsis and Populus CBF regulons.

In their CBF regulon comparison, they are using a very limited Arabidopsis CBF regulon list of genes. The Arabidopsis CBF3 regulon from Maruyama et al (2004) only surveyed 8,000 genes, and found 38 genes cold up regulated and CBF3-upregulated. In the present study, the Arabidopsis CBF regulon used was much larger set of genes (169 up- and 58 down-regulated genes). The CBF regulon consisted of genes being regulated by CBF2 overexpression (20) and by cold in different experiments (Atgeneexpress) using the ATH1 Affymetrix array that represent around 22K genes (almost its entire genome). Benedict, Skinner et al. 2006 also mention that their *Populus* CBF regulon had greater disagreement with the AtCBF2 regulon identified in Vogel, Zarka et al. 2005. It has been reported that overexpression of AtCBF1, AtCBF2 or AtCBF3 regulates very similar sets of genes in Arabidopsis suggesting that there are no CBF-specific regulon differences (26). Therefore, the differences between *Populus* and the Arabidopsis CBF2 regulon (20) suggest differences between species. All this suggests that the *Populus* and Arabidopsis CBF regulons are not as strongly conserved as they claimed.

Preliminary studies suggest that overexpression of *AtCBF3* in Sc did not increased freezing tolerance of Sc lines in non acclimating conditions. However, after a period of cold acclimation, the freezing tolerance of *35S::AtCBF3* Sc

transgenic lines increases 4°C compared to WT cold acclimated plants (unpublished data). This suggests that At*CBF3* alone is not sufficient to impart freezing tolerance in Sc, but it may act in concert with other factors that are present in the cold. Overexpression of *AtCBF3* in St has been reported to have a small increase (about 2°C) in freezing tolerance under non-cold acclimating conditions (59), suggesting that CBF could be sufficient to impart freezing tolerance in St. These experiments need to be repeated in parallel to have a better understanding of the effect of *AtCBF3* overexpression in Sc and St. Pino, et al. 2008 reported that *AtCBF1* overexpression in Sc increases freezing tolerance in non acclimating conditions by about 4°C, suggesting that *AtCBF1* is sufficient to increase freezing tolerance in Sc; and overexpression of *AtCBF1* in St had a small increase in freezing tolerance (about 2°C) in non acclimating conditions. In the future it would be interesting to test by microarrays which genes are differentially expressed by *AtCBF1* overexpression. It would be also interesting to know if endogenous overexpression of *ScCBF1* and *StCBF1* in Sc and St respectively, had any effect on freezing tolerance.

In some species there is genetic evidence for the role of CBF in freezing tolerance. In wheat, a cluster of eleven *CBF* genes have been mapped to the *Frost resistance-2* (*Fr-A^{m}2*) locus (84). This locus was mapped at the peak of two overlapping quantitative trait loci (QTL), one for frost survival and the other for differential expression of the cold regulated gene *COR14b*. Similarly, in barley, a cluster of six *HvCBF*s genes mapped to the Fr-H2 cold tolerance QTL (85). These are evidence that support the important role of CBFs in freezing

tolerance. However, to date, there is no genetic evidence that proves CBFs to be in a loci associated with freezing tolerance in *Solanum* species. There is genetic evidence that suggests that there is independent genetic control of non-acclimated freezing tolerance (NAFT) and cold acclimation capacity (CAC) in *Solanum* species (86). Two wild diploid *Solanum* species, *S. commersonii* (freezing tolerant and able to cold acclimate) and *S. cardiophyllum* (freezing sensitive and unable to cold acclimate) were crossed. By analysis of the two segregating backcross populations, it was observed that the NAFT and CAC were not correlated in any of the two backcross segregating populations. Two QTLs for NAFT and two for CAC have been identified. The QTLs for NAFT and CAC were found at separate genomic regions (87). The genes in these QTL responsible for these two traits have not been identified yet. Future experiments will be crucial to understand the role of CBF in freezing tolerance in *Solanum* species.

As this thesis was written, the potato whole genome became available (http://www.potatogenome.net/index.php/Main_Page). With this, many more future studies can be done to comprehend better the differences between the Sc and St cold transcriptomes. For instance, a more rigorous analysis of the complete cold transcriptomes from Sc and St can be done using deep sequencing techniques such as Illumina or 454 sequencing. Additionally, the promoters of the cold regulated ESTs identified in this study can be searched for enriched motifs using different bioinformatic tools. Once identified, these potential motifs can be tested in vivo for cold regulation. Cold-responsive motifs

from Sc and St could be fused to a reporter gene and transformed into Arabidopsis to test the conservation of their cold-response.

MATERIALS AND METHODS

Plant growth, cold treatments and RNA extraction

Solanum commersonii and *Solanum tuberosum* cv. Umatilla wild type plants were maintained under greenhouse conditions (Pino et al. 2007). Wild type plants and transgenic lines used in experiments were transferred to a Percival model MB60B growth chamber (Percival Scientific, Inc., Perry, IA) under a 16h photoperiod, 350 μmol m^{-2} s^{-1} PAR at 25°C. Three biological replicates for each wild type species were grown for 3 weeks under these conditions (each biological replicate consisted on three plants). Eight hours after dawn, wild type plants were transferred to an environmentally controlled cold room maintained at 2°C, under a 16h photoperiod with 50 μmol m^{-2} s^{-1} light intensity and leaf tissue was harvested after 2, 8, 24, 72, and 168 h. Warm controls were maintained at 25°C under normal growth photoperiod and tissue was harvested at 2, 8, and 24h in the light for use as reference control samples. In the case of the 72 and 168h cold samples the 24h warm control was used as reference since 72 and 168h warm plants were already flowering. The leaf tissue of transgenic lines was collected eight hours after dawn.

Total RNA was isolated from leaf tissue using RNeasy Plant Mini Kits (Qiagen, Valencia, CA). For real time PCR experiments, samples were treated with RNAse-free DNAse (Qiagen, Valencia, CA) using the on-column DNAse digestion method provided by the manufacturer.

RNA labeling and hybridization of potato microarrays

cDNA microarray experiments were conducted using the 10K potato cDNA microarray (TIGR, http://www.jcvi.org/potato/sol_ma_microarrays.shtml). 20 μg of RNA were labeled by the indirect labeling aminoallyl method. The slides were hybridized using the indirectly labeled aminoallyl probes hybridization method (http://www.jcvi.org/potato/sol_ma_protocols.shtml). To avoid bias due to dye-related differences, labeling dyes for each sample pair (cold/warm or transgenic line/wild type) were swapped in one of the three independent hybridizations (three biological replicates for cold treatments and two or three transgenic lines for the CBF regulon experiment).

Data processing and analysis

The TIFF images were quantified using Genepix 3.0 (Axon Instruments, Union City, CA). The software automatically flags spots that cannot be found in one of the channels. Spots with aberrant shapes were checked manually and flagged as bad. Spots with lower signal intensity than the background (spots with >=55% of the pixels with lower signal intensities than background) were also flagged as bad. All these "bad" flagged clones were excluded from further analysis.

The data were normalized using the print tip loess method in the Limma package (Smyth 2004). A list of differentially expressed (DE) clones were

ranked based on their p value, using a false discovery rate to correct for multiple testing. The results were selected by $p<0.05$ or else as indicated in the text. Average fold change (FC) was calculated for the two duplicates of each clone on the array. In cases where one of the duplicates didn't pass the p value cut off or the duplicate was flagged as a "bad quality spot", the value of the other duplicate was used.

Hierarchical clustering was performed using Cluster software (88), using normalized log ratios. Gene Onthologies (GO) for St EST clones were obtained from TIGR.

Quantitative real time PCR (qRT-PCR)

RNA, using an amount that fell within the linear range for all genes tested (generally 100-250ng), was reverse transcribed using a reverse transcription system (Promega, Madison, WI) according to the manufacturer's instructions. The 20μL final reaction was diluted to 200μL. A 1 μL aliquot of each cDNA was used in a real time PCR (qRT-PCR) reaction, with the addition of 0.4 μM of each primer and Fast SYBR Green master mix (Applied Biosystems, Foster City, CA) to make a final reaction volume of 10μL. The qRT-PCR reactions were performed using a FAST 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The primers used are listed in Table 2.1.

Data were analyzed by ANOVA using SAS program 9.1 (SAS Institute Inc., Cary, NC) with mixed procedures; when appropriate, least significant difference was used for multiple comparisons.

TABLE 2.1: Primers used in real time PCR.

Name	Sequence	Target
CL_RT_15	GGCCTTGTATAATCCCTGATGAATAAG	At Ubiquitin 10
CL_RT_16	AAAGAGATAACAGGAACGGAAACATAGT	At Ubiquitin 10
MC_122	TGCCCATAAACCCTTTTTGT	St 60S (clone STMCK67)
MC_123	AACAATGGCGGCTAAGAAGA	St 60S (clone STMCK67)
MC_132	TGGGCTCATAATCTCGAATC	St CAF1a (clone STMDD59)
MC_133	GCTTGAAAACAACACCAGGAA	St CAF1a (clone STMDD59)
MC_201	GATCAGATCGAAACGACCTCGTA	St ZAT10 (PUT32825/45213)
MC_202	ATCTTCGGAAATAATTGGTTGTTGT	St ZAT10 (PUT32825/45213)
MC_203	TGCGCGTGACTTTGACCTAA	St ZAT10 (PUT22122/22120)
MC_204	AGTCAATGGTCAGATCCAATTGG	St ZAT10 (PUT22122/22120)
MC_205	GGTTCCGAGGTCGATCTGGTA	St Myb73 (PUT69025)
MC_206	AAGCACGTATGCTCCACTTGAG	St Myb73 (PUT69025)
MC_207	CCCACCACAATTATTCAAACGA	St ZAT12 (PUT68089)
MC_208	GAAGGTGCTAGTAGTGGTGAATTGG	St ZAT12 (PUT68089)
MC_212	GCTTGATGCTTCTGCTGATGTG	St CZF1 (PUT12601)
MC_213	AGATCAGGTCAACAGCTCGTTTC	St CZF1 (PUT12601)
MC_214	TCACCCCACCTGCATTACAG	St CZF1 (PUT25814/25815)
MC_215	CCCGAGCGTTTAGAGATGTCTT	St CZF1 (PUT25814/25815)
MC_216	TTAGTCTGGAAAATGACTTGTGATTCA	St CDF3 (clone STMCY67)
MC_217	GAGACGACCACCGGAAGTATCT	St CDF3 (clone STMCY67)
MC_323	GGTTTGGTTAGGCACATTTAACG	St RAV1 (PUT3404/3405)
MC_324	GGCCGCGACGTCGTAA	St RAV1 (PUT3404/3405)
SJG_80	TTCCGTCCGTACAGTGGAAT	At CBF3
SJG_81	AACTCCATAACGATACGTCGTC	At CBF3

Inference of putative orthologous groups (pOGs)

A list of pOGs between the putative unique transcripts (PUT) sequences assembled from Expressed Sequence Tags of St (PlantGDB, http://www.plantgdb.org/, version 157a) and Arabidopsis protein sequences was obtained from Shinhan Shiu's laboratory, Plant Biology Department, Michigan State University.

pOGs have been previously established using protein sequences from four plant species with complete genomes (*Arabidopsis thaliana*: TAIR6, *Populus trichocarpa*; v1.1, *Oryza sativa japonica*; version 2, *Physcomitrella patens*; version 1.1) (89). From all these 4 genomes, a best matching protein for PUT was identified by Blast, using only matches with E-values lower than 10^{-5}. A PUT was assumed to be in the pOG of its best matching protein from these four species, only if the evolutionary distance between PUT and the rice member(s) and PUT and the Arabidopsis member(s) in the pOG is less than the distance between rice and the Arabidopsis members. The evolutionary distances of all sequences were calculated using the protdist program in the Phylogeny Inference Package (PHYLIP) with Gamma correction (PHYLIP version 3.6, Felsenstein, J., 2005, distributed by the author, Department of Genome Sciences, University of Washington, Seattle). A simplified version of the process is shown in Fig 2.12. First, based on blast search, reciprocal best matches (double pointed arrows) or single match (one way arrow) are obtained between Arabidopsis proteins and potato translated PUTs. In the figure example based on the blast results, two possible scenarios for phylogenetic trees can be described. Then protdist calculated the phylogenetic distances (d) and based on those, phylogenetic trees are generated. All the genes subsequent to a speciation event (red dot) will belong to the same pOG.

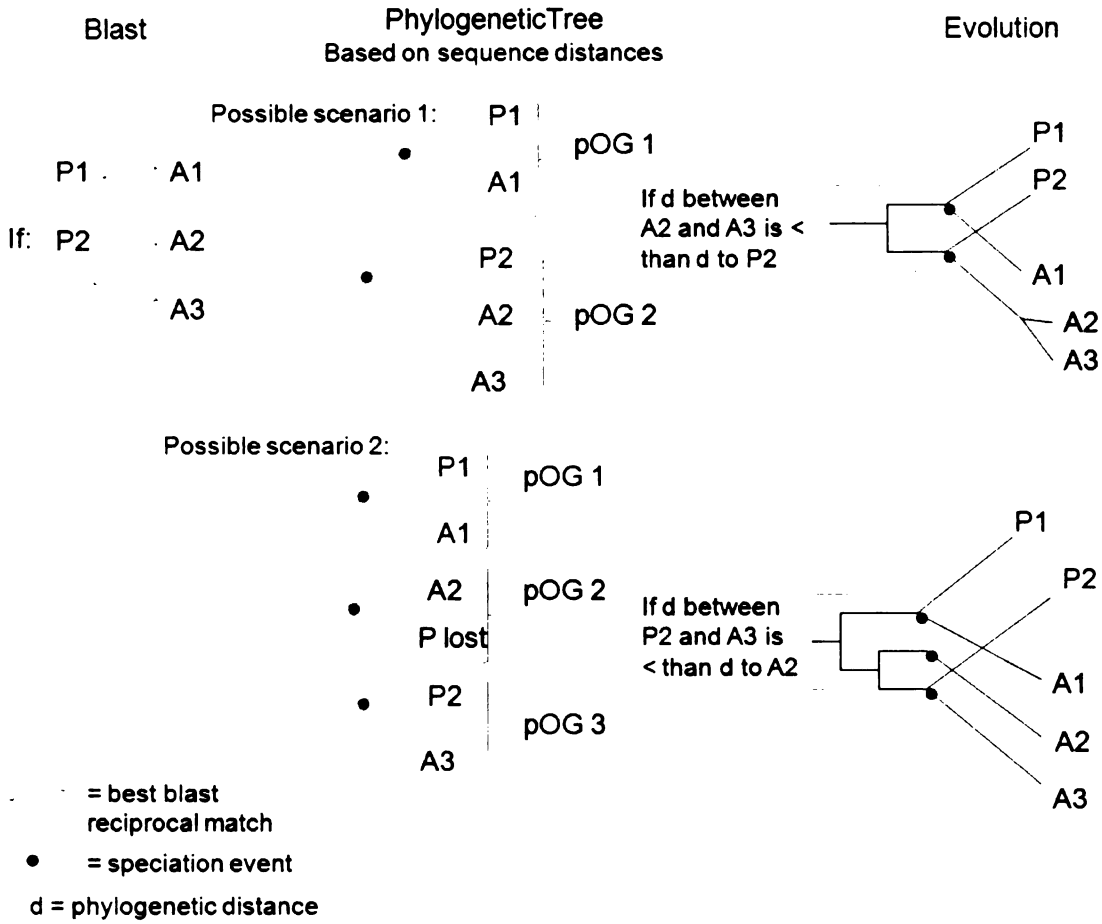

Fig 2.12: Representation of putative orthologous groups (pOGs) identification. pOGs between Arabidopsis proteins (A) and potato translated PUTs (P). Pointed line, branch of the tree leading to a gene that has been lost in evolution (P lost).

CHAPTER THREE

REGULATION OF CONSERVED TRANSCRIPTION FACTORS BY THE CIRCADIAN CLOCK, CYCLOHEXIMIDE TREATMENT AND MECHANICAL AGITATION

INTRODUCTION

CBFs are a group of AP2/ERF transcription factors that are quickly induced in response to low temperature (27). CBF1-3 transcription factors regulate the expression of more than a hundred genes in Arabidopsis known as the CBF regulon (19,20,83). Expression of these genes leads to an increase in freezing tolerance (26,28,29).

Solanum tuberosum (St) (common potato) is a freezing sensitive species and it is unable to cold acclimate. Its wild relative, *Solanum commersonii* (Sc), is able to cold acclimate and has a moderate level of freezing tolerance. These species have conserved *CBF* genes. There are 4 *Sc CBF*s and 5 St *CBF*s genes; each species has two cold-induced CBFs (*CBF1* and *CBF4*) (43).

In Arabidopsis, there is a group of transcription factors that are early cold induced with similar kinetics to CBF1-3 genes (20). These genes are *ZAT12*, *ZAT10*, *RAV1*, *CZF1* and *MYB73*. These transcription factors are likely candidates to configure the low temperature transcriptome of Arabidopsis. It has previously been shown that in addition to their low temperature response,

CBFs and these other factors are also induced in response to mechanical agitation and inhibition of protein synthesis (cycloheximide treatment) (20,27,38). Moreover, the *CBF2* promoter and some of these transcription factors have two regulatory sequences ICEr1 and ICEr2 (Induction of *CBF* expression region) that are involved in gene induction by cold, mechanical agitation and cycloheximide treatment. It is thought that there is a regulatory link between these different responses, but it is yet to be discovered in Arabidopsis (38).

In addition to these responses Arabidopsis CBF3 has also been shown to be regulated by the circadian clock (39). The Arabidopsis circadian clock is an internal oscillator that maintains an endogenous period of 24h. Its signaling networks enhance the plant's responses to its rhythmic environment. Environmental signals can regulate the phase and period of the circadian clock. A consequence of circadian control is that stimuli of the same strength applied at different times of the day can result in responses of different intensities, phenomenon known as "gating" (42,90).

Harmer et al., showed that under warm temperature, *AtCBF3* transcripts undergo circadian cycling, with a peak at ZT4 (Zeitgeiber Time, hours after dawn) and a trough at ZT16 (39). Given this circadian regulation of *AtCBF3* under warm temperatures, the question was raised whether the circadian clock also gated the expression of *CBF1-3* in response to low temperature. Fowler et al. showed that indeed it did (40). When Arabidopsis plants were entrained in 12:12 h photoperiod and then transferred to continuous light, the accumulation

of each *CBF1- 3* after exposure to 4°C cycles depending on the time during the subjective day or night that the plants were transferred to cold. Furthermore, disruption of the circadian clock by overexpression of Circadian Clock Associated 1 (*CCA1*), a Myb-related transcription factor member of the Arabidopsis clock (41,42), also disrupted the *AtCBF*s' cycling (40).

In addition, two other Arabidopsis early cold-induced transcription factors were also gated by the clock: RAV1, which encodes an AP2/B3 domain transcription factor (91) and ZAT12, which encodes a zinc-finger domain transcription factor (92). *RAV1* showed cycling with the same phase as *CBF* and *ZAT12* had the opposite phase (40).

Circadian gating of CBFs has also been suggested in tomato. When *Solanum lycopersicum* (tomato) and its wild relative *Solanum pimpinellifolium* were entrained under a 16:8 L:D photoperiod, *CBF1* expression cycled, reaching higher responsiveness during the light period in both species. When plants were shifted to constant dark or constant light CBF transcripts continued to cycle, however the peaks and troughs did not correspond to those observed in plants grown under a normal 16:8 L:D photoperiod (43).

Given that the Arabidopsis early cold induced transcription factors are also cold induced in Sc and St, the main goal of the experiments described in this chapter was to test if there was conservation in the response of these genes to these other environmental cues or if there were differences in the regulation of these genes between Sc and St that could account for their differences in freezing tolerance. We wanted to test if these transcription factors

were also responsive to mechanical agitation and cycloheximide treatment in Sc or St. We also wanted to know if the cold induction of these genes was gated by the circadian clock in Sc or St. Four early cold-induced transcription factors with orthologs in all 3 species were selected: *CBF1*, *ZAT10*, *RAV1*, and *CZF1*. The expression of the Sc and St genes in response to mechanical agitation and cycloheximide (CHX) treatment was tested by qRT-PCR. In addition, circadian experiments were conducted to test if these transcription factors were cold gated by the circadian clock in Sc or St.

ScCBF1 and ScZAT10 were induced by mechanical agitation in Sc, and the four genes tested were induced by cycloheximide treatment in Sc. In the case of St, only *StZAT10* was responsive to mechanical agitation and only *StCZF1* was responsive to cycloheximide treatment, but both St genes were induced to much lower levels than the ones observed in the Sc genes. The circadian experiments suggest that these genes are cold-gated by the circadian clock in Sc and St.

Together these results suggest that the regulatory link between cold, mechanical agitation and cycloheximide treatment appears to be conserved in Sc, but is not so clear if there is conservation to these other treatments in St. The circadian clock gating of these transcription factors appears to be conserved in the two *Solanum* species.

RESULTS

Regulation of conserved transcription factors by mechanical agitation and inhibition of protein synthesis.

From the previous chapter, some transcription factors were identified to be conserved in their response to low temperature. Their transcripts accumulated early upon cold treatment in Arabidopsis, Sc and St. The question was raised whether these transcription factors were also conserved in their response to other environmental perturbations in Sc and St, or if there were differences in the regulation of these Sc and St genes.

Four transcription factors that are early cold-induced in Arabidopsis as well as in Sc and St were selected: *CBF*, *RAV1*, *ZAT10*, and *CZF1*. The Arabidopsis genes are also induced by mechanical agitation and inhibition of protein synthesis (CHX treatment). In order to test if the St and Sc orthologs of these four transcription factors were also responsive to these treatments, they were tested for their response to mechanical agitation and CHX treatment. The putative potato orthologs with cold-induced kinetics closer to that observed for the Arabidopsis orthologs were chosen (See Fig 2.7, chapter 2). Primers that target the St PUT sequences (Putative Unique Transcript, PlantGDB website) were generated and used to detect St and Sc transcript by real time PCR (qRT-PCR).

Given the high similarity of some orthologs, primers that target both PUT3404 and 3405 (*RAV1*), both PUT32825 and 45213 (*ZAT10*), and both PUT25814 and 25815 (*CZF1*) were designed. The *CBF* genes (5 in St and 4 in Sc) are highly similar, therefore primers that primarily, but not exclusively, amplify *ScCBF1 and StCBF1* were used (Table 2.1, chapter 2).

To test mechanical agitation responsiveness, plants grown in magenta boxes were dropped approximately 6" every 2 seconds for 15 minutes, and then incubated with no agitation for 0, 15 or 30 minutes. Tissue was collected from 2 plants at each time point and the experiment was repeated once. Figure 3.1 shows results of mRNA accumulation in response to mechanical agitation. The four gene tested showed higher transcript levels in Sc than in St. Only *ScCBF1* and *ScZAT10* showed statistically significant (ANOVA, $p<0.05$, $n=2$) accumulation by mechanical agitation in Sc. *ScCBF1* mRNA accumulation reached its peak just after the treatment has been stopped (0 minutes after mechanical agitation) (5 fold compared to no shake control) and then declined after 15 minutes (3 fold), going back to the levels of no shake control after 30 minutes of incubation with no agitation. The same kinetics was observed for *ScZAT10*, but the induction was only 3 fold.

The only St gene that had statistically significant (ANOVA, $p<0.05$, $n=2$) transcript accumulation upon mechanical agitation treatment was *StZAT10* (Fig 3.1). The kinetics of transcript accumulation of *StZAT10* was similar to that of *ScZAT10*, reaching 3 fold at 0 minutes after treatment. All the other genes showed no statistically significant difference between samples.

To test responsiveness to inhibition of protein synthesis, three week old plants were treated with 10 μg/mL of CHX. Tissue was harvested after 0, 1, 2, 4, 8, and 24h of treatment (2 plants per time point and the experiment was repeated once). Fig 3.2 shows the results of mRNA accumulation under CHX treatment. All 4 gene transcripts had higher levels in Sc compared to St. The four Sc gene transcripts accumulated significantly (ANOVA, $p<0.05$, $n=2$) in response to CHX treatment. *ScCZF1*, *ScZAT10*, and *ScRAV1* accumulated slowly after CHX treatment reaching their peak accumulation after 4 hours of treatment by 66, 72, and 7 FC respectively. *ScCBF1*, however, reached its peak (20 FC) quicker after 1 h of treatment, declining to 11 FC after 4h of treatment. All of them reached their normal (no treatment control) levels after 8 h of treatment.

In the case of St genes, only *StCZF1* showed statistically significant differences between CHX treatment and control samples (ANOVA, $p<0.05$, $n = 2$) (Fig 3.2). The induction of *StCZF1* was lower (3FC at 4h) as compared to *ScCZF1* (66FC at 4h). All the other genes showed no statistically significant differences between CHX treatment and the control.

Together, the results of this section indicate that the four genes that are conserved in terms of their cold induction between Arabidopsis and the two *Solanum* species (*CBF1*, *ZAT10*, *RAV1*, and *CZF1*), are also conserved in their response to CHX treatment in Sc. Only one of these four genes, *StCZF1*, was significantly induced by CHX treatment in St; however it accumulated to a smaller level compared to *ScCZF1*.

Only two of the four cold-induced conserved transcription factors, *ScCBF1* and *ScZAT10*, are significantly induced in Sc in response to mechanical agitation. Only one of the four St counterparts, *StZAT10,* is significantly induced in St.

It can be concluded that, besides the conservation in their cold response, it appears to be some degree of conservation in the responses of these transcription factors to mechanical agitation and CHX treatment between Arabidopsis and Sc. However, the conservation in St is not so clear.

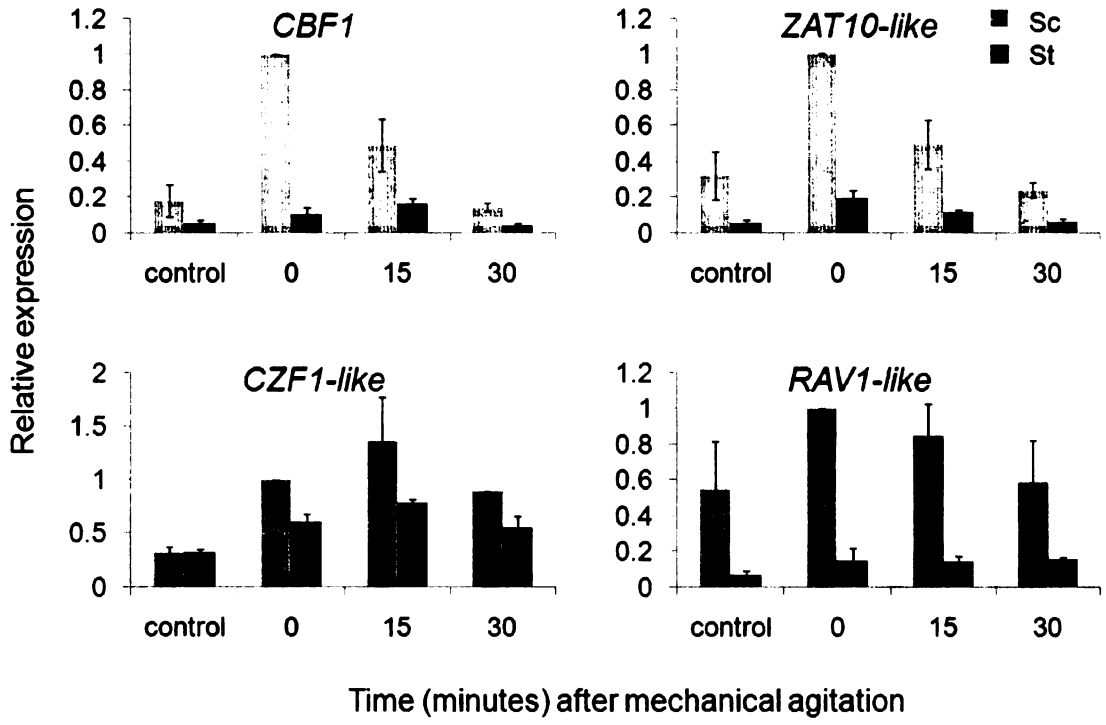

Fig 3.1: Transcript accumulation of conserved cold-induced transcription factors in response to mechanical agitation. Three weeks old plants grown in magenta boxes under continuous light were dropped approximately 6" every 2 seconds for 15 minutes. Tissue was collected from 2 plants each time, 0, 15, and 30 minutes after the mechanical agitation treatment. qRT-PCR analysis determining the transcript levels of Sc (grey) and St (black) genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of Sc 0 minute sample was set to 1. Error bars indicate SE.

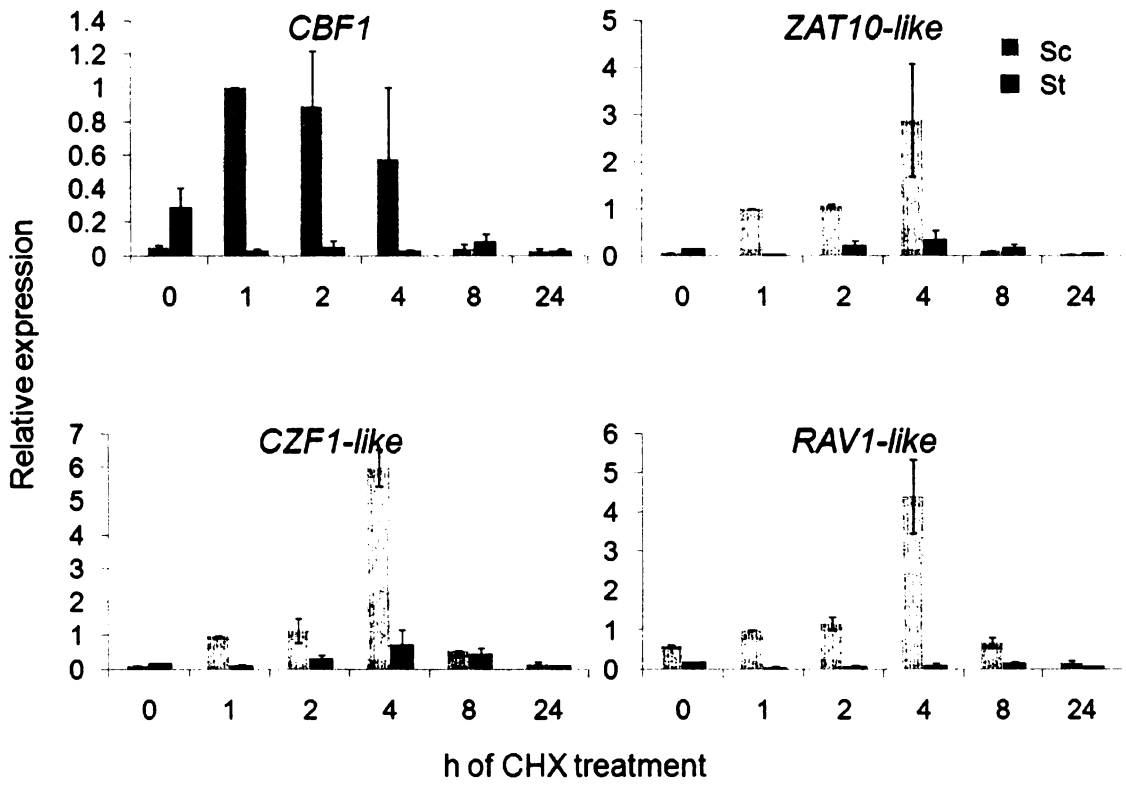

Fig 3.2: Transcript accumulation of conserved cold-induced transcription factors in response to cycloheximide treatment. Plants were grown under continuous light on liquid MS medium. After 3 weeks, cycloheximide was added to a final concentration of 10 μg/mL. Tissue was collected from 2 plants each time after 0, 1, 2, 4, 8, and 24h of cycloheximide treatment. qRT-PCR analysis determining the transcript levels of Sc and St genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of Sc 1h sample was set to 1. Error bars indicate SE.

Regulation of conserved transcription factors by the circadian clock

Fowler, et al., 2005 have shown that in Arabidopsis, the low temperature response of the *CBF*s is gated by the circadian clock. It was also found that *RAV1*, other early cold-induced transcription factor, was gated by the clock, with the same phase than that of the *CBF*s.

In order to explore in more detail the conservation in the regulation of these cold-induced transcription factors, circadian experiments were conducted with Sc and St plants to determine if the four conserved genes described in the previous section, were also gated by the circadian clock in these species.

Sc and St plants were grown for 3 weeks under a 12:12 h (L:D) photoperiod and then switched to continuous light at dawn (ZT0). Plant tissue was harvested at different times after dawn (ZT) under warm conditions. Additionally, plants were transferred to 4°C at the same ZT, and were maintained in the cold for 1 or 4h. Two biological replicates were done for each experiment.

The same primers for *ScCBF1* and *StCBF1* described in the previous section, were used here to test transcript accumulation by qRT-PCR. Fig 3.3a shows that *ScCBF1* transcript accumulation cycles in warm conditions (grey line) (ANOVA, $p<0.05$, $n=2$). The cycling pattern can be observed only from the second day and on. There are peaks at ZT28 and ZT52, during subjective day and troughs around ZT16 and ZT40, during subjective night. The same pattern of transcript accumulation was observed when Sc plants were transferred to the

cold for 1h (Fig 3.3a, black line). The pattern of cycling after 4h of cold (Fig 3.3b, black line) is similar, with peaks during subjective day and troughs during subjective night, however the peak during the second day was observed later, at ZT34. The transcripts accumulated to a higher level when transferred to cold for 1h or 4h compared to warm. Given that the cold stimulus applied at different times of the day produces different intensities of *ScCBF1* transcript accumulation, indicates that the cold response in *ScCBF1* is gated by the circadian clock.

The transcript level of *StCBF1,* like *ScCBF1*, cycles under warm conditions (Fig 3.3c). There is a statistically significant difference between samples at different ZTs (ANOVA, $p<0.05$, $n=2$). Peaks are observed at ZT10, 34 and 52 (all during the subjective day) and troughs at ZT22 and 46 (at the end of the subjective night). This suggests that *StCBF1* is under circadian regulation in the warm. When plants are transferred to cold for 1h, there is higher level of *StCBF1* transcript compared to warm, however, the peaks at ZT10 and 52 disappear (Fig 3.3c). In the case of plants that have been transferred to the cold for 4h (Fig 3.3d), the transcript levels are similar to those observed at warm temperatures and there is no statistically significant difference between peaks and troughs. The cycling observed at 1h cold during the second day suggests that *StCBF1* may also be gated by the circadian clock. Given that the low temperature responses of *AtCBF*s are gated by the circadian clock, the results shown here suggest conservation in the CBF cold response in the two *Solanum* species studied.

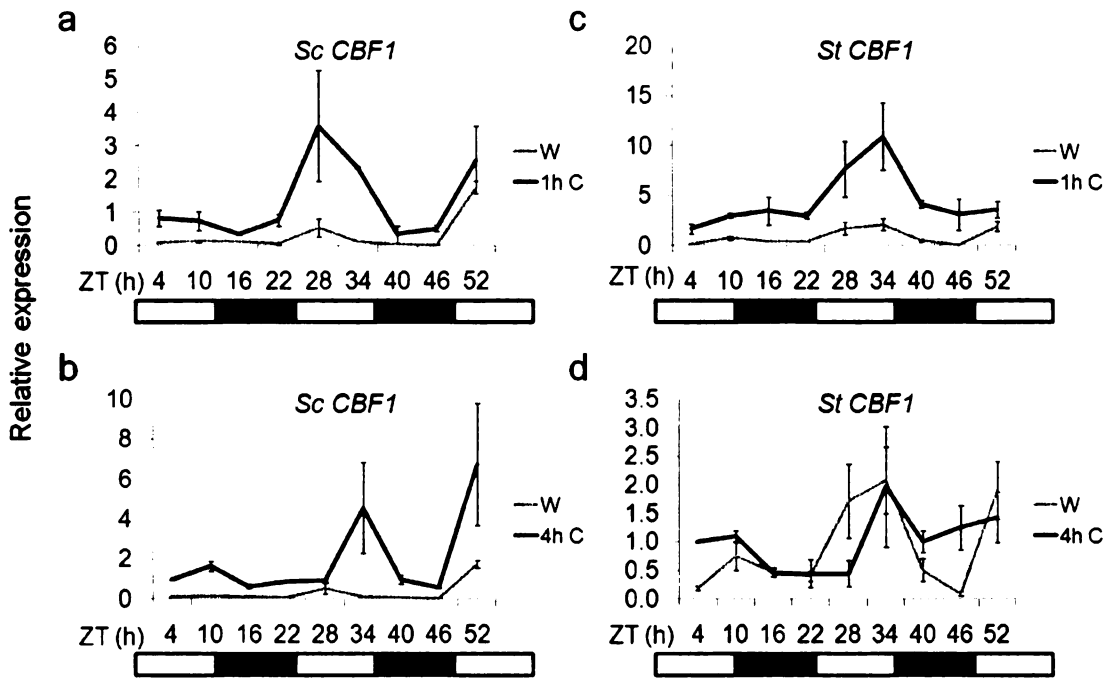

Fig 3.3: Circadian clock regulation of *CBF1*. Wild type *S. commersonii* (Sc) and *S. tuberosum* (St) plants were grown under 12:12 photoperiod for 3 weeks and then switched to continuous light at ZT0. Plant tissue was harvested at the different ZT shown (W: warm temperature). Additionally, plants were transferred to 4°C at the same ZT, and were maintained at 4°C for 1h (a, c) and 4h (b, d). qRT-PCR analysis determining the transcript levels of Sc (a-b) and St (c-d) genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of ZT4 4hC sample was set to 1. Error bars indicate SE. White box indicates subjective day and black box subjective night.

To test *ScRAV1* and *StRAV1* transcript accumulation under circadian conditions, primers that target PUT3404 and 3405 were used. The level of *ScRAV1* transcript is higher at 1h and 4h of cold compared to warm (fig 3.4a and b). This transcript shows cycling at warm, 1h cold and 4h cold. However, the cycling at 1h cold was not statistically significant (ANOVA, $p>0.05$, $n=2$). In the three cases, the peaks occur roughly at the end of the subjective day period, and the troughs during the subjective night which is similar to that observed for *AtRAV1* (40). These results suggest that *ScRAV1*, like *AtRAV1*, is also gated by the clock.

StRAV1 also shows cycling under warm conditions (Fig 3.4c), with peaks during the subjective day and troughs during the subjective night, however there was not a statistically significant difference between the peaks and troughs (ANOVA, $P>0.05$, $n=2$). When St plants were transferred to cold for 1h, there was higher accumulation of *StRAV1* transcript, but there was no statistically significant cycling (Fig 3.4c). When plants were transferred to cold for 4h, there is no cold-induction of this transcript, but there was a statistically significant difference between the peaks during subjective day and troughs during subjective night, indicating that after 4h of cold this transcript is cycling (ANOVA, $p<0.05$, $n=2$) (Fig 3.4d). There is no sufficient data to conclude whether the cold-induction of *StRAV1* is gated by the clock or not.

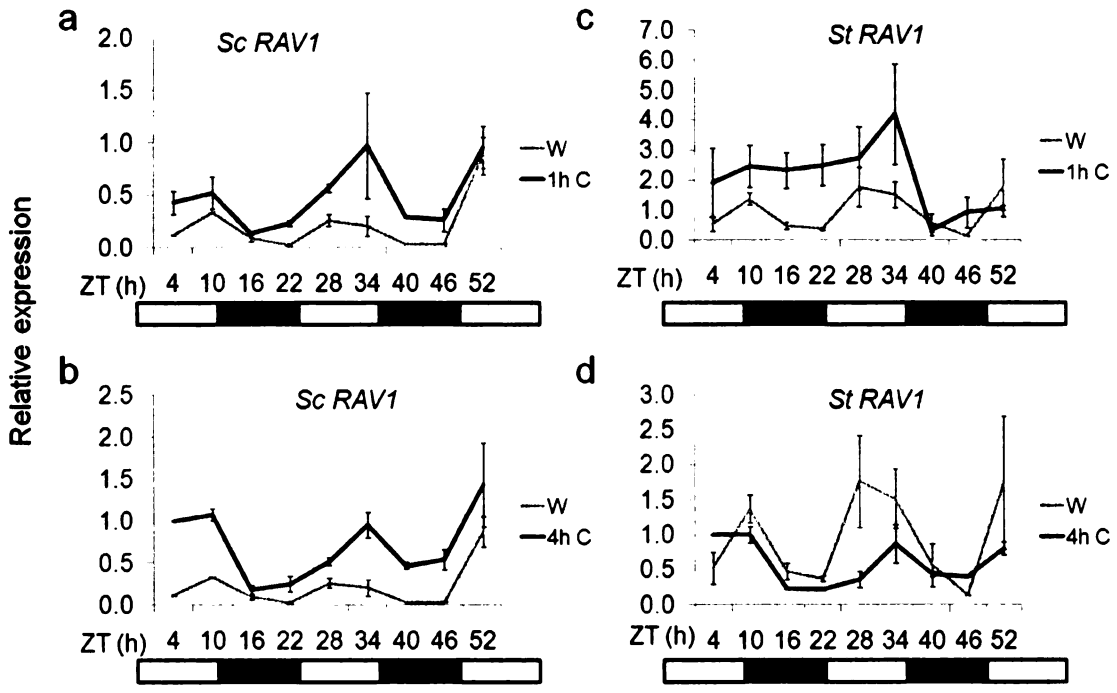

Fig 3.4: Circadian clock regulation of *RAV1*. Wild type *S. commersonii* (Sc) and *S. tuberosum* (St) plants were grown under 12:12 photoperiod for 3 weeks and then switched to continuous light at ZT0. Plant tissue was harvested at the different ZT shown (W: warm temperature). Additionally, plants were transferred to 4°C at the same ZT, and were maintained at 4°C for 1h (a, c) and 4h (b, d). qRT-PCR analysis determining the transcript levels of Sc (a-b) and St (c-d) genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of ZT4 4hC sample was set to 1. Error bars indicate SE. White box indicates subjective day and black box subjective night.

Preliminary studies suggest that *AtCZF1* and *AtZAT10* may also be gated by the clock in Arabidopsis (Dong, unpublished data). To determine whether *CZF1* and *ZAT10* were cold-gated or not in Sc and St, their transcript accumulation was tested in the circadian experiment.

ScCZF1 and *StCZF1* transcript accumulation was also tested under circadian conditions (Fig 3.5). Primers that target PUT32825 and 45213 were used for qRT-PCR. The *ScCZF1* transcript is cold-induced at 1h and 4h (Fig3.5a and b). The *ScCZF1* transcript levels cycles in warm and cold (1h and 4h) conditions (ANOVA, $p<0.05$, n=2) (Fig 3.5a and b). In all cases there were peaks during the subjective day and troughs during the subjective night, a pattern that is similar to that observed for *ScRAV1*. These results indicate that the low temperature induction of *ScCZF1* is gated by the circadian clock.

In the case of *StCZF1*, the induction by 1h and 4h of cold was only around two fold. The cycling of this transcript was statistically significant only under warm conditions and 4h cold (ANOVA, $p<0.05$, n=2) (Fig 3.5d). In these two cases, the phase of the cycling was the same as the one observed for *ScCZF1*. Given these results, it is possible that the lack of *StCZF1* cycling at 1h cold may be due to an error in the handling of the St 1h cold samples, rather than a real absence of cycling just after 1h cold (Fig 3.5c). Therefore, these results suggest that *StCZF1* low temperature induction is also gated by the clock.

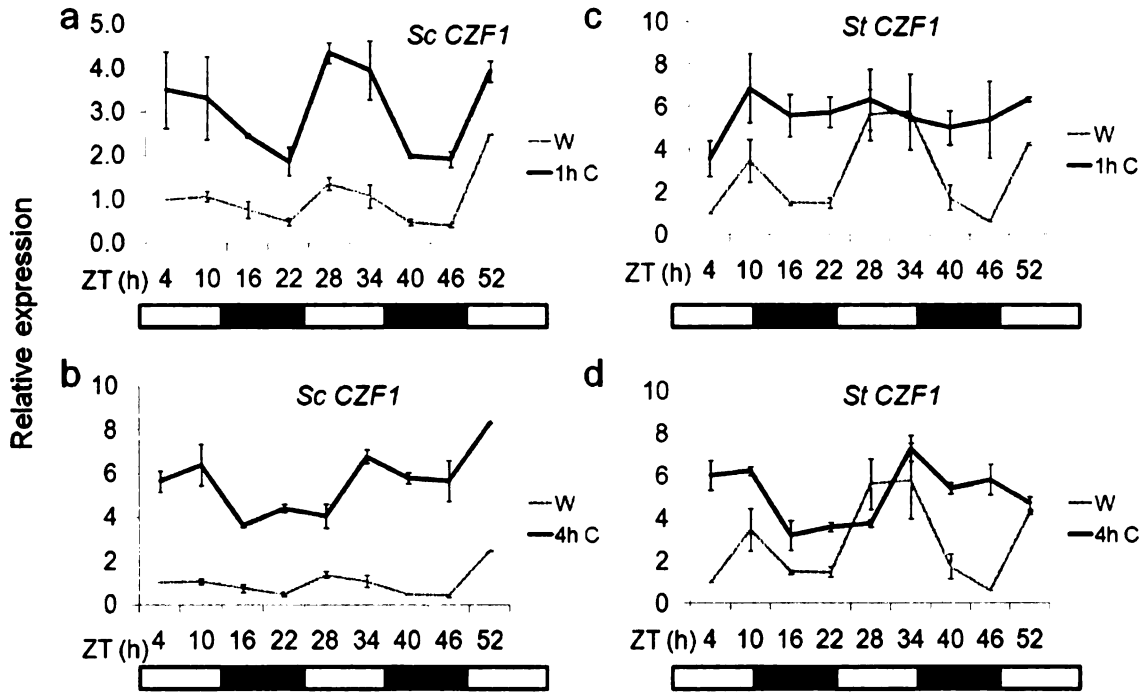

Fig 3.5: Circadian clock regulation of *CZF1*. Wild type *S. commersonii* (Sc) and *S. tuberosum* (St) plants were grown under 12:12 photoperiod for 3 weeks and then switched to continuous light at ZT0. Plant tissue was harvested at the different ZT shown (W: warm temperature). Additionally, plants were transferred to 4°C at the same ZT, and were maintained at 4°C for 1h (a, c) and 4h (b, d). qRT-PCR analysis determining the transcript levels of Sc (a-b) and St (c-d) genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of ZT4 4hC sample was set to 1. Error bars indicate SE. White box indicates subjective day and black box subjective night.

Finally, the *ScZAT10* and *StZAT10* genes were tested for circadian regulation. Primers that target PUT32825 and 45213 were used for qRT-PCR. *ScZAT10* transcript accumulates to a higher level in 1h and 4h cold treated samples compared to warm samples (Fig 3.6a and b). This transcript showed cycles with peaks during subjective day and troughs during subjective night, the same phase as *ScRAV1* and *ScCZF1*. Even though the cycling at 1h cold is not statistically significant (ANOVA, $p>0.05$, $n=2$), peaks and troughs can also be observed at the same ZT as the ones at warm and 4h cold. These results suggest that the *ScZAT10* low temperature response is gated by the clock.

In the case of *StZAT10*, there is also cold-induction at 1h and 4h, and there is cycling with the same phase as *ScZAT10*, that is peaks during subjective day and troughs during subjective night (Fig 3.6c and d). Also in this case, the cycling was statistically significant only under warm and 4h cold (ANOVA, $p<0.05$, $n=2$). These results suggest that *StZAT10* is also gated by the clock.

From these results it was concluded that the low temperature responses of three of the four conserved early cold-induced genes selected (*CBF1*, *CZF1*, and *ZAT10*) are gated by the circadian clock in St and Sc. It can also be concluded that the cold gating of *RAV1* is conserved between Sc and Arabidopsis. In the case of *CBF1*, their cold-gating response is conserved in these two *Solanum* species as well as in Arabidopsis. There are no obvious differences between the cold gating of these transcription factors between Sc and St that could account for their differences in freezing tolerance. Rather, it

appears that the imput of the circadian clock in cold stress response is conserved in the two *Solanum* species.

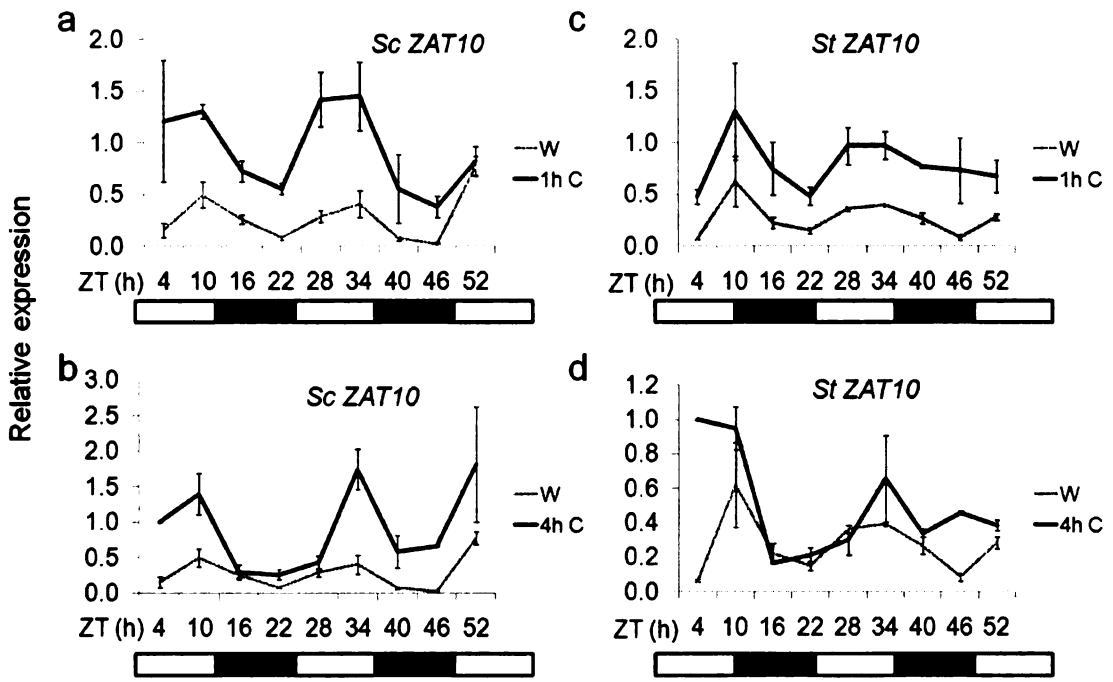

Fig 3.6: Circadian clock regulation of *ZAT10*. Wild type *S. commersonii* (Sc) and *S. tuberosum* (St) plants were grown under 12:12 photoperiod for 3 weeks and then switched to continuous light at ZT0. Plant tissue was harvested at the different ZT shown (W: warm temperature). Additionally, plants were transferred to 4°C at the same ZT, and were maintained at 4°C for 1h (a, c) and 4h (b, d). qRT-PCR analysis determining the transcript levels of Sc (a-b) and St (c-d) genes. Average values of two different experiments are shown. Relative expression level of each transcript were normalized using the potato 60S gene (clone STMCK67) as internal reference. Relative expression of ZT4 4hC sample was set to 1. Error bars indicate SE. White box indicates subjective day and black box subjective night.

DISCUSSION

In order to further explore the conservation of early cold induced transcription factors in Sc, St and Arabidopsis, the main goal of this chapter was to study the responses of four transcription factors (CBF1, RAV1, ZAT10, and CZF1) to mechanical agitation, inhibition of protein synthesis, and circadian control. Given that these four transcription factors are responsive to mechanical agitation and CHX treatment (inhibition of protein synthesis) in Arabidopsis, accumulation of the St and Sc transcripts upon the different treatments was tested by qRT-PCR.

Given that the same primers were used to test transcript accumulation of both species, it can be concluded that the gene transcript levels were always higher for the Sc transcripts than to those of St. If some differences in hybridization efficiency of the primers were to happen, the primers could have had less homology to the Sc genes (the Sc genes are unknown) and that could result in less hybridization efficiency, but given that in all the cases the Sc transcripts were higher than the St transcripts, this suggests that primers hybridized to Sc transcripts as well as to St transcripts.

In this study the transcript of *ScCBF1*, *ScZAT10* and *StZAT10* were shown to significantly accumulate after mechanical agitation. The lack of significant accumulation of the other gene transcripts tested suggests that the mechanical agitation response is not that well conserved between Arabidopsis and the *Solanum* species studied. However, this experiment was done only

twice therefore more replicates of this experiment should give more statistically significant results.

To test transcript accumulation in response to inhibition of protein synthesis, CHX treatment was performed. The four gene transcripts tested were significantly induced in Sc. Only *StCZF1* was significantly induced by CHX treatment in St; however to a smaller level. *StCBF1* showed high levels of transcripts in no treatment controls (0, Fig 3.2). It is known that the levels of *StCBF1* are almost undetectable in non-inducing (warm) conditions. In this experiment the level of *StCBF1* in the no treatment control is higher than any other time point of CHX treatment. Given that some induction of *StCBF1* can be observed by mechanical agitation (Fig 3.1) it is possible that undesired agitation of no treatment control plants is responsible for this high level of *StCBF1*.

It is unknown if inhibition of protein synthesis by CHX treatment occurs with the same efficiency in both Sc and St. It is possible that the Sc plants had better adsorption of the CHX than St. This was not tested.

It has been previously observed that unstable transcripts like *CBF1-3*, which have a half life of 7.5 min at warm temperatures (38), are associated with a mechanical agitation response and with clock-controlled genes (93). Given that *CBF1-3* and *RAV1* cold-induction are gated by the circadian clock in Arabidopsis (40), the low temperature responses of the four conserved early-induced genes were tested to determined if they were also gated by the circadian clock in Sc and St.

The results presented in this study indicate that three (*CBF1*, *CZF1*, and *ZAT10*) of the four transcription factors that are early cold-induced in St, Sc and Arabidopsis are gated by the circadian clock in the two *Solanum* species studied. The gating of *RAV1* cold-induction is conserved in Sc and Arabidopsis. *ScCBF1* cold-induction is gated by the circadian clock. The lack of a peak at ZT4 may be because plants are in the transition from 12:12 photoperiod to continuous light. In the first 12 h into the continuous light, plants don't sense yet they are in continuous light. It is possible they are still behaving as in 12:12h photoperiod and it is unknown whether in this condition *ScCBF1* peaks at ZT4 or earlier. If it peaks earlier, the peak would have been missed in this experiment. It has been observed that, for instance, *CCA1* peaks at ZT0 under a 12:12h photoperiod, but when is transferred to continuous light it peaks later (94).

The *StCBF1* transcript is circadian regulated in warm conditions. When transferred to cold for 1h the peak and trough can only be observed during the second day (peak at ZT34 and trough at ZT46). After 4h of cold there is apparent cycling, however the peaks (ZT10 and ZT34) and troughs (ZT22 and ZT40) are not statistically significant. This suggests that *StCBF1* may also be gated by the circadian clock. More replicates of the experiment should give more statistically significant results.

Previously, Pennycooke et al. (2008) showed that *S. lycopersicon* (common tomato) and *S. pimpinellifolium* (wild tomato), two *Solanum* species that are chilling and freezing sensitive, have *CBF1* genes that are regulated by

light and the circadian clock (43). Therefore, it would not be unexpected that *StCBF1*, another *Solanum* species that is freezing sensitive, would also be gated by the clock.

The other three transcription factors studied (RAV1, ZAT10, and CZF1) also showed gating of their low temperature response in Sc, however in St the gating of RAV1 cannot be concluded. There are peaks during the subjective day and troughs during the subjective night for all of these cold gated genes.

ScRAV1 has a cycling pattern that has the same phase as *AtRAV1* (40), which suggest that the gated cold response of *RAV1* is conserved.

The cold-induction of *StCZF1* was only around two fold (Fig 3.5c and d). However, in chapter 2 (Fig 2.7) there was much more high cold induction of this transcript. This difference could be due to the fact that in the experiments of chapter 2, plants are grown in 16:8h photoperiod and then transferred to cold at ZT8, but in this circadian experiment the plants were entrained in 12:12h photoperiod and then transferred to continuous light, where the temperature is dropped at the different ZTs. This different growth conditions might have had this effect on the cold-induction of this gene. It is unknown if Arabidopsis *ZAT10* and *CZF1* cold responses are gated by the circadian clock. Preliminary data suggest that they might be (Dong, unpublished data). If these results are confirmed, it will suggest conservation in the cold-induction gating of these genes too.

MATERIALS AND METHODS

Plant growth and experimental conditions

Single node cuttings of *Solanum tuberosum* cv. Umatilla and *Solanum commersonii* were grown on half strength MS medium supplemented with 20g/L sucrose and 7g/l Plant Agar (Sigma), pH 5.6 in Magenta GA7 vessels with six plants per vessel. Plants were grown in an Enconair growth chamber ("Bigfoot" GC-20) at 25°C under a 12:12h L:D photoperiod (light intensity 100 µ mol m^{-1} s^{-1}), for 3 weeks before sampling. Two plants were randomly selected for each treatment and each treatment was repeated once for both species. The top portions (approximately 5 cm) of plants were collected and the lower sections and root systems were discarded. Samples were placed in 15 ml falcon tubes, immediately submerged in liquid nitrogen and stored in a -80°C freezer. For collections during the dark period, samples were collected with very minimal indirect light. Samples that coincided with the transition from light to dark or dark to light were collected immediately prior to the transition.

For the circadian experiments, three week old plants grown in a 12:12 L:D photoperiod were transferred to continuous light (100 µmol m^{-1} s^{-1}). Two replicates from both species were collected at 4, 10, 16, 22, 28, 34, 40, 46 and 52h after the beginning of continuous light (warm samples). In addition, 3 week old plants were also transferred to continuous light at ZT0. In this case, plants were transferred to a 4°C walk-in cold room with continuous light (100 µmol m^{-1}

s^{-1}) at ZT4, 10, 16, 22, 28, 34, 40, 46 and 52. After 1h and 4h of cold, two replicates were collected for each ZT.

For the mechanical agitation treatment, 3 week old plants of both species grown under continuous light in Magenta vessels were secured together in a cardboard box and dropped approximately 6" every 2 seconds for 15 minutes. Samples were collected at 0, 15 and 30 minutes after mechanical treatment. Two replicates of two plants from each species were randomly selected from each vessel and frozen in liquid nitrogen immediately. A control without agitation was included.

For the inhibition of protein synthesis experiment, plants were grown under continuous light on liquid half strength MS medium supplemented with 20g/l sucrose, pH 5.6 on a filter paper bridge held above the level of the liquid medium. Capillary action maintained a constant supply of culture medium to the plants on the filter paper. After 3 weeks of growth, cycloheximide (CHX) was mixed into the existing liquid medium to give a final concentration of 10µg/ml. Two replicates of two plants from each vessel were collected and frozen in liquid nitrogen for each treatment. Samples were collected after 0, 1, 2, 4, 8 and 24 hours after the beginning of CHX treatment. A control without CHX in the medium was included.

RNA extraction and qRT-PCR

RNA extraction and qRT-PCR were done as described in chapter two. Primers used are listed in Table 2.1.

APPENDIX

Table A1: 40pOG cold-induced in *S. commersonii* (Sc) *S. tuberosum* (St) and Arabidopsis (At).

St up	Sc up	At up	At description
STMDJ69	STMGE83 STMDJ69	AT2G18900	transducin family protein / WD-40 repeat family protein
STMIW06	STMIW06	AT4G25990	chloroplast import apparatus CIA2-like
STMIU11	STMIU11	AT5G60680 AT2G28400	unknown protein unknown protein
STMCG52	STMGV17 STMCG52	AT2G45660	AGL20 (Agamous-like 20)
STMCN22	STMCN22	AT5G65280	GCL1 (GCR2-like 1); catalytic
STMEK16	STMIQ63 STMEK16	AT3G12670	EMB2742 (embryo defective 2742)
STMIV71 STMIY51	STMIV71 STMIY51	AT1G27760	interferon-related developmental regulator family protein / IFRD protein family
STMJG77 STMIU74	STMIU74	AT5G01880	zinc finger (C3HC4-type RING finger) family protein
STMEP26	STMEP26	AT5G26920	calmodulin binding
STMEO27	STMEO27	AT2G33210 AT3G23990	chaperonin putative HSP60 (Heat shock protein 60)
STMIU32 STMID24	STMIU32 STMID24	AT3G53230	cell division cycle protein 48 putative (CDC48)
STMIQ26 STMJI56 STMGA34	STMIQ26 STMJI56 STMGA34	AT1G01470	LEA14 (Late embryogenesis abundant 14)
STMDH66 STMJL22	STMJL22	AT4G35940	unknown protein
STMEI36 STMEQ55	STMEI36	AT1G31660	unknown protein
STMET41	STMET41	AT1G25400	unknown protein
STMGF95	STMGF95	AT1G51700	ADOF1 (Arabidopsis dof zinc finger protein 1)
STMIY82	STMIY82	AT1G52890 AT4G27410	ANAC019 (Arabidopsis NAC domain containing protein 19) RD26 (responsive to dessication 26)
STMDO86	STMDS75 STMDO86	AT3G16810	APUM24 (Arabidopsis pumilio 24)
STMEW81	STMEW81	AT5G62360	invertase/pectin methylesterase inhibitor family
STMCB90	STMCB90	AT5G62350	protein invertase/pectin methylesterase inhibitor family protein (DC 1.2 homolog)
STMHE19	STMHE19	AT5G20830	SUS1 (sucrose synthase 1)
STMDP77	STMDP77	AT4G29780	unknown protein

Table A1 continued

STMGH65	STMHG34	AT4G30290	ATXTH19 ATXTH20 (Xyloglucan
STMJJ17	STMJJ17	AT5G48070	endotransglucosilase hydrolase 19 and 20)
STMHA92	STMHA92	AT1G67970	AT-HSFA8 (Arabidopsis thaliana heat shock transcription factor A8)
STMHS29	STMHS29	AT1G42440	unknown protein
STMGG79	STMGG79	AT3G55510	unknown protein
STMCX87	STMCX87	AT5G16010	3-oxo-5-alpha-steroid 4-dehydrogenase family protein / steroid 5-alpha-reductase family protein
STMDU38	STMHN39	AT1G80270	DNA-binding protein putative
	STMDU38		
STMHO64	STMHO64	AT2G17270	mitochondrial substrate carrier family protein
STMIH78	STMIH78	AT4G27940	mitochondrial substrate carrier family protein
STMGL16	STMGL16		
STMED50	STMED50	AT4G33905	peroxisomal membrane protein 22 kDa putative
		AT2G14860	peroxisomal membrane protein 22 kDa putative
STMGR56	STMGR56	AT4G28450	transducin family protein / WD-40 repeat family protein
STMIP59	STMIP59	AT1G32860	glycosyl hydrolase family 17 protein
STMHT66	STMHT66	AT4G31140	glycosyl hydrolase family 17 protein
STMIO48	STMIO48	AT4G00640	unknown protein
STMGU17	STMGU17	AT4G12000	unknown protein
STMHS17	STMHS17	AT3G11410	ATPP2CA (Arabidopsis protein phosphatase 2CA)
		AT1G07430	protein phosphatase 2C putative
STMJO29	STMJO29	AT1G20450	ERD10/LTI45 (early responsive to dehydration 10)
		AT1G20440	COR47 (cold regulated 47) ERD14 (early
		AT1G76180	responsive to dehydration 14)
STMJO47	STMJO47	AT3G22840	ELIP1 (early light-inducible protein) chlorophyll
STMIX48	STMIX48	AT4G14690	binding ELIP2 (early light-inducible protein 2) chlorophyll binding
STMHO88	STMHO88	AT5G07990	TT7 (transparent testa 7)
STMHT73	STMHT73		
STMGJ81	STMGJ81	AT1G53645	hydroxyproline-rich glycoprotein family protein

Table A2: 13pOG cold-repressed in *S. commersonii* (Sc), *S. tuberosum* (St), and Arabidopsis (At).

St down	Sc down	At down	At description
STMDV46	STMDV46	AT1G09750	chloroplast nucleoid DNA-binding protein-related
STMIV36	STMIV36	AT2G39470	PPL2 (PSBP-like protein 2)
STMCX38	STMCX38	AT3G16150	L-asparaginase putative
STMER63	STMDB57 STMER63 STMDB57	AT3G23730	xyloglucan:xyloglucosyl transferase putative
STMGO23	STMGO23	AT4G14540	CCAAT-box binding transcription factor subunit B (NF-YB) (HAP3)
STMCR16	STMCR16	AT1G70410	carbonic anhydrase putative; carbonate dehydratase
STMCL01	STMCL01	AT3G01500	putative CA1 (carbonic anhydrase 1)
STMCV75	STMCV75		
STMIV24	STMIV24		
STMCK44	STMCK44		
STMEP82 STMCS89	STMCS89	AT1G48600	phosphoethanolamine N-methyltransferase 2 putative (NMT2)
STMCD65	STMCD65	AT5G56870	BGAL4 (beta-galactosidase 4)
STMGX24	STMGX24	AT5G35790	G6PD1 (glucose-6-phosphate dehydrogenase 1)
STMJH69	STMJH69	AT3G15840	PIFI (post-illumination chlorophyll fluorescence increase)
STMIM55	STMIM55	AT1G32080	membrane protein putative
STMDB78 STMCQ55	STMCQ55	AT1G73330	ATDR4 (Arabidopsis thaliana drought-repressed 4)
STMJD18	STMJD18	AT4G25260 AT4G12390	invertase/pectin methylesterase inhibitor family protein PME1; pectinesterase inhibitor

Table A3: 27 pOG cold-induced in *S. commersonii* (Sc) and Arabidopsis (At), but not in *S. tuberosum* (St).

Sc up	At up	At description
STMEF80 STMEF80	AT3G49320	unknown protein
STMDJ58	AT5G39410	binding / catalytic
STMCN38	AT3G08950	electron transport SCO1/SenC family protein
STMIU24	AT3G16720	ATL2 (Arabidopsis Toxicos en Levadura 2)
STMCM56	AT3G27880 AT1G23710 AT1G70420	unknown protein
STMDH61 STMDH61	AT4G37090	unknown protein
STMCN51 STMCS66 STMEQ25 STMCS25	AT1G13930	unknown protein
STMIN87	AT2G14560	unknown protein
STMHS67	AT3G46460	UBC13 (ubiquitin-conjugating enzyme 13)
STMES89	AT1G73630	calcium-binding protein, putative
STMCY67	AT5G62430 AT3G47500	CDF1 (cycling dof factor 1), CDF3 (cycling dof factor 3)
STMEU37	AT4G25470 AT4G25480	CBF2 (freezing tolerance QTL 4) DREB1A (dehydration response element B1A)
STMDE93 STMDE93	AT3G13940	DNA binding / DNA-directed RNA polymerase
STMDP46	AT1G04240	SHY2 (short hypocotyl 2)
STMDD59	AT3G44260	CCR4-NOT transcription complex protein, putative
STMEQ58	AT2G28720	histone H2B, putative
STMJN45	AT5G67480	BT4 (BTB AND TAZ domain protein 4)
STMJF94	AT3G55120	A11/CFI/TT5 (transparent testa 5)
STMCV31 STMGD36	AT5G20180	ribosomal protein L36 family protein
STMIQ74	AT4G27520	plastocyanin-like domain-containing protein
STMEY77	AT3G55430	glycosyl hydrolase family 17 protein / beta-1,3-glucanase
STMDZ38	AT3G54030	protein kinase family protein
STMEY96	AT2G25625	unknown protein
STMCG80	AT3G56090	ATFER3 (ferritin 3)
STMEY27	AT4G18530	unknown protein
STMGX29	AT1G76930	ATEXT4 (extensin 4)
STMHS45	AT2G39130	amino acid transporter family protein

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