AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Solanum species differ in their degree of frost tolerance and cold acclimation capacity. Cultivated potato species of *Solanum tuberosum L.* are frost-sensitive, incapable of cold acclimation, and have a maximum freezing tolerance of –3°C. *Solanum commersonii* Dun is frost-tolerant and can survive to –5ºC pre-acclimation and –10ºC post-acclimation. Breeding attempts to improve potato freezing tolerance and cold acclimation capacity have been largely unsuccessful in *S. tuberosum*. *Arabidopsis CBF* genes encode cold-induced transcription factors that are involved in plant cold acclimation. In this study, *S. tuberosum* (cv. Umatilla) and *S. commersonii* (PI243503 clone13), were transformed with three *Arabidopsis CBF* genes (*AtCBF1-3*) driven by either the constitutive *35S* or stress-inducible *rd29A* promoter to assess the role CBFs play in, and their effects on, potato freezing tolerance and cold acclimation capacity. Constitutive *AtCBF1* and *AtCBF3*

overexpression increased freezing tolerance in *S. tuberosum* by 2°C, and in *S. commersonii* by 4°C, while *AtCBF2* failed to increase freezing tolerance. Cold acclimation capacity was improved for *S. commersonii*, but was absent from *S. tuberosum*. During cold treatment, leaves of wildtype *S. commersonii*, but not *S. tuberosum*, showed a significant thickening due to palisade cell lengthening and enlargement of intercellular spaces. Ectopic *AtCBF1* activity mimicked cold acclimation by increasing proline and total sugar content in *S. commersonii* in the absence of cold. An increased chlorophyll content of transgenic *S. commersonii* leaves coincided with an enhanced photosynthetic capacity that was maintained during cold treatment. However, constitutive expression of all three *AtCBF* genes caused a variety of negative phenotypic alterations, including the reduction or elimination of tuber production, limiting their agronomic potential. The stress-inducible rd29A::*AtCBF* transgene versions had identical gains in freezing tolerance capacity while minimizing the negative phenotypic effects and allowing essentially normal tuber production levels. Ectopic *AtCBF* transgene expression was confirmed to induce expression of cold-regulated genes likely involved in potato frost tolerance under warm conditions. Collectively these results suggest an endogenous CBF pathway is involved in potato frost tolerance and cold acclimation. Cold-inducible overexpression of a *CBF* transgene may be a practical approach to improving frost tolerance while minimizing detrimental effects on tuber production in potato.

Ectopic Overexpression of *Arabidopsis CBF* Genes Enhances Freezing Tolerance of Two Potato Species

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María-Teresa Pino

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Maria-Teresa Pino, Author

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Dr. Eun-Jun Park was involved with plant transformation of Chapter 2.

Dr. Patrick M. Hayes was involved with the review of manuscripts of Chapter 2 and Chapter 3.

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DEDICATION

To my beloved husband for his unconditional love and support and to my little daughters Camila and Victoria for refreshing my life every day. And to God, the Eternal Father who gives me life and strength to accomplish my goals.

ECTOPIC OVEREXPRESSION OF *ARABIDOPSIS CBF* GENES ENHANCES FREEZING TOLERANCE OF TWO POTATO SPECIES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The incidence of frost has a significant impact on agricultural operations worldwide, causing considerable losses in crop productivity and limiting the geographical distribution of important crop species. Although grown in numerous climates, cultivated potato (*Solanum tuberosum L.)* is a frost-sensitive species. In most of its production areas, low temperatures can significantly reduce yields and hard frosts can sometimes completely destroy an entire plantation. Whereas no cultivars of *S. tuberosum* appear capable of cold acclimation, other tuber-bearing species, e.g., *S. acaule* and *S. commersonii Dun,* can survive at about –5 ºC, and, after becoming fully cold-acclimated, can tolerate temperatures as low as –10.0 °C (Chen and Li, 1980; Costa and Li, 1993).

To solve this problem of injury in potato, research has been conducted to transfer tolerance genes from frost-hardy wild species to frost-sensitive cultivated crops. However, when this has been attempted via traditional breeding such efforts have proven time-consuming and have not significantly increased tolerance in cultivated potatoes (Cardi et al., 1993; Estrada et al., 1993; Iovene et al., 2004). Progress has been slow mainly because frost tolerance and cold acclimation

capacity are complex genetic traits involving many genes for which their function is not fully understood.

New, promising biotechnology approaches have been directed at determining the mechanisms involved with transduction of the cold signal and regulation of gene expression by low temperatures. Acclimation entails action by a large number of cold-regulated (*cor*) genes. Identification of the transcriptional activators for dehydration-responsive-element-binding (DREB) factors or c-repeat-binding factors (CBF), and their roles in coordinately regulating the expression of *cor* genes, has significantly advanced our understanding of how plants adapt to low temperatures, and have provided a new means for improving freezing tolerance in crops (Thomashow, 1999, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000; Fowler and Thomashow, 2002). The use of *Arabidopsis CBF/DREB* genes, or homologous genes from other species, to enhance cold tolerance has been demonstrated in many plant species, including *Brassica napus* (Jaglo et al., 2001), tomato (Hsieh et al., 2002a, b), and tobacco (Kasuga et al., 2004).

Solanum species differ in their degree of frost tolerance and cold acclimation capacity. As the first step in determining the role of the CBF cold-response pathway in improving this crop, two potato species (wild *S. commersonii* and cultivated *S. tuberosum*) were transformed here with *Arabidopsis* transcription factors (*AtCBF1* through *3*) under the control of two different promoters. This study had several objectives, with the first being to investigate whether the overexpression of *AtCBF1 - 3* could improve frost tolerance and/or the cold

acclimation capacity in potato plants, and, if so, to identify the *AtCBF1 - 3* induced changes closely associated with this increased tolerance in the resultant transgenics. Another objective was to examine whether constitutive overexpression of the *AtCBF1 - 3* genes would induce morphological alterations in the plant and tuber phenotypes, and, if so, whether a stress-inducible rd29A promoter could minimize any negative effects on transgenic plant growth in potato, as has been reported previously with *Arabidopsis* (Kasuga et al., 1999) and tobacco (Kasuga et al., 2004).

Literature Review

Potato Cold Hardiness

The Significant Impact of Frost on Potato Production

Cultivated potato (*Solanum tuberosum*) is the fourth most important food crop (after rice, corn, and wheat), and is widely cultivated around the world. In all of its production areas frost can reduce yields and tuber quality. Within the temperate zones, frost injuries are encountered mainly in early spring and late fall. In the Andean highlands of South America, where frost can occur at any time during the growing season, potato production can be seriously diminished (Chen and Li, 1980; Barrientos et al., 1994; Vega and Bamberg, 1995). Likewise, frosts can be devastating in the Mediterranean region where early potatoes are a high-profit crop. This is because it is common to plant early-season potatoes from late

fall through early spring, periods when low temperatures can cause significant losses in production (Iovene et al., 2004).

All cultivars of *S. tuberosum* are frost-sensitive and are seemingly incapable of cold acclimation compared with other, wild, tuber-bearing *Solanum* species (Chen and Li, 1980; Costa and Li, 1993). Freezing temperatures, i.e., <-2.5 ºC, can damage the foliage of *S. tuberosum*, shorten the growing season, and reduce yields (Chen and Li, 1980). Frost injury in most plant tissues results from the severe cellular dehydration that follows extracellular ice formation, causing profound damage to cellular membranes and protein denaturation (Palta and Li, 1980; Toivio-Kinnican et al., 1981; Steponkus, 1984; Thomashow, 1999). Other consequences of freezing-induced cellular dehydration include the generation of reactive oxygen species (ROS) that then damage other cellular components (Guy, 1990; McKersie, 1991; Thomashow, 1999). ROS accumulations arise from failures in electron transfer reactions, which are connected to damage in Photosystem II and decreased photosynthetic efficiency (O'Kane et al., 1996, McKersie et al., 2000). Photosynthesis rates are substantially decreased by low temperature and severe frost, especially when plants are exposed to high-intensity light following a freezing event (Steffen and Palta, 1989). Within this stress period, photosynthesis is momentarily reduced in *S. commersonii* but can be recovered when the stress is released, whereas photosynthetic activity in S. *tuberosum* is severely decreased during freezing stress, with damage very often being irreversible (Seppanen and Coleman, 2003). During cold acclimation, photosynthesis can interact with other

processes, such as sugar-signaling pathways, to regulate the adjustment to low temperatures (Ensminger et al., 2006).

Multiple Changes in Potato Plants during Cold Acclimation

Many biochemical and morphological changes occur in conjunction with the acquisition of enhanced freezing tolerance during cold acclimation in potato plants. These include alterations in their levels of carbohydrates, proteins, nucleic acids, amino acids, growth regulators, phospholipids, and fatty acids (Li, 1984).

Cold acclimation is associated with the synthesis of cryoprotective polypeptides (Artus et al., 1996; Steponkus et al., 1998) and the accumulation of compatible solutes with cryoprotective properties (Gilmour et al., 2000; Taji et al., 2002), such as free amino acids (e.g., proline), quaternary ammonium compounds (glycinebetaine), and carbohydrates (sucrose). These substances may play an important role in increasing internal osmotic pressure and preventing the loss of water from cells during freezing-induced dehydration (Nanjo et al., 1999; Thomashow, 1999).

The accumulation of free prolines in the leaves, shoots, and roots of angiosperms is one of the most common responses to stress, such as that induced by low temperatures (Chu et al., 1974). Changes in proline content during cold acclimation have been reported in perennial ryegrass (Draper, 1972), barley (Chu et al., 1974), alfalfa (Paquin, 1977), winter rape and winter wheat (Stefl et al., 1978), and annual bluegrass (Dionne, 2001b). van Swaaij et al. (1985) have evaluated the effect of cold acclimation and wilting in potato leaves, and have found that proline

contents can increase by 3- to 10-fold, without any change in their water status. Exogenous application of proline also increases frost tolerance in potato (van Swaaij et al., 1985). This rise in proline content may be more related to drought stress than to cold stress, although that association has not been demonstrated in *Solanum* (Levy, 1983). Studies with *Arabidopsis* also have shown that photoperiod and the conditions for initiating cold acclimation are highly associated with proline accumulations and enhanced freezing tolerance (Wanner and Junttila, 1999), and that proline levels in *Arabidopsis* plants ectopically expressing *Arabidopsis CBF* (c-repeat-binding factor) genes in conjunction with increased cold tolerance are similar to those measured in cold-acclimated plants (Liu et al., 1998; Gilmour et al., 2000).

Another common occurrence during cold acclimation is the accumulation of sugars, which act as effective cryoprotectants *in vitro* (Carpenter and Crowe, 1988) and confer protection to cell membranes under such stress (Sanitarius, 1973; Strauss and Hauser, 1986; Livingston and Henson, 1998; Vijn and Smeekens, 1999; Taji et al., 2002). Acclimation-related changes in sugar levels have been reported in many plant species (Gilmour et al., 2000; Hincha et al., 2000; Dionne et al., 2001a). For *Solanum* species, the contents of both free sugars and starch rise during cold acclimation, with the greatest increase occurring in *S. commersonii* (Chen and Li, 1980). Nevertheless, the accumulation of sugars cannot entirely explain the differences in cold acclimation capacity among *Solanum* species, as evidenced by increased sugar levels in *S. tuberosum* without any concomitant cold

acclimation when that species is grown under low temperatures (Chen and Li, 1980).

Guy (1990) has demonstrated that a specific subset of proteins is synthesized during cold acclimation. Furthermore, proteins in the dehydrin family are accumulated to high levels not only in response to low temperatures, but also during the late stages of embryogenesis, following exogenous applications of abscisic acid (ABA), or under drought stress (Close, 1996, 1997). COR, LEA, and similar soluble proteins that accumulate in cold-acclimated plants seem to be critical to the mechanism for developing freezing tolerance (Thomashow, 1998; Iba, 2002). Synthesis of soluble proteins also is highly correlated with freezing tolerance in some tuber-bearing *Solanum* species (Chen and Li, 1980). For example, cold acclimation in *S. commersonii* induces the production of several new polypeptides (Tseng and Li, 1987, 1990; Ryu and Li, 1994), a response that has also been reported in cell cultures of that species following ABA treatment (Lee et al., 1992).

The composition of lipid membranes is altered during cold acclimation in a wide range of plants, and many of those changes in membrane fluidity and composition are thought to be associated with the development of freezing tolerance (Palta and Li, 1980; Williams et al., 1988; Palta et al., 1993; Steponkus et al., 1993; Welti et al., 2002). Palta et al. (1993) have compared membrane lipids between *Solanum commersonii*, a freezing-tolerant species capable of cold acclimation, and *S. tuberosum*, which is freezing-sensitive and incapable of such

acclimation (Palta et al., 1993). Although both species show lower levels of palmitic acid and cerebrosides, but greater amounts of free sterols and sitosterol plus higher ratios of unsaturated to saturated fatty acids, only *S. commersonii* has an increase in phosphatidylethanolamine and linoleic acid, a decrease in linolenic acid content, plus a lower sterol to phospholipid ratio, and a higher acylated steryl glycoside to steryl glycoside ratio. Those results indicate that changes in lipid contents associated with increased freezing tolerance during cold acclimation are distinct from the differences in lipids in the non-acclimating state.

Chilling affects patterns of leaf growth and cell ultrastructure in some plant species, which suggests that such morphological characteristics may play an important role in the development of freezing tolerance (Kaku, 1973; Palta and Li, 1979; Ristic and Ashworth, 1993). Frost-tolerant potato plants exhibit significant changes in their leaf structure and cell wall thicknesses when grown at low temperatures, with double or triple palisade layers being observed in species that are capable of cold acclimation but not in the leaves of freezing-sensitive plants (Chen et al., 1977; Palta and Li, 1979; Estrada, 1982). Likewise, leaf cells enlarge due to the increased thickness of their mesophyll cells in conjunction with changes in the cell ultrastructure of *Arabidopsis* (Ristic and Ashworth, 1993) and winter oilseed rape (Stefanowsna et al., 1999, 2002).

Changes in Gene Expression Associated to Potato Frost Hardiness

Cold acclimation is associated with fluctuations in the expression of genes that are probably responsible for many biochemical and physiological changes

(Guy et al., 1985; Thomashow, 1999; Chinnusamy et al., 2006). In *Arabidopsis*, the expression of hundreds of genes is altered in response to low temperature, with many being regulated by the CBF cold-response pathway and functioning prominently in the cold acclimation process (Chinnusamy et al., 2006; van Buskirk and Thomashow, 2006).

In *S. commersonii*, altered gene expression during the development of freezing tolerance can be induced by cold acclimation or exogenous ABA application (Chen et al., 1983; Tseng and Li, 1987, 1990; Ryu and Li, 1994). Zhu et al. (1993) isolated four cDNA clones of ABA-responsive genes with high homology to tobacco osmotin. A cDNA clone encoding cyclophilin (CyP) has also been obtained from *S. commersonii* (Meza-Zepeda et al., 1998), again demonstrating that the level of cyclophilin mRNA increases in plants exposed to low temperatures, abscisic acid (ABA), drought, or wounding. That gene also responds to salicylic acid and pathogen challenges, playing a role in tolerating cold and other types of stress (Meza-Zepeda et al., 1998). cDNA encoding a putative RNA-binding glycine-rich protein (SCRGP-1) from a *S. commersonii* gene is induced by low temperatures, ABA, wounding, or drought in both *S. commersonii* and *S. tuberosum*, suggesting that the SCRGP-1 protein participates in the adaptation process leading to increased freezing tolerance (Baudo et al., 1999).

Rorat et al. (1997, 1998) isolated 24 cDNA clones (*Ssci*) corresponding to cold-induced mRNAs from a cDNA library of *Solanum sogarandinum*, a frost-hardy species similar to *S. commersonii* in its capacity for cold acclimation. Among

those clones, *Ssci*1, *Ssci*12, *Ssci*17, and *Ssci*20 show high homology with genes encoding S-adenosyl-L-methionine decarboxylase, TAS14 protein (dehydrin), glucosyl transferase, and the 22-kD PSBS protein from Photosystem II, respectively. These are the only ones with higher transcript levels when *S. sogarandinum* plants are cold-treated. Detailed northern blot analysis has revealed that the levels of transcripts that hybridize with *Ssci*17 and *Ssci*20 cDNAs are closely correlated with cold acclimation (Rorat et al., 1998).

Attempts to Transfer Frost Hardiness Genes from Wild Potato Species to Cultivated Potato

Although plants of *S. tuberosum* (2n = 4x = 48) are killed at temperatures below –3°C, and cannot be cold-acclimated, wild potato species, such as *S. acaule, S. commersonii, S. boliviense, S. chomatophilum, S. multidissectum, S. megistacrolobum*, and *S. sanctae-rosae*, survive when exposed to much lower freezing temperatures, i.e., –4.0 to –6.0 °C, and can be cold-acclimated after a period of chilling (Chen and Li, 1980; Costa and Li, 1993). The most cold-hardy of these is *S. commersonii* (2n = 2x = 24), a tuber-bearing, wild potato endemic to Argentina, Paraguay, and Uruguay that can tolerate temperatures as low as –10.0 °C after cold acclimation (Chen and Li, 1980).

Some attempts have been made to transfer frost hardiness genes from wild to cultivated potato species via traditional breeding. However, inserting the specific genes associated with cold acclimation and freezing tolerance is challenging. As an alternative, potato breeders have used somatic fusion, embryo rescue, and bridge

strategies to overcome the natural barriers from interspecific crossing between wild and cultivated species. However, linkage drag still limits the use of wild potatoes because many exotic genes and undesirable traits, e.g., a high alkaloid content or long stolons, can be transferred along with the acquisition of cold hardiness. Therefore, successful breeding schemes require time-consuming backcrosses, evaluations, and phenotypic selections to obtain an improved, cultivated phenotype (Cardi et al., 1993; Estrada et al., 1993; Pavek and Corsini, 2001; Iovene et al., 2004).

A study of the inheritance of freezing tolerance in the F1 generation and backcrosses between *S. commersonii x S. cardiophylum* has demonstrated that desirable tolerance and cold-acclimation traits are under independent genetic control and determined by a small number of genes (Stone et al., 1993; Valverde and Chen, 1999). Characterization of somatic hybrids between frost-tolerant *S. commersonii* and frost-sensitive *S. tuberosum* have revealed no appreciable increase in their extent of cold hardiness, with only a small improvement in freezing tolerance after cold acclimation but not in its absence (Cardi et al., 1993; Nyman and Waara, 1997; Palta et al., 1997). For example, offspring of the somatic hybrid *S. commersonii x S. tuberosum* is freezing-sensitive when not cold-acclimated (similar to a cultivated potato) but increases its freezing tolerance after that acclimation occurs (Chen et al., 1996). Furthermore, freezing tolerance has been characterized in selfed and back-crossed progenies derived from that same somatic hybrid (Chen et al., 1999). Aneuploid hybrids resulting from *5X x 4X*

crosses of S. *commersonii x S. tuberosum* also have been analyzed for their degree of tolerance and cold-acclimation capacity (Iovene et al., 2004). Their killing temperature (LT_{50}) under non-acclimated conditions does not differ from that of the crop-potato cultivars. In contrast, the LT_{50} for cold-acclimated genotypes ranges between the values determined for their wild and cultivated parents, with some hybrids displaying a capacity higher than 3 ºC (Iovene et al., 2004).

Some attempts have been made to enhance freezing tolerance in potato through gene transfer. For example, transgenic plants of *S. commersonii* that highly express sense and antisense genes for an osmotin-like protein (pA13) show improved tolerance to late blight but not to low temperatures, whereas transgenic plants expressing antisense genes for the osmotin-like protein have no alterations in either late blight or freezing tolerance (Zhu et al., 1996). Finally, *S. tuberosum* plants transformed with a fish antifreeze protein gene exhibit only a marginal increase in their cold tolerance (Wallis et al., 1997).

Molecular Biology of Cold Acclimation

Cold-sensing Mechanisms

New molecular approaches have been directed toward understand the mechanisms involved in cold-stress signaling and gene regulation by low temperatures. Changes in membrane fluidity may be the primary cold-stress sensor, leading to an increase in cytosolic calcium (Ca^{2+}), which subsequently inhibits protein phosphatase

activity (PP2A) and activates a series of phosphorylation/dephosphorylation events. These processes can then induce the expression of several cold-regulated (*cor*) genes, including CBF transcription factors (Plieth et al., 1999; Orvar et al., 2000; Sangwan et al., 2001; Chinnusamy et al., 2006; van Buskirk and Thomashow, 2006). In conjunction with calcium, cold-induced reactive oxygen species induce a kinase cascade response (AtMEKK1-MKK2-MPK4/6) that is required for cold acclimation in plants (Kovtun et al., 2000, Chinnusamy et al., 2004, 2006; Teige et al., 2004).

Abscisic acid serves as a secondary cold signal and possibly plays an important role in this acclimation (Chen et al., 1983; Xiong et al., 2001). ABA may transduce cold-stress signals through second messengers such as H_2O_2 and Ca^{2+}, and may also induce the expression of *CBF1 - 3* genes, although at a much lower level than that observed under low temperatures. Thus, this plant growth hormone may activate ICE1-CBF- dependent and -independent pathways, thereby maintaining the expression of *cor* genes during prolonged chilling (Knight et al., 2004).; However, the precise role of ABA is not entirely clear because, even though many cold-responsive genes are positively regulated by its application, most are also induced by cold in the absence of ABA (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight et al., 2004; Chinnusamy et al., 2006).

CBF Regulation

ICE1 (Inducer of CBF Expression 1) is a master regulator of cold acclimation, encoding a MYC-type basic helix-loop-helix (bHLH) transcription

factor that binds to a MYC cis-element in the AtCBF3 promoter and induces the expression of a C-repeat (CRT)-binding factor (*AtCBF3*) upon cold stimulus (Chinnusamy et al., 2003). ICE1 and ICE1-like proteins may be involved in the cold-responsive CBF-dependent and -independent pathways that induce the expression of *cor* genes (Chinnusamy et al., 2003, 2006; Zarka et al., 2003; van Buskirk and Thomashow, 2006). The ICE1-CBF pathway is possibly regulated negatively via ubiquitination, which may be mediated by the *HOS1* (*high expression of osmotically responsive*) gene. HOS1, which might negatively regulate CBF transcription by inducing the degradation of ICE1, is a RING finger protein with ubiquitin E3 ligase activity that interacts with ICE1 and represses the expression of *CBF*s and their downstream genes (Dong et al., 2006).

*CBF*s also self-regulate their transcription. For example, C_2H_2 zinc finger transcriptional repressors are positively regulated by *CBF*s and negatively regulated by the *LOS2* (*low expression of osmotically responsive*) gene. The ICE1-CBF pathway then positively regulates the expression of cysteine-2 and histidine-2 (C_2H_2) zinc finger transcriptional repressors, which are under the negative control of LOS2, a bi-functional enolase (Novillo et al., 2004; van Buskirk and Thomashow, 2006).

Other Cold-response Pathways

Arabidopsis has additional cold-response pathways that involve the ZAT12 cold-response pathways, as well as HOS9 and HOS10 transcription factors (van Buskirk and Thomashow, 2006). In the ZAT12 pathway, overexpression of ZAT12

(a zinc-finger protein) not only represses the expression of 15 genes that are down-regulated in response to low temperature but also diminishes the expression of 9 genes normally induced in response to low temperature, some of them may lead to increased freezing tolerance. The CBF2 and ZAT12 regulons share some common genes that are up-regulated and down-regulated by low temperatures, which suggests that these two pathways overlap. In addition, the constitutive overexpression of ZAT12, or one of the ZAT12 regulons, helps to negatively regulate the CBF1 and CBF3 cold-response pathway (Vogel et al., 2005).

Two transcription factors -- HOS9 (a homeodomain type) and HOS10 (a R2R3 myeloblastosis type) -- play pivotal roles in the regulation of *cor* genes and freezing tolerance, in a CBF-independent manner. Mutants of Hos9-1 and Hos10-1 show enhanced induction of *RD29A* and several other known cold-responsive genes, but not of the *CBF* genes (Zhu et al., 2004, 2005). The exact functioning of HOS9 and HOS10 is still not completely understood. Although both Hos9-1 and Hos10-1 plants express *cor* genes at higher levels than do the wild-type (WT) plants, they continue to exhibit less freezing tolerance than the WT, both before and after cold-acclimation treatments.

Transformation with *CBF/DREB* Genes and Enhanced Freezing Tolerance in Crop Plants

Transcriptional activator *CBFs*, also known as *DREB* (dehydration-responsive-element-binding) factors, play an important role in counteracting abiotic

stresses, including freezing and drought (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Medina et al., 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). In *Arabidopsis*, the CBF pathway has a much greater effect on cold acclimation than any other pathway (van Buskirk and Thomashow, 2006).

*CBF*s contain an AP2/EREBP DNA-binding domain that can bind to a cold and dehydration-responsive regulatory sequence known as a CRT/DRE element (C-repeat/dehydration responsive element). This element contains the conserved CCGAC core sequence common to promoter sequences in many cold-responsive genes (Horvath et al., 1993; Nordin et al., 1993; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al., 1995). Many target stress-inducible genes of *CBF* have been identified, using both cDNA and Gene Chip microarrays. Most contain the CRT/DRE element or a related sequence in their promoter region (Kasuga et al., 1999; Fowler and Thomashow, 2002; Maruyama et al., 2004; Chinnusamy et al., 2006). The majority of these genes are up-regulated in response to chilling and *CBF* overexpression. Transcripts of *CBF1*, *CBF2*, and *CBF3* are detectable within 15 min after plants are exposed to low temperature (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Medina et al., 1999), which suggests that the inducer of *CBF* expression is present at warm temperatures, but is then activated by a post-transcriptional mechanism in response to chilling (van Buskirk and Thomashow, 2006).

Orthologous genes of *CBF*s have been identified in a wide range of herbaceous and perennial plants, indicating that a CBF cold-response pathway may

be highly conserved among species (Owens et al., 2002; Dubouzet et al., 2003; Kitashiba et al., 2004; Skinner et al., 2005; Benedict et al., 2006). Constitutive overexpression of *CBF1 - 4* in *Arabidopsis* induces the expression of *cor* genes, and enhances cold tolerance in the absence of any low-temperature stimulus (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Haake et al., 2002; Maruyama et al., 2004). Transgenic *Arabidopsis* plants overexpressing *CBF3* show greater tolerance to freezing, salt, and drought (Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000; Maruyama et al., 2004). Several of the biochemical changes observed in cold-acclimated *Arabidopsis* can also be found in transgenic *Arabidopsis* plants that constitutively over-express *CBF3*, including altered contents of proline and sugars, e.g., glucose, fructose, sucrose, and raffinose (Gilmour et al., 2000).

Photosynthetic activities become adapted to low temperatures in conjunction with *cor* expression. In barley, *COR14*, whose expression is regulated by a *CBF/DREB* transcription factor, accumulates in the chloroplast stroma in response to light and low temperature (Crosatti et al., 1995, 1999; Ensminger et al., 2006). Furthermore, the overexpression of two *Brassica* CBF/DREB1-like transcription factors in canola plants enhances their freezing tolerance (Savitch et al., 2005). Interestingly, overexpression of these *CBF*-like transcription factors induces the expression of genes involved in chloroplast development and photosynthetic capacity, such that those lines have higher rates of photosynthesis under low temperatures than do non-transformed WT plants (Savitch et al., 2005).

Exploiting the overexpression of *CBF*s is an effective approach to improving stress tolerance in many plant species (Table 1.1) (Holmberg and Bulow, 1998; Bajaj et al., 1999; Zhang et al., 2000; Zhang and Blumwald, 2001). For example, *AtCBF1* overexpression in canola (*Brassica napus*) plants activates the expression of *cor* genes and improves tolerance at non-acclimating temperatures (Jaglo et al., 2001). Transgenic tomato plants that constitutively over-express *AtCBF1* have enhanced tolerance to oxidative or chilling stress but not to freezing (Hsieh et al., 2002a, b). Tobacco plants overexpressing *CBF3/DREB1A* also exhibit improved tolerance to drought, salt, or cold (Kasuga et al., 2004). Finally, compared with WT plants, poplars that ectopically express *AtCBF1* show increased freezing tolerance in their non-acclimated leaves and stems (Benedict et al., 2006).

All of these previous studies demonstrate that *CBF* genes can be used to improve abiotic stress tolerance in agriculturally important crops. Nevertheless, the constitutive overexpression of *Arabidopsis CBF* genes can also result in undesirable phenotypical alterations in the transgenics. For example, plants that constitutively over-express *CBF3/DREB1A* show improved freezing tolerance but also manifest severe growth retardation and developmental delays in their flowering under normal growing conditions (Liu et al., 1998; Gilmour et al., 2000). Similar outcomes have been reported with transgenic tomato and tobacco plants over-expressing *AtCBF1* or *AtCBF3,* respectively (Hsieh et al., 2002a,b; Kasuga et al., 2004). Therefore, it has been proposed that the stress-inducible rd29A promoter, rather than a constitutive 35SCaMV promoter, be used for the

overexpression of *CBF* genes to avoid the occurrence of those changes (Kasuga et al., 1999, 2004). In fact, *Arabidopsis* and tobacco studies have shown that such replacement can minimize the negative effects on transgenic plant growth (Kasuga et al., 1999, 2004).

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Table 1.1 Examples of transgenic plants that over-express CBFs, resulting in enhanced cold tolerance

Gene	Promoter	Origin	Transgenic species	Phenotypic expression	References
CBF1	35SCaMV	*Arabidopsis*	*Arabidopsis*	Increased freezing tolerance	Jaglo-Ottosen et al. (1998)
CBF1	Rd29 and 35SCaMV	*Arabidopsis*	*Arabidopsis*	Increased tolerance to cold, drought, and salinity	Kasuga et al. (1999)
CBF1	35SCaMV	*Arabidopsis*	Tomato	Enhanced tolerance to oxidative and chilling stresses	Hsieh et al. (2002a, b)
CBF1	35SCaMV	*Arabidopsis*	Strawberry	Increased freezing tolerance	Owens et al. (2002)
CBF1	ABA/stress inducible	*Arabidopsis*	Rice	Enhanced stress tolerance	Lee et al. (2003; 2004)
CBF1	35SCaMV	*Arabidopsis*	Poplar	Increased freezing tolerance	Benedict et al. (2006)
CBF3	35SCaMV	*Arabidopsis*	*Arabidopsis*	Increased freezing tolerance	Liu et al. (1998); Gilmour et al. (2000)
CBF3	35SCaMV	Rice	*Arabidopsis*	Increased tolerance to cold, drought, and salinity	Dubouzet et al. (2003)
CBF3	Rd29 and 35SCaMV	*Arabidopsis*	Tobacco	Increased drought and cold -stress tolerance	Kasuga et al. (2004)
CBF3	Ubi1	*Arabidopsis*	Rice	Increased stress tolerance	Oh et al. (2005)
CBF4	35SCaMV	*Arabidopsis*	*Arabidopsis*	Increased freezing tolerance, *cor* gene expression	Haake et al. (2002)
CBF1, *CBF2*, *CBF3*	35SCaMV	*Arabidopsis*	Canola	Increased freezing tolerance	Jaglo-Ottosen (1998), Jaglo et al. (2001)
CBF	35SCaMV	Sweet cherry	*Arabidopsis*	Increased freezing and salt tolerances	Kitashiba et al. (2004)

CHAPTER 2

ECTOPIC *AtCBF1* OVEREXPRESSION ENHANCES FREEZING TOLERANCE AND INDUCES COLD ACCLIMATION-ASSOCIATED PHYSIOLOGICAL MODIFICATIONS IN POTATO

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Abstract

We compared the physiological alterations that occur in freezing-sensitive *Solanum tuberosum* L. cv Umatilla and freezing-tolerant *Solanum commersonii* Dun (PI243503 Clone 13) during cold exposure, and the effect of ectopic *Arabidopsis CBF1* overexpression on these alterations. Ectopic *AtCBF1* overexpression yielded a significant freezing tolerance gain of 2°C for *S. tuberosum* and up to 4°C for *S. commersonii* relative to wildtype. Transgenic *S. commersonii* lines displayed improved cold acclimation capacity, while transgenic *S. tuberosum* lines were still incapable of cold acclimation. During cold treatment, leaves of wildtype *S. commersonii*, but not *S. tuberosum*, showed significant thickening due to palisade cell lengthening and intercellular space enlargement. Ectopic *AtCBF1* overexpression induced palisade cell elongation and increased leaf thickness in the absence of cold in both *S. commersonii* and *S. tuberosum*. Ectopic AtCBF1 activity also mimicked cold treatment by increasing proline and total sugar

content in the absence of cold in *S. commersonii*. Relative to wildtype, transgenic *S. commersonii* leaves were darker green, confirmed to contain higher chlorophyll and displayed greater photosynthetic capacity, suggesting the plants may have higher productivity potential. These results suggest that the endogenous CBF pathway is involved in many of the structural, biochemical and physiological alterations associated with cold acclimation in potato.

Keywords: *Solanum*, *commersonii*, *tuberosum*, *CBF*, freezing tolerance, leaf structure, anatomy.

Introduction

The agricultural range of many important crop species is limited by their maximum freezing tolerance capacity and damage resulting from freezing stress can result in considerable crop-productivity loss. Most freezing-tolerant plant species are capable of cold acclimation, a process whereby a plant increases its overall freezing tolerance during exposure to low but non-freezing temperatures (Chen and Li, 1980a). Many important plant species and varieties however are freezing-sensitive and/or incapable of cold acclimation. Considerable research effort has therefore been focused on understanding the basis of plant freezing tolerance and the differences between freezing-tolerant and freezing-sensitive species.

Cultivated potato (*Solanum tuberosum*) comprises the majority of the economically and agronomically important potato cultivars. While produced in diverse climates including where frost events occur, *S. tuberosum* is a frost-sensitive species incapable of cold acclimation, having a maximum freezing tolerance of about –3°C both before and after exposure to low temperatures (Chen and Li, 1980a). Even a brief exposure to frost can significantly reduce *S. tuberosum* yields, while hard frosts can completely destroy entire fields. Gains in freezing tolerance capacity of even a few degrees would be of considerable benefit. Some wild potato species (e.g., *S. acaule* and *S. commersonii*) are much more frost hardy than *S. tuberosum. They are* capable of cold acclimation, and are a potential gene source for breeding of *S. tuberosum* varieties with increased freezing tolerance. *S. commersonii* can survive to about –5ºC pre-acclimation, and to as low as –10ºC after becoming fully cold-acclimated (Chen and Li, 1980a; Costa and Li, 1993). Breeding efforts to date using wild potatoes to increase *S. tuberosum* freezing tolerance capacity have been proven time consuming and have yielded neither a significant increase in freezing tolerance nor cold acclimation capacity (Cardi et al., 1993a,b; Estrada et al., 1993; Iovene et al., 2004). Part of these results are explained because many wild species are sexually incompatible with cultivated potato due to differences in endosperm balance number (EBN) effective ploidy (Johnston et al. 1980). This incompatibility can be circumvented by ploidy level manipulation or somatic hybridization, however some somatic hybrids were also found to be chilling sensitive i.e. more sensitive than *S.*

tuberosum, and developed chlorosis during cold acclimation (Nyman and Waara 1997). Further use of somatic hybrids in breeding programs is problematic as most of them are male sterile (Cardi et al. 1993a, b; Nyman and Waara 1997).

In addition to crossing barriers between cultivated potato and wildtype potato, the inheritance of freezing tolerance and acclimation capacity is complex and it is best explained by an additive-dominance model, involving changes in the expression of numerous genes (Guy et al., 1985; Sutka and Veisz, 1988; Thomashow, 1990; Tseng and Li, 1990; Stone et al., 1993; Chinnusamy et al., 2006). Microarray studies demonstrated that the expression of over 500 genes in *Arabidopsis* is altered in response to cold (Vogel et al., 2005). These gene expression changes result in modification of many structural, biochemical, and photosynthetic properties which subsequently facilitate an increase in the plant's tolerance to freezing stress (Guy, 1990).

In most plant species, one commonly observed biochemical change is the accumulation of compatible solutes that confer cryoprotective properties (Wanner and Junttila, 1999; Gilmour et al., 2000; Iba, 2002). Studies in a variety of plant systems have reported increases in sugar content during cold treatment, suggesting sugar accumulation and cold acclimation are associated (Livingston and Henson, 1998; Hincha et al., 2000; Dionne et al., 2001a). In potato, Chen and Li (1980b) found that both free sugars and starch increase during cold acclimation. While the exact role of increased sugar content has not been determined, it appears sugars may help stabilize cellular membranes by protecting them against freeze-induced

damage. In addition to sugars, other compatible solutes involved in freezing tolerance are amino acids and amino acid derivatives. Proline in particular seems to play a major role and its accumulation in response to cold is observed in practically all plant species, including potato (van Swaaij et al., 1985; Dionne et al., 2001b; Iba, 2002). Genes specifying soluble polypeptides (e.g., dehydrins) are also typically induced to high levels by cold (Guy, 1990) and increased soluble polypeptide contents have been correlated with freezing tolerance in some *Solanum* species (Chen and Li, 1980b).

Changes in leaf and cell structural characteristics may also play an important role in cold acclimation and freezing tolerance (Kaku, 1973; Palta and Li, 1979; Ristic and Ashworth, 1993; Stefanowska et al., 1999; Iba, 2002). Changes in palisade layer number have been observed in cold-hardy potatoes (Palta and Li, 1980), while cold acclimation-associated enlargement of leaf mesophyll cells occurs in *Brassica napus* (Stefanowska et al., 1999). Changes in the photosynthetic apparatus also occur during cold treatment, with photosynthetic competence being substantially reduced by low temperature (Flexas et al., 1999). Frost damage causes significant alterations in photosynthetic efficiency and in particular when damaged plants are exposed to high light intensities following cold stress (Steffen and Palta, 1989). Studies in potato show that during freezing stress, photosynthesis is inhibited transiently in *S. commersonii,* whereas it is highly reduced in *S. tuberosum* and causes irreversible damage (Seppänen and Coleman, 2003).

A majority of the recent advancements in elucidating the molecular bases of these processes were made using the model plant *Arabidopsis*. A major breakthrough was the identification of the CBF transcriptional regulatory factors *CBF1-3*, which are cold-induced and control the cold-responsive expression of a major regulon of genes that increase the cold tolerance of a plant (reviewed in van Buskirk and Thomashow, 2006). The *Arabidopsis CBF1* gene (*AtCBF1*) has been used to increase freezing tolerance in a number of diverse plant species, including *Brassica napus* (Jaglo et al., 2001), strawberry (Owens et al., 2002), and poplar (Benedict et al., 2006) among others. Transgenic ectopic expression of *CBF* genes under warm conditions activates a suite of genes that results in an increase in the freezing tolerance of the plant without a cold stimulus. Ectopic CBF expression induces many of the biochemical changes normally observed during exposure to cold, allowing insight into which processes may involve the CBF-response pathway. CBF factors appear to be ubiquitously present in plants regardless of freezing tolerance capacity, and analysis of the EST sequence database reveals that at least four distinct *CBF* genes are encoded for in potato (J. Skinner, unpublished data).

We are interested in determining why *S. tuberosum* is deficient in freezing tolerance capacity in relation to it wild relative *S. commersonii*, what freezing tolerance pathways and modifications are missing or disrupted, and what the molecular basis of these alterations may be. In the current study, we evaluated the similarities and differences in the physiological modifications that occur during

cold acclimation between freezing-sensitive *S. tuberosum* and freezing-tolerant *S. commersonii*. We also transformed both species with the well-characterized *AtCBF1* gene under control of a constitutive promoter to evaluate whether freezing tolerance was enhanced, which adaptative responses might be part of a CBF-response pathway, and if there were differences in response to ectopic *CBF* expression between these two closely-related *Solanum* species.

Materials and Methods

Plant materials, transformation, and transgenic line identification

A 35S::*Arabidopsis CBF1* (*AtCBF1*) cDNA constitutive expression operon was ligated as a *Hin*dIII cassette into the *Hin*dIII-cut binary vector pGAH to yield pGAH-35S::*AtCBF1* (Benedict et al., 2006). Plantlets of *S. commersonii* (PI 243503 clone 13) and *S. tuberosum* L (cv. Umatilla) were propagated *in vitro* on sucrose-supplemented (20g/l), hormone-free Murashige and Skoog (MS) medium-Agar 7g L^{-1} at 25ºC with constant illumination (95-100µmol $m^{-2}s^{-1}$, cool white fluorescent lights). The pGAH-35S::*AtCBF1* plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105 and suspensions grown overnight (28°C, 240 rpm) in liquid YEP plus 50 mg L^{-1} kanamycin to an OD_{600}=0.5–0.7. Cells were collected by centrifugation (2500 rpm, 10 min), resuspended in liquid MS-2% sucrose medium (pH 5.2), and used to transform young leaf and stem explants of both potato species as described below.

Explants of *S. commersonii* were pre-cultivated in MS-2% sucrose medium (pH 5.7) with 5 mg L^{-1} 2iP and 2 mg L^{-1} IAA for two days, incubated 15 min (RT, 50 rpm) in the bacterial suspension plus 20 mg L^{-1} acetosyringone, then co-cultivated on MS-2% sucrose medium (pH 5.2) supplemented with 5 mg L^{-1} 2iP, 2 mg L^{-1} IAA, and 20 mg L^{-1} acetosyringone for 2-3 d at 25°C in the dark. Next, explants were washed three times in MS-2% sucrose medium (pH 5.7) with 250 mg L^{-1} cefotaxime, blotted dry on sterile paper towels for 30 s, then transferred to callus induction medium: MS-2% sucrose medium (pH 5.7) supplemented with 5 mg L^{-1} 2iP, 2 mg L^{-1} IAA, 200 mg L^{-1} cefotaxime, and 50 mg L^{-1} kanamycin. Similarly, explants of *S. tuberosum* were pre-cultivated in MS-2% sucrose medium (pH 5.7) with 2 mg L^{-1} BAP and 0.1 mg L^{-1} IAA for two days, incubated 15 min (RT, 50 rpm) in the bacterial suspension plus 20 mg L^{-1} acetosyringone, then co-cultivated on MS-2% sucrose medium (pH 5.2) supplemented with 2 mg L^{-1} BAP, 0.1 mg L^{-1} IAA, and 20 mg L^{-1} acetosyringone for 2-3 d at 25°C in the dark. Next, explants were washed three times in MS-2% sucrose medium (pH 5.7) supplemented with 250 mg L^{-1} cefotaxime, blotted dry on sterile paper towels for 30s, then transferred to callus induction medium: MS-2% sucrose medium (pH 5.7) with 2 mg L^{-1} BAP, 0.1 mg L^{-1} IAA, 200 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin. Explants of both species were transferred to fresh callus induction medium every three weeks and regenerated shoots transferred to hormone-free MS-2% sucrose medium containing the same antibiotic concentrations (200 mg L^{-1} cefotaxime, 50 mg L^{-1}kanamycin). Kanamycin-resistant rooted shoots were propagated *in vitro* and leaves of rooted

plantlets were analyzed for transgene integration via PCR using the primers 35S-P.001 (5'-cacgtcttcaaagcaagtgg-3') and AtCBF1.002 (5'-ccttcgctctgttccggtgtataaat-3').

Plant Growth Conditions

Rooted explants of independent transgenic pGAH-35S::AtCBF1 lines (referred to as p35S::AtCBF1 lines hereafter) and untransformed controls were transferred to Sunshine SB40 mix (Sun Gro Horticulture Inc., Bellevue, WA) with controlled-release fertilizer (Osmocote, The Scotts Company, Marysville, OH) and maintained under greenhouse conditions (16/8h day/night photoperiod, 400-480 $\mu molm^{-2}s^{-1}$ light intensity supplemented with 300-400 $\mu molm^{-2}s^{-1}$ light supplied via SUN System III lamps (Sunlight Supply, Inc, Vancouver, WA) at 25±3ºC) prior to transfer to experimental conditioning treatments. Plants were fertilized weekly with foliar fertilizer (J.R. Peters, Allentown, PA). Unless noted otherwise, plants used in experimental trials were transferred from the above greenhouse conditions to a Percival Model MB60B growth chamber (16/8h photoperiod, 350µmol $m^{-2}s^{-1}$PAR at 25ºC) for three days to acclimate to the controlled environmental conditions before the collection of experimental warm plant material. For cold-treated plants, following the three day controlled environmental conditioning; plants were transferred to a cold room maintained at 2°C (16 h photoperiod; Very High Output Phillips CW/VHO fluorescent bulbs, 75 µmole $m^{-2}s^{-1}$ light intensity) for two weeks, unless specified otherwise, before harvesting of plant material.

Northern and gene expression analysis

Thirteen independent transgenic *S. commersonii* lines, 19 independent transgenic *S. tuberosum* lines and untransformed control plants growing under greenhouse conditions were directly screened for transgene expression via northern blot analysis. RNA was extracted from leaf tissue using RNeasy Plant Mini kits (Qiagen, Valencia, CA) and 20 μg total RNA per sample was electrophoretically separated and transferred to a nylon membrane as previously described (Skinner et al., 2005). Blots were probed in Ultrahyb solution (Ambion Inc., Austin, TX) and washed following the manufacturer's guidelines; labeled probes were generated using a High Prime Labeling Kit (Roche Biochemicals, Indianapolis, IN). The AtCBF1 probe excluded the conserved AP2 domain and consisted of only the C-terminal domain and 3' UTR to minimize cross hybridization to endogenous potato *CBF*s. A cloned potato ubiquitin fragment, amplified via the primers StUbiq.001 (5'-gcagttggaggacggacgt-3') and StUbiq.002 (5'-ggccatcttccaactgtttcc-3'), was used as a loading control probe. Probed blots were exposed and scanned using an MD-SI PhosphorImager system (Amersham Biosciences, Piscataway, NJ).

Controlled freeze tests

Freezing tolerance of wild type and transgenic plants was determined via controlled freezing tests (Sukumaran and Weiser, 1972) on leaf tissue of warm and two week cold-treated plants. For each sample and temperature point evaluated, three independent experiments were conducted using three replicate samples per experiment. Briefly, three 10 mm leaf discs were collected from fully expanded third and four leaves per sample assayed and placed in 16×120 mm test tube. Tubes

were incubated at -1ºC in a cooling bath (NESLAB, Model LT-50DD, Newington, NH) for 1 h. Ice nucleation was initiated by adding an ice chip to each tube, samples maintained at -1.5 ºC for an additional 1 h, and then the temperature was lowered 1ºC/h. Sample tubes were removed at -2, -4, -6, -8 -10, -12, and -14°C, and slow-thawed overnight at 2°C. Freezing injury of thawed leaf samples was assessed by determining electrolyte leakage using a YSI Model 35 conductance meter (Yellow Springs, OH). Following conductivity measurements, all samples were frozen at -20°C for 24 h, thawed at room temperature, and total conductivity determined. LT_{50} values (temperature causing 50% electrolyte leakage) were plotted as a function of freezing temperature. For the time course study, LT_{50} values were determined as above on samples following 0, 1, 2, 4, 7, 14 and 21 days cold treatment.

Plant morphological and histological analysis

Leaf structural analysis was conducted on fully expanded third and four leaves of warm and two week cold-treated plants. Three leaves were collected from each of three individual plants (nine leaves total) for each line or control evaluated; three independent experimental replications were performed. For each individual leaf harvested, ten cross sectional segments were prepared. Briefly, sections were cut through the leaf midrib, fixed in FAA, and dehydrated in a graded ethanol (50% through 95%) series. Samples were transferred to a 1:1 plastic infiltration: 95% ethanol solution, vacuum infiltrated for 12 h (20-25 inches Hg), then infiltrated as above with pure infiltration solution. Leaves were embedded in Technovit 7100

glycol methacrylate plastic (Energy Beam Sciences, East Granby, CT) and rotary microtome-prepared 4-5 micron sections mounted on glass slides. Sections were stained in 0.5% Toluidine Blue O dissolved in citrate buffer (pH 4.2). Cell and leaf thicknesses were visualized using a Nikon light microscope (Model LABOPHOT-2) at 40X magnification and quantitative measurements of cell structure, palisade cell length (μm), and total leaf thickness (μm) determined using a mounted micrometer.

Carbohydrate and proline analysis

Total soluble sugar and proline content were determined from leaf tissue of fully expanded third and four leaves of warm and two week cold-treated plants. For both assay types, three leaves were collected from each of three independent plants of each sample analyzed; three independent replications were performed per sample. Leaf tissue was collected, immediately frozen and macerated in liquid N_2, then stored at -80°C until analysis. Carbohydrate (20 mg lyophilized tissue per sample) and proline (30 mg lyophilized tissue per sample) analyses were conducted as described in Gilmour et al. (2000); carbohydrate analysis was done using the phenol-sulfuric acid method (Dubois et al, 1956).

Pigment Analysis

Pigment analysis was conducted using leaf tissue of fully expanded third and four leaves of warm and two week cold-treated plants. For each sample analyzed, 10 mm leaf discs were collected from three leaves each of three independent plants, with three discs harvested per leaf (27 leaf discs total per

sample); three independent replications were performed per sample. For chlorophyll analysis, the fresh weight of each leaf disc sample set was determined, ground into a fine powder in liquid N_2 before adding to 80% (v/v) acetone and vortexing for 1 min. Particulate matter was pelleted by centrifugation (10,000 x *g*) for 3 min and the absorbance of the clarified supernatant determined at 653.4 nm (Chl *a*), 665.4 nm (Chl *b*), and 470 nm (carotenoids); pigment concentrations (μg/g FW) were calculated as described by Lichtenthaler (1988). For anthocyanin analysis, leaf disc fresh weight was determined, ground into a fine powder in liquid N_2 before adding to 3M HCl:H_2O:MeOH (1:3:16) and vortexing for 1 min. Particulate matter was pelleted by centrifugation (10,000 x *g*) for 3 min and the absorbance of the clarified supernatant determined at 530 nm and 653 nm; anthocyanin concentration (μg/g FW) was calculated as in Gould et al. (2000).

Gas exchange and chlorophyll fluorescence

Measurements of photosynthetic parameters were conducted as in Schittenhelm et al. (2004) and Seppänen and Coleman (2003) on fully expanded second leaves of warm and two week cold-treated plants. For each sample type analyzed six replications were performed; assessment order of plants and leaf measurements was randomly done for each replication. The response of net photosynthesis (A) to both photosynthetically active radiation (PAR) and to intercellular CO_2 concentration (C_i) was determined. The light response curve (A/PAR curve) was determined as the rate of net photosynthesis at 0, 250, 500, 1000 and 1500 $\mu molm^2s^{-1}$ PAR using an external leaf CO_2 concentration (C_a) of

400 ppm. Net photosynthetic response to intercellular CO_2 concentration (A/C_i curve) was determined at different C_a levels (0, 85, 170, 370, 780, 1200 ppm) using a saturation point of 1500 µmol $m^{-2}s^{-1}$ PAR. Gas exchange measurements were conducted using a 400 ppm C_a and a 1500 µmol saturation point $m^{-2}s^{-1}$ PAR. Both net photosynthetic response (A/C_i) and gas exchange rates were determined using an automated cuvette unit (2.5 cm^2 leaf area, 25ºC constant air temperature) of an open gas Ciras-1 Exchange System (PP System, Hitchin, UK). For chlorophyll fluorescence measurements, leaves were dark adapted for 30 min at the plant growth treatment temperature (2ºC or 25ºC) and the Fv/Fm ratio measured using a pulse modulated fluorometer (Type FMS1, Hansatech, England) as directed.

Statistical analyses

Data was statistically analyzed using analysis of variance (ANOVA) and the differences of value means were compared using Duncan's Multiple Range test. Associations between LT_{50} and cold treatment period, A and PAR, and A and C_i, were determined using regression analysis. All statistical analyses were performed using the SAS statistical program (SAS, 2000).

Results

Ectopic *AtCBF1* over-expression causes transient and stable morphological alterations in transgenic potato plants

To examine the phenotypic differences in how *S. commersonii* and *S. tuberosum* adapt to cold and which processes may be CBF-dependent, we

generated transgenic lines of each species ectopically expressing *AtCBF1* for comparison with wildtype plants. We verified the presence of the 35S::*AtCBF1* transgene cassette in 13 independent *S. commersonii* and 19 independent *S. tuberosum* lines via PCR, then employed northern expression analysis to determine the subset of lines ectopically expressing the transgene. In *S. commersonii*, 11 of the 13 lines showed detectable *AtCBF1* transgene expression (Figure 2.1B), while only three of the 19 *S. tuberosum* lines displayed detectable expression in contrast (Figure 2.1C).

We selected all three of the *S. tuberosum* (T1.2, T1.11, and T1.15) and ten of the eleven *S. commersonii* (C1.1, C1.2, C1.3, C1.4, C1.6, C1.7, C1.9, C1.10, C1.11, C1.15) AtCBF1-expressing lines for phenotypic comparisons with wildtype plants. Phenotypically, the three *S. tuberosum* 35S::AtCBF1 lines displayed slight growth retardation in tissue culture, while non-expressing lines were similar to wildtype (data not shown). Following transplantation to soil, the expressing lines recovered within a few weeks and were similar in growth phenotype to wildtype plants by week four (Figure 2.2A). While the color, shape, and size of the leaves were similar to wildtype plants (Figure 2.2A), all three lines were delayed about 2-3 weeks in flowering relative to wildtype (data not shown). Ectopic *CBF* expression had a more pronounced and sustained effect in *S. commersonii* by contrast (Figure 2.3). In tissue culture, many transgenic lines displayed growth retardation, a prostrate growth habit, and altered plant and leaf morphological characteristics relative to wildtype (Figure 2.3A). Following transplantation to soil, the majority of the

transgenic lines recovered and displayed a relatively wildtype-like growth habit. Lines C1.1, C1.9, and C1.11 were an exception and exhibited sustained growth retardation and dwarfing (Figure 2.3C). Despite recovery of a nearly normal growth habit in soil for most of the *S. commersonii* lines, altered leaf characteristics relative to morphology, color, and size were retained compared to wildtype (Figure 2.3B). Additionally, transgenic lines were delayed in flowering by about 2-3 weeks relative to wildtype, while line C1.9 failed to flower (data not shown).

AtCBF1 over-expression increases potato freezing tolerance

In an analysis of freezing tolerance and cold acclimation capacity, wildtype *S. commersonii*, a freezing-tolerant potato species, displayed a gain in freezing tolerance of about 4°C (-6°C pre-acclimation to -10°C post-acclimation) after two weeks of cold acclimation (Figure 2.4A). Freezing-sensitive wildtype *S. tuberosum*, as expected, was unable to cold acclimate and displayed a much lower freezing tolerance capacity of only about -3°C, both before and after two weeks of cold treatment (Figure 2.2B). A time course study through three weeks cold treatment showed that most of the *S. commersonii* freezing tolerance gain was obtained during the first two days of cold treatment, with only a minor additional gain occurring over the next 19 days (Figure 2.4C). *S. tuberosum* on the other hand, showed no gain in freezing tolerance over this same time period (Figure 2.2C), confirming that *S. tuberosum* lacks the ability for cold acclimation.

In *Arabidopsis* and other plant species, ectopic *CBF* transgene expression bypasses the need for cold acclimation to increase whole plant freezing tolerance

(van Buskirk and Thomashow, 2006). We evaluated whether *AtCBF1* over-expression could promote a freezing tolerance gain in either the freezing-tolerant and/or freezing-sensitive potato species. Controlled freeze tests on 35S::AtCBF1 *S. tuberosum* lines grown at 25°C demonstrated a gain in freezing tolerance of about 2ºC (Figure 2.2B). Similar to wildtype *S. tuberosum*, this gain was unaffected by cold treatment (Figure 2.2B-C), indicating that introduction of a known functional *AtCBF1* gene is insufficient to restore or introduce a cold acclimation response in this species. Similarly, *AtCBF1* transgene expression resulted in a gain in freezing tolerance of about 2° to 4°C for non-acclimated (25°C-grown) *S. commersonii* lines relative to wildtype (Figure 2.4A). The two highest 35S::AtCBF1-expressing lines (C1.6, C1.7) exhibited a significantly greater gain in freezing tolerance post-acclimation relative to all other lines, while the remaining low and medium expressing lines all similarly displayed a lower gain. We evaluated three of the transgenic *S. commersonii* lines in a three week cold treatment time course study. In contrast to *S. tuberosum* (Figure 2.2C), all three 35S::AtCBF1 *S. commersonii* lines increased in freezing tolerance over the time course, with the two high expressing lines (C1.6, C1.7) maintaining an ~4°C gain in capacity relative to wildtype after three weeks (Figure 2.4C).

Cold treatment and *AtCBF1* over-expression affect potato leaf cell structure

As changes in leaf and cell structural characteristics are implicated in freezing tolerance and leaf phenotype was affected in transgenic *S. commersonii* lines (Figure 2.3), we investigated the anatomy of wildtype and transgenic leaves before

and after cold-treatment. A comparative cross-sectional analysis revealed that in *S. commersonii* wildtype plants, two weeks cold acclimation resulted in a significant thickening of leaves to nearly double that of the warm controls (Table 2.1). This thickness increase was a result of palisade cell length elongation and an increase in intercellular spaces due to a more loosely packed spongy parenchyma matrix relative to warm controls (Table 2.1, Figure 2.5). In contrast, wildtype *S. tuberosum* leaves were unaltered by cold-treatment relative to thickness and cell structure (Table 2.1, Figure 2.6).

Analysis of leaf characteristics in three transgenic *S. commersonii* lines revealed that ectopic *AtCBF1* expression induced similar changes under warm conditions as observed in cold acclimated wildtype controls. Specifically, leaf thickness and palisade cell length increased beyond those of cold-acclimated wildtype plants (Table 2.1, Figure 2.5). Likewise, the intercellular spacing and spongy parenchyma packing were altered as in cold-acclimated wildtype plants. Cold treatment of the transgenic *S. commersonii* lines further enhanced these characteristics, though not all gains were significant relative to those observed under warm conditions. In the transgenic *S. tuberosum* lines, *AtCBF1* expression also induced both a significant thickening of the leaf and increase in palisade cell length prior to cold treatment; cold treatment did not result in significant additional gains for these characteristics (Table 2.1, Figure 2.6). In general, intercellular spacing and spongy parenchyma packing were unaltered in transgenic *S. tuberosum* regardless of warm or cold treatment.

Ectopic *AtCBF1* expression induces biochemical changes associated with cold acclimation in *S. commersonii*

Proline and sugars are two cryoprotectant metabolites that are commonly induced in plant systems in response to cold regardless of cold acclimation capacity. As *S. commersonii* and *S. tuberosum* are closely related species contrasting for cold acclimation capacity, we assessed how cold affected these metabolite levels and whether the AtCBF1 transgenic plants mimicked any cold-based alterations that occurred. We selected one low (C1.4) and two high (C1.6, C1.7) *S. commersonii AtCBF1*-expressor and the three *S. tuberosum* lines for characterization of proline and total sugar levels. In both *S. commersonii* wildtype and transgenic plants harboring *AtCBF1* two week cold treatment causes a significant increase in proline content (Table 2.2); lines C1.6 and C1.7 even under warm conditions (25°C) increased proline content in about 2 fold in relation to untransformed wildtype. Cold treatment resulted in further significant increases in proline content in all transgenic lines. Similar results were obtained for sugar content where total sugars increased following cold treatment in wildtype *S. commersonii*. In the three transgenic lines, sugar content was significantly greater in warm-grown plants than even the wildtype cold-treated levels (Table 2.2); cold-treatment resulted in further sugar content gains for the transgenic lines. Wildtype and transgenic *S. tuberosum* did not show significant changes in either metabolites with cold treatment or with *AtCBF1* overexpression.

Ectopic *AtCBF1* expression increases photosynthetic capacity of *S. commersonii*

In addition to structural alterations (Figure 2.6), the leaf color of 35S::AtCBF1 *S. commersonii* lines was typically a darker green relative to wildtype, suggesting changes in pigment content had occurred. We evaluated the response of chlorophyll, carotenoid, and anthocyanin pigment content to both low temperature and ectopic *AtCBF1* expression in wildtype plants and in the same three lines examined above (Table 2.3); *S. tuberosum* was not evaluated as leaves were not visually altered between wildtype and transgenic plants (Figure 2.2B). *S. commersonii* transgenic lines C1.6 and C1.7 showed significant differences in total chlorophyll (Chl *a+b*) in relation to untransformed wild types; this differences were essentially due to differences in *Chla.* Two week of cold treatment did not cause differences in total chlorophyll *(a+b)* neither in Chl*a*; however, cold treatment decreased significantly the chl *a:b* ratio in wildtype and in all three transgenic lines. The carotenoid content was not significantly altered with two weeks of cold acclimation in *S. commersonii* wildtype and transgenic lines, except for line C1.6 which showed a significant increase in carotenoid content after two week cold treatment. Anthocyanin content in all transgenic *S. commersonii* lines was significant lower than wildtype plants, non significant differences were observed between plants grown either under warm conditions (25°C) or after two weeks cold treatment.

We also assessed a variety of photosynthetic parameters in *S. commersonii* transgenic lines and wildtype to see if any were altered (Table 2.4). Wildtype plants and transgenic lines were significantly photoinhibited by cold as expected. Relative to wildtype, the transgenic lines had similar Fv/Fm values in both the warm and cold, indicating that ectopic *AtCBF1* expression had no effect on photoinhibition.

The photosynthetic rate of the transgenic lines was significantly higher under warm conditions; however after two weeks under cold treatment only line C1.7 showed a significant higher photosynthesis rate. Similar to previous parameter, lines C1.6 and C.7 showed higher stomatal conductance and higher internal CO_2 concentration. We tested one of the superior lines, C1.6, and found the increase in photosynthetic rate was superior to wildtype plants over both a photosynthetically active radiation range and a CO_2 concentration range (Figure 2.7).

Discussion and Conclusions

In the current study, we looked at phenotypic adaptations that the freezing-tolerant potato species *S. commersonii* undergoes, how these compared with the agronomically important but freezing-sensitive potato species *S. tuberosum*, and what effects ectopic *CBF* expression had on the ability to induce these alterations in the absence of a cold stimulus. We employed the well-characterized *AtCBF1* gene as it has been shown to function in a diverse array of plant backgrounds, including *Solanaceous* species (Hsieh et al., 2002a,b). Two common phenotypic effects indicative of transgenic *CBF* over-expression are delayed flowering and stunted

plant growth (Liu et al., 1998; Gilmour et al., 2000; Haake et al., 2002). Lines of both *S. commersonii* and *S. tuberosum* actively expressing the *AtCBF1* transgene displayed one or both of these phenotypes, indicating the transgene was expressing a functional product in these lines.

The most obvious confirmation that the transgene was altering the phenotype of the two potato species was the increase in freezing tolerance that was acquired in the absence of a cold stimulus. Potatoes prefer cooler temperatures and are predominantly grown in the relatively cool climates of the northern temperate zone and Andean tropical highlands. In these locales, frost is typically encountered during the early spring and late fall and can often be a major limiting factor for potato production (Chen and Li, 1980a; Costa and Li, 1993; Barrientos et al., 1994; Vega and Bamberg, 1995). During the growing season in these regions, frost events usually occur in the -3° to -4°C temperature range (Carrasco et al., 1997; Hijmans et al., 2003). *S. tuberosum* varieties, comprising the bulk of potato production, cannot survive temperatures below -3°C on average (Chen and Li, 1980a), and thus a gain of just 1-2°C in freezing tolerance could have a major impact relative to a good vs. poor potato harvest. In *S. tuberosum*, the gain in freezing tolerance due to *AtCBF1* overexpression was about 2°C, with plants capable of surviving a frost of nearly -5°C. This represents a desirable agronomic gain and *S. tuberosum* engineered to express higher *CBF* levels could therefore have a potential positive impact on potato production in frost- prone areas.

Likewise, *AtCBF1* expression under warm conditions also led to a freezing tolerance increase in *S. commersonii* of 2°C to 4°C. Growth under cold temperature led to a further gain in freezing tolerance for the *S. commersonii*, but not the *S. tuberosum*, transgenic AtCBF1 lines. Ectopic over-expression of *CBF* genes, including *AtCBF1*, in other freezing tolerant plants such as *Arabidopsis* and *B. napus*, results in the same trend – an increase in freezing tolerance under warm conditions relative to untransformed, and a further gain in freezing tolerance following growth under cold conditions (Gilmour et al., 2000; Jaglo et al., 2001; Gilmour et al., 2004; Savitch et al., 2005). Ectopic overexpression of *CBF* genes yields an increase in freezing tolerance by inducing CBF-regulon genes conferring cryoprotective adaptations in the absence of a cold stimulus. While the CBF cold response pathway has a prominent role in cold acclimation, microarray studies in *Arabidopsis* have established that additional (i.e., CBF-independent) pathways, such as ZAT12, contribute to an increased freezing tolerance capacity (Fowler and Thomashow, 2002; Vogel et al., 2005; van Buskirk and Thomashow, 2006). Likewise, the *eskimo1* mutation results in constitutive freezing tolerance without affecting expression of the CBF-dependent *cor* genes (Xin and Browse, 1998). There are two probable bases for the additional freezing tolerance gain in transgenics following cold acclimation and the effect may be a combination of these two scenarios: (i) cold induces the CBF-independent systems which then contribute toward total freezing tolerance, and/or (ii) cold induces endogenous *CBF* genes which stimulate the CBF gene regulon to even higher levels.

In contrast, no additional gain in freezing tolerance was observed for the transgenic *S. tuberosum* lines following cold treatment. Studies have traditionally suggested that potato was domesticated from multiple independent origins; however recent molecular work has concluded a monophyletic origin for potato is likely (Spooner et al., 2005). Relative to freezing-sensitive *S. tuberosum* vs. freezing-tolerant potato species such as *S. commersonii*, it is currently unclear whether freezing tolerant species (i) are ancestral where *S. tuberosum* would represent a descendant mutant lineage impaired for the genetic traits of cold acclimation capacity and higher freezing tolerance capacity or (ii) were originally freezing-sensitive and later gained the capacities for cold acclimation and higher freezing tolerance after divergence from the last shared common ancestor with the *S. tuberosum* lineage. Based on the first hypothesis, it is possible that relative to *S. commersonii*, mutations have led to disruptions in one or more *S. tuberosum* pathways leading to increased freezing tolerance. The general CBF pathway involves cold temperature modifying the ICE gene product from an inactive to active form, this active ICE form then binds the promoter of cold-inducible *CBF* genes and induces their expression, the newly synthesized CBF products bind to promoters of multiple *cor* genes and induce their expression, and the COR gene products collectively increase the freezing tolerance of the plant (reviewed in van Buskirk and Thomashow, 2006). In tomato, a chilling- and freezing-sensitive and closely-related *Solanaceous* plant, it has been shown that at least three *CBF* genes are present, one of which is induced by cold, indicating the cold sensing pathway

through induction of CBF expression is still retained (Zhang et al., 2004). The tomato *CBF* is functional and can confer increased freezing tolerance in *Arabidopsis*, but microarray analysis established that only four of the approximately 8700 genes screened in tomato were significantly upregulated by its ectopic expression, indicating the tomato CBF regulon downstream of the CBF factor may be significantly downsized and a potential basis for the plants inability to tolerate freezing conditions (Zhang et al., 2004). As noted earlier based on analysis of the EST database, *CBF* genes are encoded and expressed in the *S. tuberosum* genome (data not shown). The ability to increase freezing tolerance capacity via ectopic *AtCBF1* expression suggests that *S. tuberosum* still retains a sufficient CBF-controlled downstream *cor* gene regulon to impart this effect. However, cold treatment did not affect this gain and ectopic *AtCBF1* expression was unable to impart a cold acclimation capacity to this species, suggesting impairment of the capacity to induce endogenous functional CBF activity and/or CBF-independent pathways in *S. tuberosum*.

While an indicator of CBF transgene activity, stunting is an undesirable phenotype which would likely limit the agricultural applications of potato over-expressing CBFs. In both potato species, this phenotype was primarily manifested during growth in tissue culture however, and lines of both species grew out of the stunted phenotype for the most part following soil transplantation. We have observed similar effects in transgenic poplar lines transformed with the same 35S::AtCBF1 construct where the plants were dwarfed during tissue culture

growth, but displayed growth similar to wildtype following a few weeks in soil (Benedict et al., 2006; data not shown). Stunting appears to be a pleiotropic side effect rather than a functional property of *CBFs*. Wang et al., (2005) found in *Arabidopsis* that while overexpression of an active AtCBF1 activation domain fused to a yeast DNA binding domain failed to induce *cor* gene expression as expected, it still resulted in stunted plant growth. This suggests the stunted phenotype is a pleiotropic byproduct of excessive functional activation domain presence (Wang et al., 2005), rather than a CBF property resulting from constitutive overexpression of downstream CBF-targeted *cor* gene products as had been hypothesized (Liu et al., 1998; Gilmour et al., 2000). Hsieh et al. (2002a) found that AtCBF1-based stunting in tomato was alleviated by exogenous GA_3 application, suggesting this pathway is affected. Three of the *S. commersonii* lines failed to grow out of the dwarfed phenotype however, exhibiting sustained dwarfing during growth in soil. While some studies have associated the degree of growth retardation with the level of *AtCBF* expression (e.g., Haake et al., 2002), this did not appear to be the case for these three lines and may instead be related to transgene positional insertion effects. Use of a stress-inducible promoter to drive ectopic *CBF* expression (Kasuga et al., 1999) is another means to avoid negative growth effects and is currently being evaluated in potato (data not shown).

We also noted differences in the frequency with which expressing lines were recovered between the two potato species. Recovery of expressing lines was highly efficient for *S. commersonii*, where 11 of 13 lines (85%) AtCBF1-transformed lines

examined showed detectable transgene expression. In contrast, only 3 of 19 (16%) AtCBF1-transformed *S. tuberosum* lines showed expression. We have observed a similar effect in poplar, where only 2 of 20 (10%) AtCBF1-transformed poplar lines showed expression (Benedict et al., 2006; data not shown). The level of *AtCBF1* expression in the three *S. tuberosum* lines was generally equivalent to the low to intermediate *S. commersonii* line expression levels, possibly suggesting *S. commersonii* is more tolerant of excessive *CBF* expression levels. Higher *AtCBF1* expression levels may inhibit *S. tuberosum* growth in tissue culture more severely than *S. commersonii* and thus only *S. tuberosum* plantlets with lower or silenced *AtCBF1* expression levels are efficiently recovered from tissue culture, whereas high expressors fail to regenerate into plantlets in a timely manner. Transformation of additional varieties is currently underway and will determine whether this is a trait common to *S. tuberosum* in general, or specific to a subset of varieties that includes the utilized Umatilla cultivar.

It was visually apparent that leaves of transgenic *S. commersonii* plants were altered based on color and size. A number of studies suggest that cold-based leaf structural and morphological alterations contribut to final freezing tolerance capacity (Kaku, 1973; Palta and Li 1979; Ristic and Ashworth, 1993). Alterations in the epidermis and palisade parenchyma cells have been associated with cold acclimation of *B. napus L.* var *olifera* leaves (Stefanowska et al., 1999, 2002) and low temperature leads to leaf cell enlargement in *Arabidopsis* (Ristic and Ashworth, 1993). In some frost tolerant potato species, the formation of double to

triple palisade layers in response to cold, as well as changes in leaf structure and cell wall thickness, have been observed (Chen et al., 1977; Palta and Li, 1979; Estrada, 1982). In *Arabidopsis*, ectopic *AtCBF1* expression was observed to lead to the novel formation of a double palisade layer (Gilmour et al., 2004). Neither *S. tuberosum* nor *S. commersonii* wildtype plants displayed formation of multiple palisade layers in response to cold as has been observed for some other potato species, and ectopic *AtCBF1* also did not induce formation of additional palisade layers. While *S. tuberosum* wildtype leaves were unaltered, cold treatment of *S. commersonii* wildtype leaves caused an increase in leaf thickness that was a result of palisade cell elongation and an increase in the intercellular spongy parenchyma spacing. In plant cells, tolerance of freezing temperatures depends on the ability of protoplasts to withstand dehydration and the volume of extracellular spaces to accommodate growing ice crystals. The larger intercellular spacing in the *S. commersonii* spongy parenchyma matrix may function in a similar capacity relative to increased freezing tolerance. Importantly, ectopic *CBF* expression mimicked these cell length and spacing alterations in the absence of cold treatment, suggesting they are a result of a CBF-dependent process in *S. commersonii*. While spongy parenchyma spacing was unaltered, ectopic At*CBF1* expression in *S. tuberosum* also resulted in thicker leaves with elongated palisade cells, suggesting ectopic *AtCBF1* expression can restore the cell lengthening capacity in *S. tuberosum*, but not the ability to increase spongy parenchyma spacing.

An increase in proline and sugar, as well as other compatible solutes with cryoprotective properties, is a nearly universal plant response to low temperatures and plays an important role in freezing tolerance by both increasing the internal osmotic pressure and preventing cellular water loss during freezing-induced dehydration (Nanjo et al., 1999; Thomashow, 1999). Proline and total sugar content increased in response to cold in both *S. tuberosum* and *S. commersonii*. Freezing tolerance and increased proline content have been previously associated in potato, with increases of 3 to 10 fold in leaf proline being noted during cold hardening (van Swaaij et al., 1985). Sugar accumulation has also been associated with cold acclimation in many plant species, including potatoes (Chen and Li, 1980b; Hincha et al., 2000; Dionne et al., 2001a). A comparison of multiple *Solanum* species found that both free sugars and starch increase during cold acclimation, with *S. commersonii* in particular showing the largest sugar content increase following cold acclimation (Chen and Li, 1980b). The transgenic *S. commersonii* lines showed largest increases in the levels of proline and sugar under warm conditions, and cold caused a further increase (Table 2.2). This is in agreement with studies in *Arabidopsis*, where ectopic *CBF* expression also leads to an increase in these two metabolite classes (Gilmour et al., 2000; Gilmour et al., 2004). *S. tuberosum* wildtypes and transgenic lines did not show significant increase in proline or sugars. *S. commersonii* lines with higher *AtCBF1* overexpression showed proline and sugar content similar to and even higher than cold acclimated wildtype plants,

suggesting that these processes are controlled by the CBF response pathway in *S. commersonii*, but not in *S. tuberosum*.

The photosynthetic capacity of plants is adversely affected by cold and freezing. Recent studies show that a large number of photosynthetic products are altered in response to low temperatures, and that ectopic *CBF* expression can affect genes involved in photosynthesis (Savitch et al., 2005; Goulas et al., 2006). In contrast to *S. tuberosum* which suffers irreversible damage, cold hardy potatoes like *S. commersonii* are able to recover from the photosynthetic inhibition imposed by freezing stress (Steffen and Palta, 1989; Seppänen and Coleman, 2003). The darker green leaves of the transgenic *S. commersonii* plants suggested pigment content, and possibly related photosynthetic parameters, were altered, and we confirmed this was indeed the case for those lines with higher *AtCBF1* overexpression but not for C1.4 which was a transgenic line with lower *AtCBF1* transcript accumulation and less cold hardier. Relative to wildtype, the cold hardier transgenic *S. commersonii* line C1.7, was more photosynthetically active under both warm and cold conditions, and the rate of photosynthesis for these transgenic lines was improved to levels nearly as great as wildtype plants under non-stressed (i.e., warm) conditions. This suggests that *S. commersonii* utilizes CBF-dependent pathways to activate protection mechanisms for the photosynthetic apparatus to counter the inhibitory effects of cold temperatures.

A minor carotenoid increase was observed in wildtype *S. commersonii* and transgenic lines in response to cold. Only line C1.6 shows significant increase in

carotenoid during cold acclimation. Kristjansdottir and Merker (1993) compared wild potato species in their sensitivity to low temperature and light stress; they found that wild potato species were more tolerant to photoinhibition than *S. tubersosum,* they associated this fact to the higher carotenoid content in wild potato species. Carotenoid content has been also positively correlated with freezing tolerance in high altitude plants (Wildi and Luz, 1996; Streb et al., 2003) and in protection against low temperature photoinhibition in *Arabidopsis* (Harvaux and Kloppstech, 2001). In the transgenic lines, carotenoid content did not differ significantly from wildtype plants, except line C1.6 suggesting that carotenoid content is not strongly affected by the CBF pathway in *S. commersonii*. Both chlorophyll and anthocyanin pigment contents were altered in leaves from those two cold hardier transgenic lines. Anthocyanins reduce photosynthetic efficiency (Burger and Edwards, 1996; Gould et al., 2000) and have been proposed to play a phytoprotective role as they can be induced by environmental factors such as cold temperatures in some systems (Krol et al., 1995; Chalker-Scott, 1999). In wildtype *S. commersonii*, anthocyanin content decreased in response to cold; however, ectopic *AtCBF1* expression caused an analogous trend, suggesting the cold-based decrease in anthocyanin level is part of the CBF response pathway. Low temperatures are noted to induce changes in leaf pigment, with chlorophyll deficiency being one of most distinctive (Haldimann et al., 1998, 1999). In agreement with the increased greening of the transgenic leaves, the chlorophyll content of the transgenic lines C1.6 and C1.7 was greater than the wildtype levels.

Significant differences in chlorophyll content were observed in *Chla* for cold hardier transgenic lines with higher *AtCBF1* overexpression. This response may also be part of a CBF-dependent process as these changes were induced in the same manner in those transgenic lines.

In tomato, tobacco, and *B*. *napus* ectopically over-expressing *CBF* genes, gains in photosynthetic parameters have been observed (Hsieh et al., 2002a; Kasuga et al., 2004; Savitch et al., 2005). For instance, AtCBF1 transgenic tomato plants are less susceptible to oxidative stress and lose less chlorophyll than wildtype (Hsieh et al., 2002a). We assayed additional parameters involved in photosynthetic efficiency and found the transpiration rate, stomatal conductance, and internal CO_2 concentration for transgenic C1.6 and C1.7 were each increased relative to wildtype plants under both warm and cold conditions. In a separate study, we reported that leaves of AtCBF1 transgenic *S. commersonii* plants have an increase in the number of stomata per unit area, which is a likely partial basis for these observed increases (Pino et al., 2006). Low temperature decreases the rate of photosynthetic electron transport and carbon fixation (Huner et al., 1998). In several species, freezing tolerance capacity is correlated with an increased photosynthetic electron transport capacity at low temperatures (Öquist and Huner, 1993; Gray et al., 1997; Streb et al., 2003). One of the cold hardier transgenic *S. commersonii* lines with higher *AtCBF1* overexpression, C1.6 line was more photosythetically active than wildtype plants under both increased light levels and increased internal CO_2 concentrations. Steffen and Palta (1986) compared the photosynthetic capacity of *S. acaule* and

S. tuberosum after cold acclimation and found a positive correlation between ability to maintain photosynthetic activity at low temperature and acclimation capacity. Contrary, Griffith et al. (1994) found that S. *commersonii* was unable to alter its sensitivity to photoinhibition during cold acclimation. Together, the ectopic *AtCBF1* overexpression at level found in lines C1.6 and C1.7 seems have a role in photosynthesis protective mechanisms

Studies in other species such as grapevine show that cold-tolerant genotypes exhibit higher photosynthetic capacity and higher stomatal conductance at low temperature (Flexas et al., 1999). We found that two of the AtCBF1-overexpressing *S. commersonii* lines displayed significantly higher stomatal conductance and a markedly elevated Ci (Table 2.4). After two weeks at 2ºC, the stomatal conductance in all over-expressing lines was significantly higher (>115molm^{-2}s^{-1}) than wildtype (62 molm^{-2}s^{-1}). Because excess light during low temperature stress can lead to photoinhibition, we evaluated the response of the transgenic *S. commersonii* to increasing light intensities during cold treatment. Transgenic *S. commersonii* maintained higher photosynthetic rates under all light intensities, and the increased photosynthetic capacity was also displayed under CO_2-enriched conditions (Figure 2.7). These results suggest *S. commersonii* plants over-expressing *AtCBF1* have higher productivity potential under higher light intensities and/or higher CO_2 conditions than wildtype plants. How the overexpression of *AtCBF1* genes influences this response is not clear. Goulas et al. (2006) found that the abundance of a large number of photosynthetic proteins are affected by both

exposure to, and time in, low temperature conditions, but found poor correlation with genes induced by ectopic *CBF* expression from microarray studies (e.g., Fowler and Thomashow, 2002). Perhaps other factors that have not been evaluated in this experiment may be being affected by *AtCBF1*. For example, chloroplast alterations and/or modification in light regulated enzyme activities (Robertson et al., 1993; Krause et al., 1988) may cause such changes. We previously noted that both chloroplast and stomata numbers are increased in transgenic *S. commersonii* over-expressing *AtCBF1* (Pino et al., 2006). A recent study in *B. napus* also found that a *Brassica* CBF transcription factor affects chloroplast development and enhances photosynthetic capacity (Savitch et al., 2005).

In conclusion, ectopic *AtCBF1* expression resulted in a freezing tolerance gain in both *S. tuberosum* and *S. commersonii*. While *S. commersonii* cold acclimation capacity was increased, ectopic *AtCBF1* expression failed to impart the ability to cold acclimate to *S. tuberosum*. The freezing tolerance gain in *S. tuberosum* may be important relative to the ability of this agronomically important species to better withstand the frost events that typically occur during the beginning and end of its growing season. Many of the cold acclimation alterations *S. commersonii* undergoes were mimicked by ectopic *AtCBF1* expression, suggesting many of the processes are regulated at least in part by CBF-responsive pathways. Furthermore, for many of the *S. commersonii* changes induced by ectopic *AtCBF1* expression under warm conditions, cold treatment resulted in additional gains for the traits, which may be due to cold-based induction of the endogenous *CBF* genes and/or

CBF-independent pathways. These results imply the CBF pathway is involved in potato freezing tolerance and that its manipulation through either breeding or genetic engineering could be used to develop more frost tolerant varieties.

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Table 2.1 Effects of cold treatment and *AtCBF1*-overexpression on leaf thickness and leaf palisade length in *S. commersonii* Dun (PI 243503 clone 13) and *S. tuberosum L.* (cv. Umatilla)[1]

Line	Temp	*S. commersonni*		*S. tuberosum*	
		Leaf thickness[2] (µm)	**Palisade length[2] (µm)**	**Leaf thickness[2] (µm)**	**Palisade length[2] (µm)**
Wildtype	25°C	117.5^{e}	38.9^{d}	185.6^{c}	68.11^{c}
	2°C	200.9^{d}	66.7^{c}	191.1^{c}	70.20^{c}
Line A[3]	25°C	215.3^{cd}	84.6^{b}	246.08^{a}	101.2^{a}
	2°C	237.1^{c}	92.3^{ab}	246.08^{a}	102.53^{a}
Line B[3]	25°C	271.7^{b}	84.6^{b}	193.53^{c}	80.75^{b}
	2°C	275.6^{b}	85.9^{ab}	196.10^{c}	80.75^{b}
Line C[3]	25°C	249.9^{bc}	79.5^{b}	206.35^{b}	78.18^{b}
	2°C	312.7^{a}	98.7^{a}	219.17^{b}	80.75^{b}

[1]Based on leaf cross sections of plants grown at 25ºC and following two weeks at 2ºC

[2]Letters indicate significant differences among lines (p-value<0.05) based on Duncan's Multiple Range Test

[3]*S. commersonii* lines C1.4 (A), C1.6 (B) C1.7 (C); *S. tuberosum* lines T1.2 (A), T1.11, and T1.15 (C)

Table 2.2 Effects of cold treatment and *AtCBF1*-overexpression on proline and total sugar content in *S. commersonii* and *S. tuberosum*[1]

Line	Temp	*S. commersonni*		*S. tuberosum*	
		Proline[1] (mg/g DW)	**Sugar[1] (mg/g DW)**	**Proline[1] (mg/g DW)**	**Sugar[1] (mg/g DW)**
Wildtype	25°C	1.2^{e}	104.0^{d}	2.4^{b}	182.6^{a}
	2°C	1.9^{cd}	149.8^{c}	2.8^{ab}	204.6^{a}
Line A[2]	25°C	1.5^{de}	171.2^{bc}	2.5^{ab}	191.6^{a}
	2°C	2.9^{b}	224.4^{a}	2.7^{ab}	207.1^{a}
Line B[2]	25°C	2.7^{b}	174.0^{bc}	3.1^{ab}	206.8^{a}
	2°C	3.6^{a}	227.2^{a}	3.4^{a}	223.5^{a}
Line C[2]	25°C	2.1^{c}	203.3^{ab}	3.0^{ab}	202.1^{a}
	2°C	3.0^{b}	227.2^{a}	3.2^{ab}	218.5^{a}

[1]Letters indicate significant differences among lines (p-value<0.05) based on Duncan's Multiple Range Test

[2]*S. commersonii* lines C1.4 (A), C1.6 (B) C1.7 (C); *S. tuberosum* lines T1.2 (A), T1.11, and T1.15 (C)

Table 2.3 Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* pigment content[1]

Line	Temp[2]	Chl *a* (µg/g FW)	Chl *b* (µg/g FW)	Chl *a*+*b* (µg/g FW)	Chl *a*:*b*	Carotenoids (µg/g FW)	Anthocyanins (µg/g FW)
C.WT	25°C	859.0^{c}	350.7^{bc}	1209.7^{cd}	2.5^{bc}	247.3^{bc}	28.8^{a}
	2°C	920.2^{c}	275.5^{c}	1195.7^{cd}	3.4^{a}	259.0^{bc}	25.3^{ab}
C1.4	25°C	787.2^{c}	489.7^{b}	1276.9^{cd}	1.7^{c}	155.5^{c}	23.4^{b}
	2°C	817.1^{c}	300.3^{bc}	1117.4^{d}	2.8^{ab}	196.8^{c}	16.2^{c}
C1.6	25°C	1347.3^{a}	692.4^{a}	2039.7^{a}	2.1^{bc}	240.8^{bc}	14.5^{c}
	2°C	1391.2^{a}	424.3^{bc}	1815.5^{ab}	3.3^{a}	383.8^{a}	10.8^{c}
C1.7	25°C	1101.5^{ab}	692.4^{a}	1794.0^{ab}	1.6^{c}	280.2^{bc}	15.2^{c}
	2°C	1137.2^{ab}	346.5^{bc}	1483.6^{bc}	3.3^{a}	331.3^{ab}	11.1^{c}

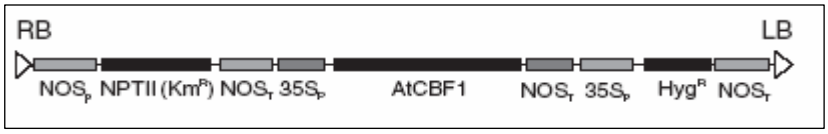
[1]Letters after values indicate significant differences among lines (p-value<0.05) based on Duncan's Multiple Range Test
[2]Plants grown at 25ºC and following two weeks at 2ºC

Table 2.4 Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* photosynthetic parameters[1]

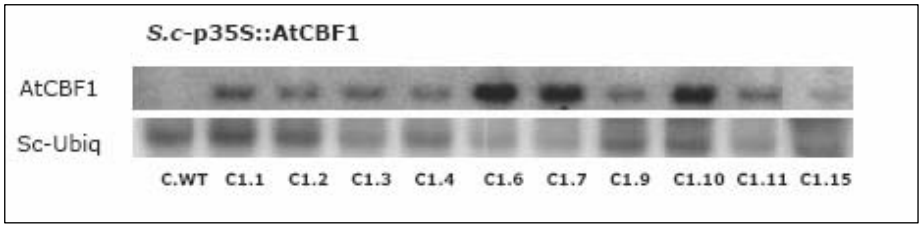
Line	Temp[2]	Fv/Fm	Photosynthesis Rate (A) µmol $m^{-2}s^{-1}$	Transpiration Rate (EVAP) mol $m^{-2}s^{-1}$	Stomatal Conductance (GS) mol $m^{-2}s^{-1}$	Internal CO_2 Concentration (C_i) ppm
C.WT	25°C	0.844^{a}	9.85^{b}	4.23^{ab}	96.3^{cd}	166.8^{b}
	2°C	0.770^{b}	6.78^{c}	2.12^{c}	62.3^{d}	168.7^{b}
C1.4	25°C	0.856^{a}	14.60^{a}	5.50^{a}	152.7^{b}	179.8^{b}
	2°C	0.795^{b}	8.45^{bc}	3.24^{bc}	115.5^{bcd}	229.7^{ab}
C1.6	25°C	0.854^{a}	15.22^{a}	5.49^{a}	235.0^{a}	232.0^{a}
	2°C	0.784^{b}	8.74^{bc}	3.34^{bc}	114.0^{bcd}	236.8^{a}
C1.7	25°C	0.855^{a}	15.28^{a}	5.49^{a}	163.0^{b}	200.5^{ab}
	2°C	0.787^{b}	9.73^{b}	3.72^{b}	132.0^{bc}	239.8^{a}

[1]Letters after values indicate significant differences among lines (p-value<0.05) based on Duncan's Multiple Range Test
[2]Plants grown at 25ºC and following two weeks at 2ºC

A



B



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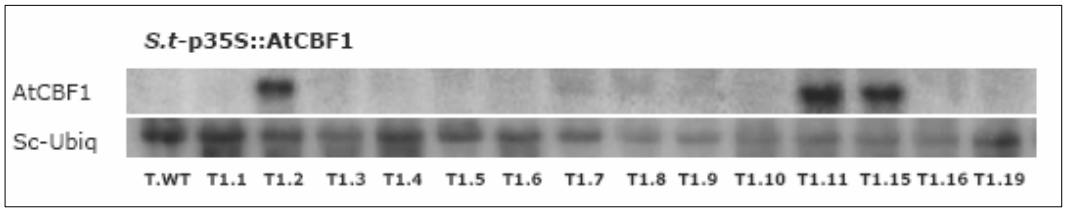

Figure 2.1 Transgenic potato lines over expressing *AtCBF1* gene. **A**, T-DNA region of expression vector used for potato transformation. **B**, Transcripts levels of *AtCBF1* transgene in *S. commersonii* Dun PI243503 clone13 (C-lines) and wildtype (C.WT*)*. **C,** Transcripts levels of *AtCBF1* transgene in *S. tuberosum* L. cv. Umatilla (T-lines) and wildtype (T.WT). Total RNA (20ug) from non acclimated plants growing at 25ºC were loaded per lane and hybridized with the indicated probe (panels B and C). A ubiquitin probe (Sc-Ubiq) was used as a loading control probe

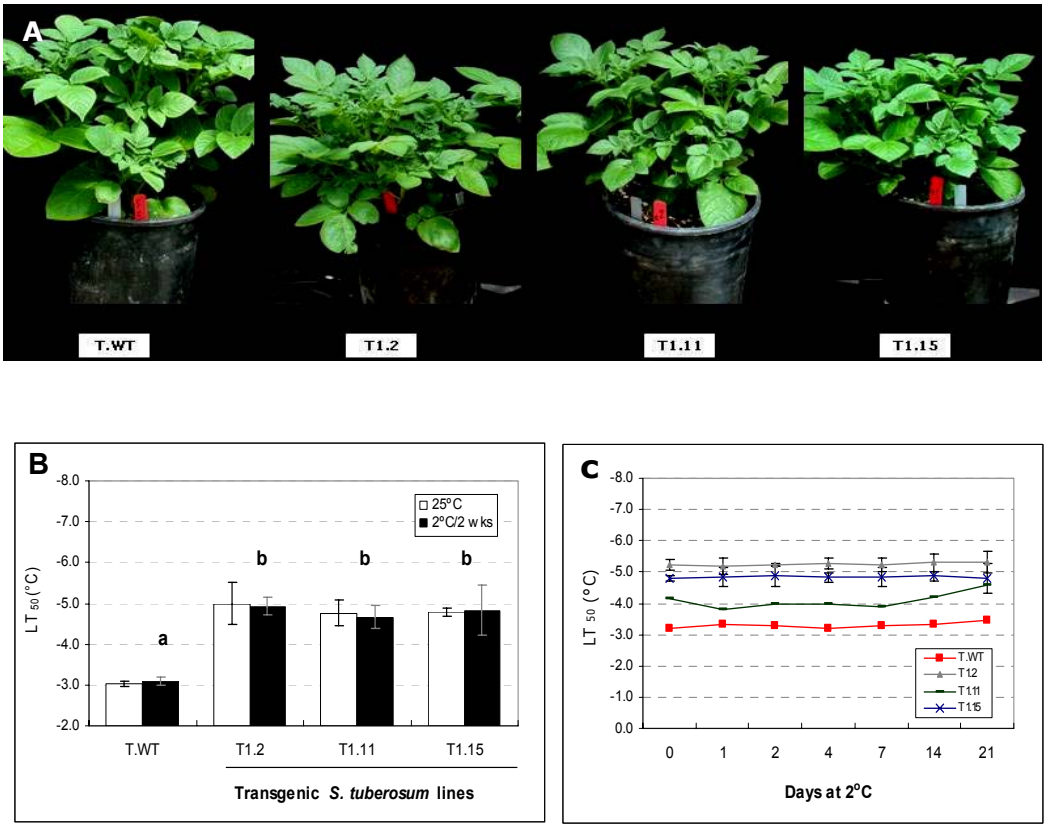

Figure 2.2 Effect of *AtCBF1* transgene overexpression on phenotype and freezing tolerance of *S. tuberosum* plants. **A,** Phenotype of wildtype (T.WT) and 35S::AtCBF1 overexpressing plants (T1.2, T1.11, T1.15 lines). **B,** LT_{50} (-°C) for wildtype and 35S::AtCBF1 overexpressing plants (T1.2, T1.11, T1.15) growing at 25°C and after two weeks cold treatment at 2ºC (different letters indicate significant differences (p-value<0.0001) according Duncan's Multiple Range Test). **C,** Time course as LT_{50} (-°C) for wildtype and 35S::CBF1-overexpressing plants (T1.2, T1.11, T1.15) after 0, 1, 2, 4, 7, 14 and 21 days cold treatment at 2ºC.

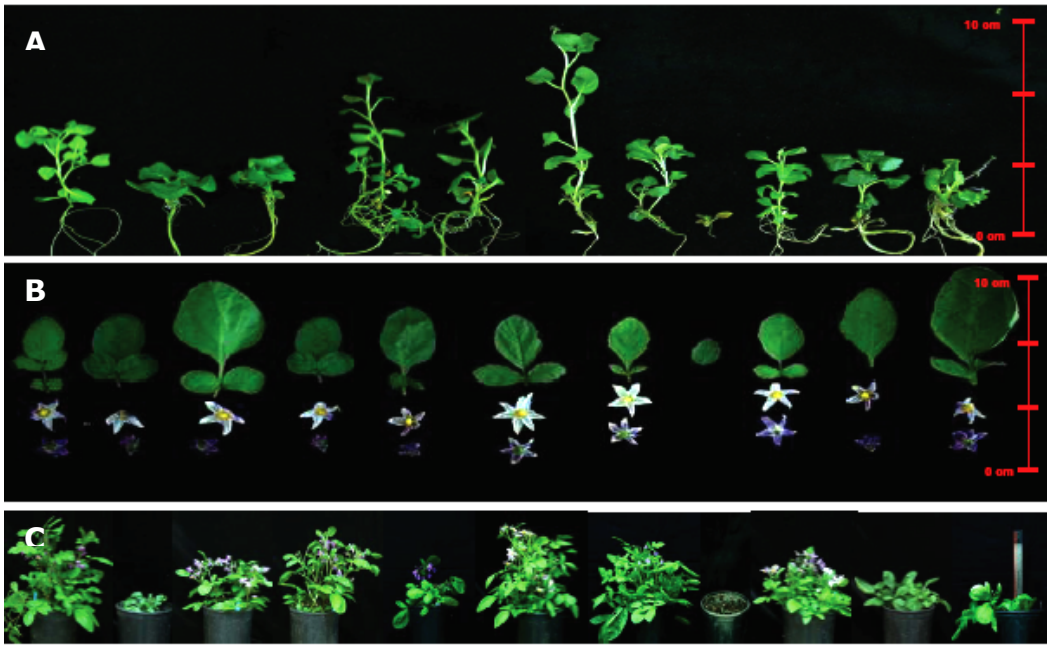

Figure 2.3 Effect of *AtCBF1* overexpression in *S. commersonii* phenotype **A**, 35S::AtCBF1 transgenic plants during tissue culture-based propagation (C- lines) relative to the wildtype (C.WT*)*. **B**, Leaves and flowers of greenhouse-growing wildtype and transgenic plants. **C**, Wildtype and transgenic plants growing in soil under greenhouse conditions for 12 weeks.

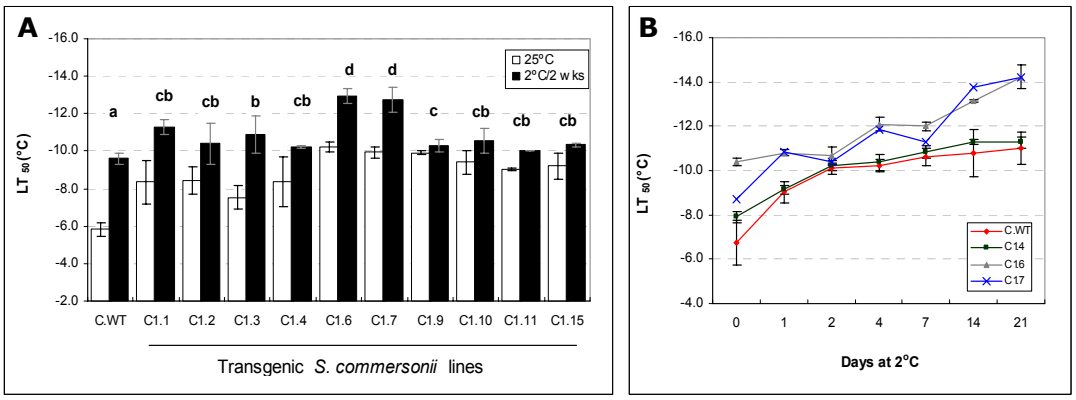

Figure 2.4 Effect of *AtCBF1* overexpression on freezing tolerance of *S. commersonii* plants. **A,** LT_{50} (-°C) of wildtype (C.WT) and 35S::AtCBF1 transgenic plants (C-lines) growing at 25°C and after two weeks cold treatment at 2ºC (Different letters indicate significant differences among lines (p-value<0.0001) according Duncan's Multiple Range Test). **B,** Time course as LT50 (-°C) for C.WT and 35S::CBF1-transgenic plants (C1.4, C1.6, C1.7) after 0, 1, 2, 4, 7, 14 and 21 days cold treatment at 2ºC.

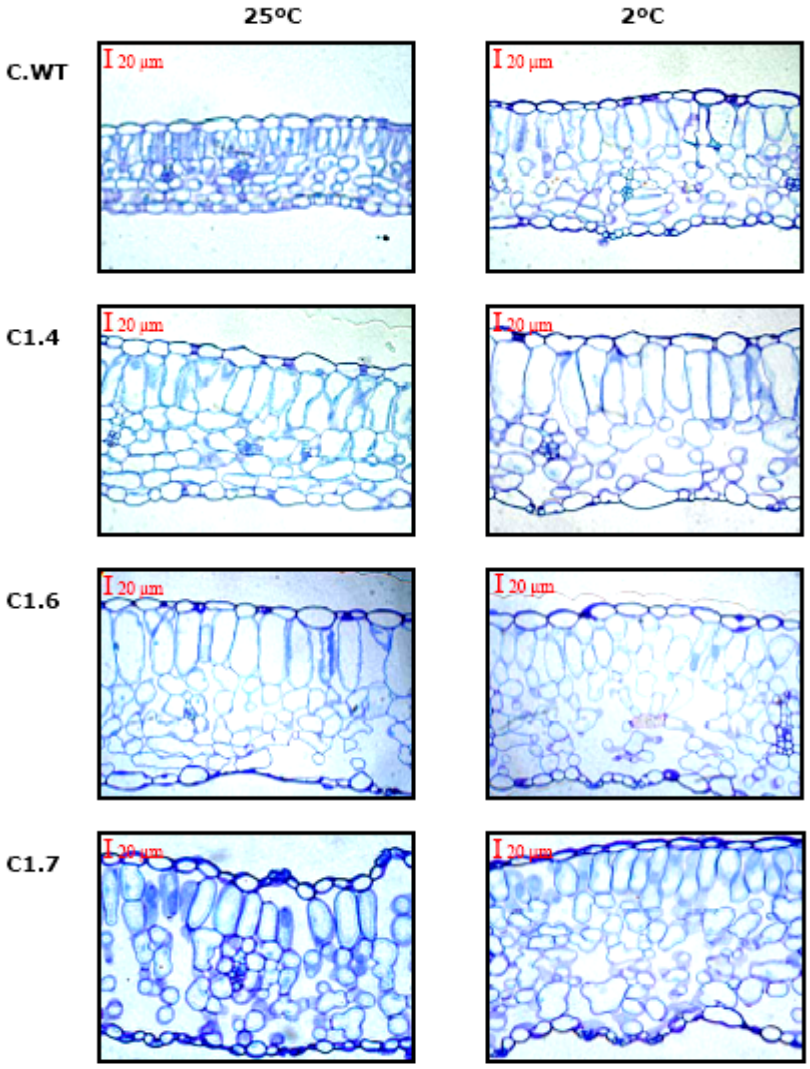

Figure 2.5 Effect of *AtCBF1* overexpression on leaf structure of *S. commersonii*. Cross sections were made of leaves from wildtype (C.WT) plants and 35S::AtCBF1 overexpressing lines (C1.4, C1.6 and C1.7) growing at 25ºC and after two weeks cold treatment at 2ºC. Light micrographs were taken at 200X.

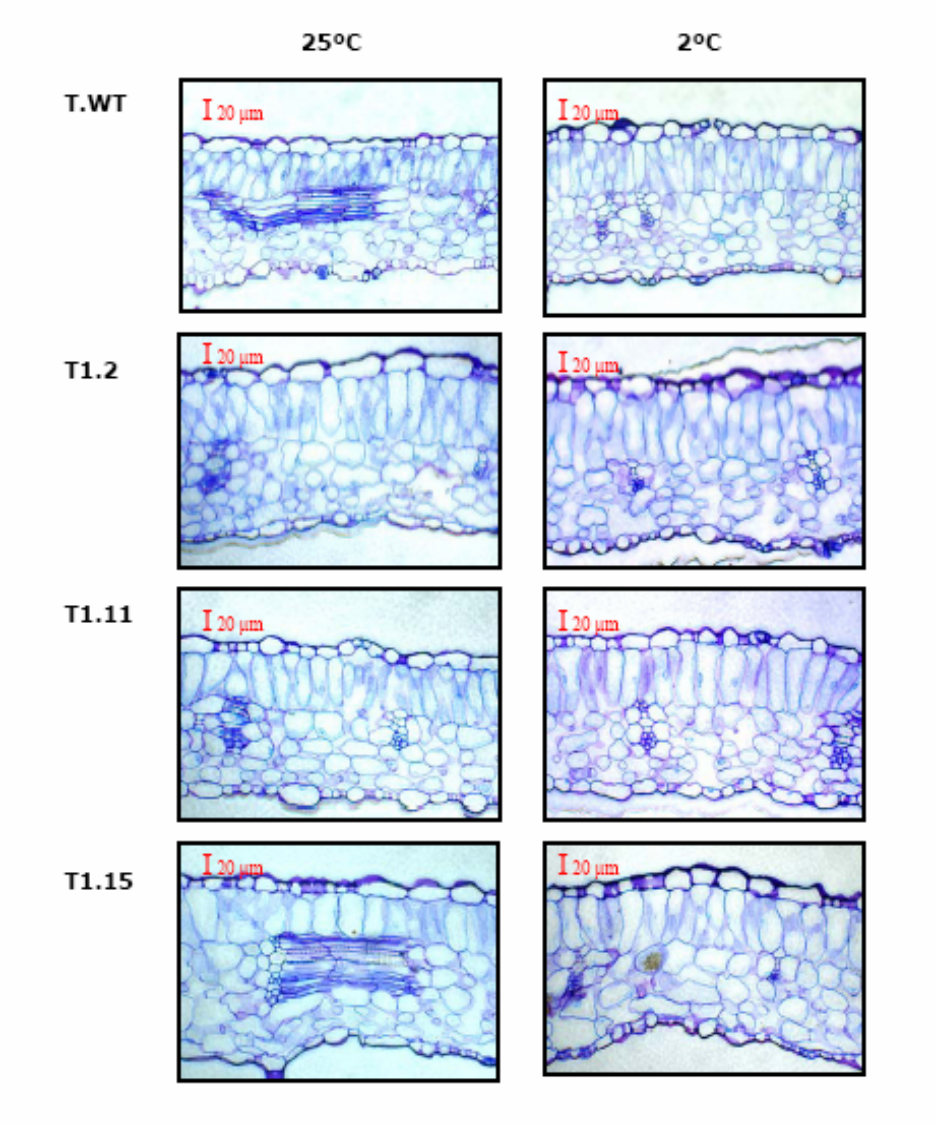

Figure 2.6 Effect of *AtCBF1* overexpression on leaf structure of *S. tuberosum*. Cross sections were made of leaves from wildtype (T.WT) and 35S::AtCBF1 overexpressing lines (T1.2, T1.11 and T1.15) growing at 25ºC and after two weeks cold treatment at 2ºC. Light micrographs were taken at 200X.

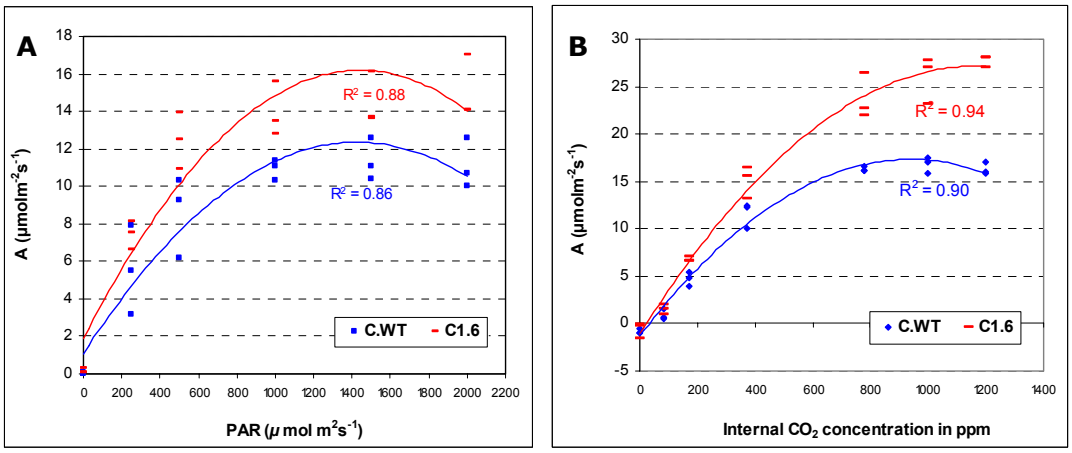

Figure 2.7 Effect of *AtCBF1* overexpression on net photosynthesis of *S. commersonii* wildtype (C.WT) plants and 35S::AtCBF1 transgenic plants line C1.6. **A**, The response of net photosynthesis (A) to different levels of photosynthetically active radiation (PAR) was determined for a CO_2 concentration outside the leaf (C_a) of 400ppm at 25ºC. **B**, The CO_2 response curve of net photosynthesis was determined to *PAR* close to the saturation point 1500 µmolm^{-2}s^{-1} at 25ºC.

CHAPTER 3

USE OF A STRESS-INDUCIBLE PROMOTER TO DRIVE ECTOPIC *AtCBF* EXPRESSION IMPROVES POTATO FREEZING TOLERANCE WHILE MINIMIZING EFFECTS ON TUBER YIELD

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Abstract

The cultivated potato *Solanum tuberosum L.* is a frost-sensitive species. The *S. tuberosum* cv. Umatilla was transformed with three *Arabidopsis CBF* genes (*AtCBF1-3*) under the control of either a constitutive or stress-inducible promoter to access their effects on freezing tolerance and tuber yield. *AtCBF1* and *AtCBF3* overexpression under both promoter types increased transgenic potato plant freezing tolerance by about 2°C, whereas *AtCBF2* failed to increase freezing tolerance. Constitutive expression of the *AtCBF* genes induced negative phenotypic alterations however, including smaller leaves, stunted plants, delayed flowering, and lack of or reduction in tuber production. While imparting the same degree of freezing tolerance, use of the abiotic stress-inducible promoter to direct *AtCBF* expression ameliorated the negative phenotypic effects and restored tuber production to levels similar to wildtype plants. These results suggest that use of a stress-inducible promoter to direct *CBF* transgene expression can yield significant

gains in freezing tolerance without negatively impacting the agronomically important traits of potato.

Keywords: Potato, *Solanum tuberosum*, *AtCBF*s, freezing tolerance, tuber, frost

Introduction

Potato is the fourth most important food crop and most important non-cereal food crop produced worldwide. Potatoes are a cool season crop that produces higher yields when grown in climates with relatively cool summer temperatures. Due to this, potato production predominantly occurs in the relatively cool climates of the northern temperate zone and Andean tropical highlands. *Solanum tuberosum*, which comprises the bulk of the economically and agronomically important potato production, is a frost-sensitive species and prone to freezing injury – particularly during the early spring and late fall – which can be a major limiting factor in potato production (Chen and Li, 1980; Costa and Li, 1993; Barrientos et al., 1994; Vega and Bamberg, 1995). Likewise, early potatoes, a high-profit specialty crop grown in the Mediterranean region, are commonly planted from late fall through early spring, and despite the mild winter conditions of the region, this period coincides with the highest probability of frost events and subsequently causes significant production losses (Iovene et al., 2004).

Frost events usually occur in the -3° to -4°C temperature range during the growing season of these various potato production regions (Carrasco et al., 1997; Hijmans et al., 2003). *S. tuberosum* varieties are frost-sensitive and incapable of

cold acclimation, having a maximum freezing tolerance capacity of about –3°C both before and after exposure to low temperatures (Chen and Li, 1980). Even a brief frost exposure can significantly reduce *S. tuberosum* yields and hard frosts can completely destroy entire potato fields, and thus improvement in freezing tolerance of just a few degrees would be of considerable benefit.

While *S. tuberosum* varieties possess minimal frost hardiness, many wild tuber-bearing *Solanum* species are much more frost hardy and tolerant of lower temperatures (Chen and Li, 1980; Barrientos et al., 1994; Vega and Bamberg, 1995). For instance, *S. commersonii* can survive to about –5ºC pre-acclimation and -10 ºC after becoming fully cold-acclimated (Chen and Li, 1980; Costa and Li, 1993). These wild potatoes have therefore been utilized as a potential gene source for increasing the cold hardiness of *S. tuberosum*. Due to the complex genetic traits that influence them (Thomashow, 1999), traditional breeding efforts to transfer the superior freezing tolerance and cold acclimation capacities of wild potatoes to cultivated *S. tuberosum* have not resulted in significant increases for these traits, and agronomically-undesirable 'wild' characteristics are often co-transfered (Cardi et al., 1993; Estrada, 1982; Estrada et al., 1993; Pavek and Corsini, 2001; Iovene et al., 2004).

While introduction of increased freezing capacity through traditional breeding has failed to meet with success to date, recent biotechnological advancements offer viable alternative strategies to meet the same end. In *Arabidopsis*, major progress has been made in determining key genes and pathways

involved in cold acclimation and freezing tolerance (reviewed by Chinnusamy et al., 2006 and van Buskirk and Thomashow, 2006). The *Arabidopsis CBF* genes *AtCBF1-3* (corresponding to *DREB1b-c-a*, respectively) encode cold-responsive transcription factors that control expression of a major regulon of cold-responsive (*cor*) genes which collectively increases the plant's freezing tolerance. These factors bind the CRT/DRE motif of *cor* genes, inducing their expression, and constitutive overexpression of *AtCBF1-3* in transgenic *Arabidopsis* leads to *cor* gene induction and a subsequent increase in whole plant freezing tolerance without a cold stimulus (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000, 2004). The capacity of ectopic *CBF* transgene activity to increase plant freezing tolerance has been demonstrated in many additional plants, including species of agricultural importance such as canola (Jaglo et al., 2001), strawberry (Owens et al., 2002), tobacco (Kasuga et al., 2004), and poplar (Benedict et al., 2006).

A major disadvantage of constitutive overexpression of *CBF* genes however is that it is often associated with undesirable phenotypes, including growth retardation and delayed flowering under normal growth conditions (Hsieh et al., 2002a,b; Gilmour et al., 2000; Kasuga et al., 2004; Benedict et al., 2006). This potentially precludes the usefulness of CBF biotechnological applications in crop species where yield would be dramatically reduced. We have noted in preliminary studies that constitutive *AtCBF1* overexpression, while leading to increases in freezing tolerance, is also associated with mild in *S. tuberosum* and strong growth

retardation in *S. commersonii* (Pino et al., 2006). Kasuga et al. (1999, 2004) demonstrated that use of the stress inducible *rd29A* promoter to drive *AtCBF* transgene expression can minimize these negative overexpression effects on plant growth. In the current study, we assessed the capacity of the *Arabidopsis AtCBF1-3* genes to increase the freezing tolerance of *S. tuberosum*, whether negative agronomic growth qualities were associated with high levels of constitutive transgene activity, and if use of a stress-inducible promoter could still increase freezing tolerance while minimizing negative effects on yield.

Materials and Methods

Constructs and plant transformation

Binary transformation constructs expressing the *Arabidopsis CBF1-3* genes (*AtCBF1*, *AtCBF2*, *AtCBF3*) driven by either the constitutive CaMV *35S* or abiotic stress inducible *rd29A* promoter were utilized for transformation of the *S. tuberosum* cv. Umatilla (Figure 3.1). Generation of the constitutively expressing 35S:*AtCBF1* construct pGAH-35S::*AtCBF1* (Benedict et al., 2006), 35S:*AtCBF2* construct pMPS11 (Gilmour et al., 2004), and 35S:*AtCBF3* construct pMPS13 (Gilmour et al., 2000) have been previously described. The rd29A-based constructs prd29A::*AtCBF1*, prd29A::*AtCBF*2, and prd29A::*AtCBF*3 were derived from pMPS8, pMPS11, and pMPS13, respectively, by excising the 35S promoter region and replacing it with an 1133 bp *rd29A* promoter fragment corresponding to basepairs 4298-5430 of accession D13044; the integrity of the *rd29A*-based

derivatives was confirmed by sequencing through the ligation junctions of the *rd29A* promoter. pMPS8, described in Gilmour et al., (2004), contains a 35S::AtCBF1 operon in the same vector backbone as pMPS11 and pMPS13. Constructs were transformed into either *Agrobacterium tumefaciens* strain EHA105 or GV3101 prior to transformation of *S. tuberosum*.

Plantlets of *S. tuberosum* used as explant source material were propagated *in vitro* on sucrose-supplemented (20g/l), hormone-free Murashige and Skoog (MS) medium agar 7g L^{-1} at 25ºC with constant illumination (95-100µmol $m^{-2}s^{-1}$, cool white fluorescent lights). Suspensions of *A. tumefaciens* harboring the respective plasmid were grown overnight (28°C, 240 rpm) in liquid YEP plus 50 mg L^{-1} kanamycin to an OD_{600}=0.5–0.7, cells were collected by centrifugation (2500 rpm, 10 min), resuspended in liquid MS-2% sucrose medium (pH 5.2), and used to transform young leaf and stem explants. Explants were pre-cultivated in MS-2% sucrose medium (pH 5.7) with 2 mg L^{-1} BAP and 0.1 mg L^{-1} IAA for two days prior to transformation, incubated for 15 min (RT, 50 rpm) in the bacterial suspension plus 20 mg L^{-1} acetosyringone, then co-cultivated on MS-2% sucrose medium (pH 5.2) with 2 mg L^{-1} BAP, 0.1 mg L^{-1} IAA, and 20 mg L^{-1} acetosyringone for 2-3 d at 25°C in the dark. Explants were then washed three times in MS-2% sucrose medium (pH 5.7) with 250 mg L^{-1} cefotaxime, blotted dry on sterile paper towels for 30s, then transferred to callus induction medium: MS-2% sucrose medium (pH 5.7) with 2 mg L^{-1} BAP, 0.1 mg L^{-1} IAA, 200 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin. Explants were transferred to fresh callus

induction medium every three weeks and regenerated shoots to hormone-free MS-2% sucrose medium containing the same antibiotic concentrations (200 mg L^{-1} cefotaxime, 50 mg L^{-1}kanamycin).

Kanamycin-resistant rooted shoots were propagated *in vitro* and leaves of rooted-plantlets analyzed for transgene integration via PCR. The *35S* promoter primer 35S-P.001 (5'-cacgtcttcaaagcaagtgg-3') and the *rd29A* promoter primer rd29A.001 (5'-caagccgacacagacacgcg-3') were used as the respective forward primer to verify integration of the three *35S*-based and three *rd29A*-based expression operons. The forward promoter primers were paired with *Arabidopsis CBF* gene reverse primers AtCBF1.002 (5'-ccttcgctctgttccggtgtataaat-3'), AtCBF2.002 (5'-catccccaacatcgcctcttc -3'), or AtCBF3.002 (5'cctccaccaacgtct cctcc3') to verify integration of the respective 35S or rd29A AtCBF expression operon.

Plant Growth Conditions

Rooted plantlets of transgenic lines and untransformed (i.e., wildtype) controls were transferred to Sunshine SB40 mix (Sun Gro Horticulture Inc., Bellevue, WA) supplemented with the controlled-release fertilizer (Osmocote, Scotts Company, Marysville, OH) and grown under greenhouse conditions (25±3ºC, 16/8h day/night photoperiod, 400-480µmol$m^{-2}s^{-1}$ light intensity) with supplemental light (300-400 µmol$m^{-2}s^{-1}$) supplied via SUN System III lamps (Sunlight Supply, Inc, Vancouvor, WA) prior to transfer to experimental conditioning treatments. Greenhouse plants were fertilized weekly with foliar

fertilizer (J.R. Peters, Allentown, PA). Plants used in experimental trials were transferred from the above greenhouse conditions to a Percival Model MB60B growth chamber (16/8h photoperiod, 350 μmol $m^{-2}s^{-1}$PAR at 25ºC) for three days to acclimate to controlled environmental conditions prior to collection of experimental warm plant material. For cold-treated plants, following the three day controlled environmental conditioning, plants were transferred to an environmentally-controlled cold room maintained at 2°C (16 h photoperiod; Very High Output Phillips CW/VHO fluorescent tubes, 75 μmole $m^{-2}s^{-1}$ light intensity) for the indicated time (24 h or two weeks unless noted otherwise) before harvesting of plant material.

Leaf tissue for expression analysis was harvested from wildtype and transgenic plants growing under the above conditions. Transgene expression confirmation was conducted on plants growing at 25°C for the three *35S*-based constitutive CBF expression constructs and 24 h cold-treated plants for the three *rd29A*-based stress-inducible CBF expression constructs. Tissue used for analysis of ectopic *CBF* transgene-induced *cor* gene expression was collected from plants growing at 25°C and after two weeks of cold treatment.

Gene expression analysis

Leaf tissue was used as the RNA source for all expression analyses. Total RNA was extracted from leaf tissue using RNeasy Plant Mini kits (Qiagen, Valencia, CA) and 20 μg total RNA was electrophoretically separated per sample and transferred to nylon membrane as previously described (Skinner et al., 2005).

Blots were probed in Ultrahyb solution (Ambion Inc., Austin, TX) and washed following the manufacturer's guidelines; labeled probes were generated using a High Prime Labeling Kit (Roche Biochemicals, Indianapolis, IN). Probe fragments to each of the three *AtCBF* transgenes were prepared that excluded the conserved AP2 domain and consisted of only the C-terminal domain and 3' UTR to minimize cross hybridization to endogenous potato *CBF*s. Potato orthologs to the ectopic CBF-responsive tomato TC116174 and TC115955 *cor* genes (Zhang et al., 2004) were isolated for expression analysis. The primers Stci18.001 (5'-gctaaaccccaaaaaaaaactcatt-3') and Stci18.002 (5'-gtccaaaagacgagtacattcac-3'), based on the TIGR Potato Gene TC103027 sequence, were used to PCR amplify and clone a 496 bp fragment of the cold-responsive potato *ci18* dehydrin-like *cor* gene (the TC116174 ortholog) from *S. tubersoum* cv. Umatilla. The *S. tuberosum* cv. Shepody EST clone CK854013 encoding the potato protease inhibitor-like *cor* gene ortholog to TC115955 was identified via searches of the GenBank EST database, obtained, and the 509 bp cDNA insert completely sequenced. The insert fragments to the above two potato *cor* genes were isolated and used as probes as above. Probed blots were exposed and scanned using an MD-SI PhosphorImager system (Amersham Biosciences, Piscataway, NJ).

Controlled freeze tests

Freezing tolerance of wild type and transgenic plants was determined via controlled freezing tests (Sukumaran and Weiser, 1972) on leaf tissue of warm and two week cold-treated plants. For each sample and temperature point evaluated,

three independent experiments were conducted using three replicate samples per experiment: Test tubes for each experimental set were arranged in a randomized design. Briefly, three 10 mm leaf discs were collected from fully expanded third and fourth leaves per sample assayed and placed in 16×120 mm test tube. Tubes were incubated at -1ºC in a NESLAB bath (Model LT-50DD, Newington, NH) for 1 h. Ice nucleation was initiated by adding an ice chip to each tube, samples were maintained at -1.5 ºC for an additional 1 h, and then the temperature lowered 1ºC/h. Sample tubes were removed at -2, -4, -6 and -8ºC, and slow-thawed overnight at 2°C. Freezing injury of thawed leaf samples was assessed by determining electrolyte leakage using a YSI Model 35 conductance meter (Yellow Springs, OH). Following conductivity measurements, all samples were frozen at -20°C for 24 h, thawed at room temperature, and total conductivity determined. LT_{50} values (temperature causing 50% electrolyte leakage) were plotted as a function of freezing temperature. In conjunction with one of the trials, replicate leaf disc samples of wildtype and prd29A::AtCBF3 potato plants were subjected to the treatment conditions and collected for RNA isolation and gene expression analysis. Cold treated samples were collected at the -1,-1.5, -2, and -4°C temperature points, while warm samples were prepared by punching leaf discs from plants growing at 25°C.

Plant biomass and tuber analyses

Wildtype and transgenic plants used for leaf morphology and foliar fresh weight analyses were *in vitro* propagated, transferred to soil (one plant per 1.5 L pot), and grown in a greenhouse in Santiago, Chile (33º 27´ S.L.) under natural day length photoperiod at 25±3ºC from October to February for 16 weeks total; plants were fertilized weekly with foliar fertilizer (J.R. Peters, Allentown, PA). Following 16 weeks of greenhouse growth, total above-ground foliar biomass (leaf and stem tissue) was collected and fresh weight determined prior to analysis and documentation of leaf morphology. Wildtype and transgenic plants for tuber yield analysis were *in vitro* propagated and transferred to soil as above, then grown in a greenhouse in Santiago, Chile from October through March for 24 weeks total under natural day length photoperiod at 25±3ºC and fertilized weekly as above. Following leaf senescence, tubers were harvested, photo-documented, then tuber yield evaluated as the (i) number of tubers per plant, (ii) individual weight of each tuber, and (iii) total tuber yield weight per plant. For both above analysis types, three independent experiments were conducted with five individual plants assessed for each transgenic line or wildtype control per experiment. Plants were arranged in a randomized design in each experiment.

Statistical analyses

Data was statistically analyzed using analysis of variance (ANOVA) and mean values compared using Duncan's Multiple Range test. All statistical analyses were performed using the SAS statistical program (SAS, 2000).

Results

Selection of constitutive and stress inducible transgenic AtCBF potato lines for analysis

We screened PCR positive transgenic lines for each of the six constructs (Figure 3.1) via RNA blotting to identify lines actively expressing the transgene. Transgene expression was evaluated in plants growing at 25°C for the three p35S:AtCBF series or following 24 hours at 2°C for the three prd29A:AtCBF series. Between five and ten independent transgenic lines on average displayed detectable levels of *AtCBF* transgene expression per construct (data not shown), from which we selected three independent expressing lines per construct for further analysis (below).

Constitutive overexpression of three *AtCBF* genes exhibits differential effects on plant phenotype and freezing tolerance

Transgene expression was readily detectable in each line assessed in further detail for each of the three constitutive 35S:AtCBF transgenics (Figure 3.2A). Based on exposure times of northern blots, expression of the *AtCBF1* transgene was much lower for all three lines relative to that of the AtCBF2 and AtCBF3 lines, suggesting it was not expressed as strongly (data not shown). Analysis of plant morphology revealed the shape and size of the leaves to be somewhat altered in each line of all three 35S:AtCBF transgenic series (Figure 3.2B). The degree of alteration varied between each construct series, but was relatively consistent among the three independent lines of a given construct (Figure 3.2B). In agreement with

the lower expression levels of the *AtCBF1* transgene, the 35S:AtCBF1 lines displayed the least degree of leaf alteration relative to wildtype leaves. Growth retardation, a common indicator of high levels of CBF activity, was also most pronounced in the 35S:AtCBF2 and 35S:AtCBF3 lines (Figure 3.2C). Again, the 35S:AtCBF1 lines showed the least growth retardation and were relatively similar to wildtype plants (Figure 3.2C); the 35S:AtCBF1 lines exhibited a somewhat more pronounced growth retardation phenotype during *in vitro* propagation, but grew out of the more stunted phenotype within the first few weeks after transfer to soil (data not shown). Lines of all three 35S:AtCBF construct sets showed some delay in flowering relative to wildtype, but in agreement with the expression level differences, the 35S:AtCBF1 lines were less delayed than the 35S:AtCBF2 and 35S:AtCBF3 lines (data not shown).

We evaluated whether the transgenic plants had increased freezing tolerance as constitutive *CBF* transgene expression bypasses the need for a cold stimulus. Under warm growth conditions, all three lines of both the 35S:AtCBF1 and 35S:AtCBF3 constructs showed a significantly increased freezing tolerance to about-5°C, a gain of about 2°C (Figure 3.3A). Surprisingly, the 35S:AtCBF2 lines only showed a minor average increase in freezing tolerance that was not significant (Figure 3.3A). We isolated plasmid DNA from the *A. tumefaciens* glycerol stock harboring the pMPS11 transformation construct and sequenced the 35S:AtCBF2 operon, confirming no mutations were present and that a functional product should be expressed (data not shown), in agreement with the observed growth retardation

and delayed flowering phenotypes (Figure 3.2C, data not shown). Following two weeks of cold treatment, no significant increase in freezing tolerance was observed for any of the constructs (Figure 3.3A). Together, these results indicate *AtCBF1* and *AtCBF3* overexpression can effectively increase the freezing tolerance of potato, while *AtCBF2* cannot.

Constitutive overexpression of three At*CBFs* in potato inhibits tuber formation

As tubers are the major agronomic trait of potato, we evaluated if the negative pleiotropic effects of ectopic *AtCBF* expression also affected tuber yield. Despite the beneficial gain in freezing tolerance, constitutive *AtCBF* expression did cause a significant inhibition of tuber formation compared to wildtype potato plants (Table 3.1). *AtCBF3* completely abolished tuber formation, while *AtCBF2* also abolished tuber formation capacity in two of the lines. In the third AtCBF2 line, only four tiny tubers formed; these results further support the *AtCBF2* transgene is expressing a functional product. For these two transgenes, the abolishment or reduction in tuber yield corresponded with a reduction in vegetative growth (Table 3.1), where the foliar fresh weight was 676 g/plant for WT, 370±25 g/plant for AtCBF2, and 360±30 g/plant for AtCBF3 transgenic plants, respectively. The AtCBF1 lines which displayed the least growth retardation relative to wildtype (Figure 3.2C) were also the least affected by constitutive transgene expression. While significantly lower than wildtype, each of the AtCBF1 lines was capable of tuber production and produced total foliar biomass closer to wildtype levels (Table

3.1 and Figure 3.4A). For example, while about half of the tubers were greater than 75 g in wildtype plants, the majority of tubers harvested from the AtCBF1 lines were less than 75 g, with a large percentage being less than 25 g (Table 3.1).

Cold-inducible expression of *AtCBF1* and *AtCBF3* minimizes phenotypic alterations and negative effects on tuber yield

While the 35S:AtCBF1 lines were capable of tuber production, the significant decrease in tuber number and mass would be agronomically unacceptable despite the beneficial gain in freezing tolerance. In *Arabidopsis*, Kasuga et al. (1999) found that directing *CBF* transgene expression via an abiotic stress inducible promoter minimized negative effects on plant growth. We therefore generated analogous transformation constructs in which the *AtCBF1-3* genes were driven by the abiotic stress inducible *rd29A* promoter, which is responsive to cold, drought, and high salt (Yamaguchi-Shinozaki and Shinozaki, 1994). As in the 35S:AtCBF construct series, we selected three independent transgene-expressing lines for each of the three rd29A:AtCBF constructs to examine in more detail. Preliminary analysis of the rd29A:AtCBF2 transgenic lines demonstrated that despite high levels of transcript accumulation, they failed to increase freezing tolerance relative to wildtype (data not shown) and therefore the rd29A:AtCBF2 plants were excluded from further analysis. Transgenic lines of rd29A:AtCBF1 and rd29A:AtCBF3 plants accumulated high levels of *AtCBF* transgene transcript following 24 h at 2°C (Figure 3.2D), while only negligible transcript levels were detectable in prolonged exposures when grown under warm conditions (see below,

data not shown). Morphological comparisons of the rd29A:AtCBF1 and rd29A:AtCBF3 plants with wildtype revealed no major differences in the leaf morphology and plant height when grown under non-stress (i.e., warm) conditions (Figure 3.2E and 2F), indicating absence of deleterious levels of *AtCBF* transgene product

In agreement with the lack of morphological changes, the foliar biomass of rd29A:AtCBF1 and rd29A:AtCBF3 lines was not significantly different from wildtype plants (Table 3.2). Importantly, these lines retained the capacity for tuber generation (Figure 3.4B), producing similar number and yields of tubers as wildtype (Table 3.2); one rd29A:AtCBF1 line (line 6) was somewhat inhibited in tuber yield, although foliar biomass was unaffected. The main observable difference between the transgenic and wildtype plants related to tuber size, where 40% of tubers were larger than 75 g for wildtype plants, while only about 25% were for the transgenic lines (Table 3.2, Figure 3.4B).

Cold-inducible expression of *AtCBF1* and *AtCBF3* increases potato freezing tolerance

We examined the freezing tolerance of rd29A:AtCBF1 and rd29A:AtCBF3 lines under both warm growth conditions and after two weeks of cold stress (Figure 3.5A). Under non-stress conditions, lines of both constructs showed an increase in freezing tolerance of approximately 0.5-1°C, implying low, but non-deleterious, AtCBF product levels. This partial freezing tolerance increase under non-stress conditions alternatively could be an artificial byproduct of the ion leakage assay

itself, rather than being representative of the actual plant's state under the non-stress growth conditions (see below). Following the two week cold-stress treatment, lines of both constructs displayed an additional gain in freezing tolerance that was equivalent to the levels imparted by 35S:AtCBF lines – a net gain of about 2°C over WT plants.

Constitutive vs. inducible control of *AtCBF3* expression yielded a similar gain in freezing tolerance, yet the two promoters led to very contrasting capacities of ectopic CBF activity to negatively affect plant growth and tuber production. We examined expression of the *AtCBF3* transgene and two potato orthologs (see Methods) to tomato *cor* genes (Zhang et al., 2004) responsive to ectopic *CBF* expression in more detail. As expected, the *AtCBF3* transgene was detectable in both the warm and cold treated 35S:AtCBF3 lines, although the steady-state transcript level was higher in the cold-treated lines. In support of the wildtype-like growth phenotype, transgene presence was negligible – just barely detectable under prolonged exposures – in rd29A:AtCBF3 lines grown under warm conditions, but induced upon cold treatment as expected. In wildtype plants under warm growth conditions, *StPI* transcript was not detectable while *ci18* transcript was present as a weak signal. The two week cold treatment resulted in a respective minor and more substantial increase of the *StPI* and *ci18* transcript levels (Figure 3.5A). In the 35S:AtCBF3 lines, *ci18* transcript levels were somewhat increased over wildtype, particularly under warm conditions, while *StPI* transcript levels were substantially increased under both conditions. In the rd29A:AtCBF3 lines, *ci18* transcript levels

were similar to wildtype under both conditions, while *StPI* transcript, absent under non-inducing (i.e. warm) conditions, was noticeably higher during cold-treatment.

The increase in freezing tolerance of non-cold-treated rd29A::AtCBF plants suggested either a low level of *AtCBF* expression was occurring and/or *AtCBF* expression was being triggered by the controlled freezing test. We thus studied whether the controlled freezing test itself was acting as an induction source of *AtCBF* transgene expression in the non-cold-treated samples. A set of rd29A::AtCBF3 leaf disc samples were treated side by side with the duplicate ion leakage samples and the discs harvested for RNA isolation at the following temperatures: warm and -1,-1.5, -2, and -4°C temperature points (see Methods). In all three rd29A:AtCBF3 lines, *AtCBF3* transgene presence was not detected in warm control conditions, while transcript was present at -1°C. Decreasing the temperature caused slight gains in transgene level (Figure 3.5B), indicating the freeze test conditions were inducing transgene expression. Expression patterns of the two *cor* genes were similar to the above results. Specifically, the StPI *cor* gene level was noticeably higher in the three transgenic lines relative to wildtype, indicating the CBF activity provided by the *AtCBF3* transgene was inducing the expression of the StPI *cor* gene to a higher level.

Discussion and Conclusions

Damage due to frost conditions can be a major limiting factor in potato production. We assessed the ability of the *Arabidopsis AtCBF1-3* genes to increase freezing tolerance in *S. tuberosum*, as these genes have been successfully used to improve this trait in a diverse range of plant species, including agronomically important crops (Jaglo et al., 2001; Gilmour et al., 2004; Kasuga et al., 2004; Vogel et al., 2004; Benedict et al., 2006).

When utilizing the strong constitutive CaMV *35S* promoter to direct expression, potato freezing tolerance was successfully enhanced in plants ectopically overexpressing *AtCBF1* and *AtCBF3*, whereas the freezing tolerance of plants overexpressing *AtCBF2* did not increase (addressed below). A major drawback to high levels of *CBF* transgene expression however is the associated negative effects on plant growth and development, which include stunting and delayed flowering. We observed both these traits in the 35S:AtCBF lines. In potato, a crop grown for tuber production, delays in flowering would not be of major concern. However, the degree of stunting correlated with a decrease in total foliar biomass, and more importantly, high levels of constitutive *CBF* expression in potato also had major negative effects on tuber production and quality. For the 35S:AtCBF1 lines, where *CBF* transgene expression was the weakest, tuber production was severely reduced and the tubers produced were of a smaller size, while in the 35S:AtCBF3 lines, tuber production was completely eliminated (Table 3.1). Despite the lack in freezing tolerance gain, high levels of *AtCBF2* transgene

expression also inhibited tuber formation. The lack of recovery of AtCBF1 plants expressing high levels of the transgene may indicate that expression of high *AtCBF1* levels is either toxic or retards growth to such an extent that only weaker expressing lines can regenerate into shoots and be recovered in a timely fashion. It is possible that selection of AtCBF2 and AtCBF3 lines with lower transgene expression levels would also have resulted in recovery of less severely affected phenotypes. Together, these results suggest the constitutive presence of high CBF product levels is inhibitory to potato tuberization. Thus, while 35S-based expression was good for increasing potato freezing tolerance, it is unsuitable for agronomic applications because of its negative impact on tuber production.

Kasuga et al. (1999) demonstrated that driving ectopic *CBF* expression via a promoter induced by abiotic stress (i.e., a *cor* gene promoter) can alleviate the stunting phenotype, which is apparently a pleiotropic side-effect of excessive activation domain presence and not a CBF-specific property. Wang et al. (2005) found that fusion of the CBF activation domain to a yeast DNA binding domain still yields a stunted phenotype in *Arabidopsis*, even though the CBF *cor* gene regulon would not be induced. We therefore investigated whether this strategy could also eliminate the negative effects on tuber production, as the degree of stunting appeared related to the severity of negative effects on tuber production. Use of the stress-inducible *rd29A* promoter to direct *AtCBF* transgene expression in potato alleviated the stunting phenotype and led to generation of foliar biomass levels similar to wildtype. Importantly, tuber formation was also restored and did

not significantly differ from wildtype. The differential control of *AtCBF3* transgene expression had the most dramatic effect on plant morphology and tuber production of the three *AtCBF* genes tested. Whereas constitutive *AtCBF3* expression led to a significant reduction in foliar biomass, stunting, and total abolishment of tuber production, expression of *AtCBF3* under the rd29A promoter ameliorated these trends without affecting the freezing tolerance gain (below). The means by which constitutive *Arabidopsis CBF* expression interferes with tuber formation is unclear. The constant presence of excessive CBF product could delay the time to reach a sufficient growth and/or developmental level necessary for tuberization in a manner similar to the delay in flowering time. In tomato, constitutive overexpression of *AtCBF1* not only delayed flowering, but also resulted in production of fewer fruits per plant (Hsieh et al., 2002a, b). Alternatively and as hypothesized for the stunting effect (Wang et al., 2005), excess CBF activation domain presence may lower the pool of necessary transcriptional accessory factors involved in tuberization and thereby preclude or delay the tuberization process.

A similar study in tobacco using an rd29A:AtCBF3 expression operon found some lines exhibited growth retardation characteristics similar to 35S:AtCBF3 overexpression lines (Kasuga et al., 2004). We observed one of the rd29A:AtCBF1 lines (line 6; Table 3.2) had a slight decrease in tuber yield, similar to those of the 35S::AtCBF1 lines. These observations in tobacco (Kasuga et al., 2004) and potato (this study) may be due to either position effects and/or leaky expression. The *rd29A* promoter is responsive to a variety of abiotic stresses (e.g.,

cold, drought, and salt) and minor amounts of transcript are detectable under non-stressed conditions (Yamaguchi-Shinozaki and Shinozaki, 1994). Minor stresses encountered during greenhouse growth, such as drought stress, could transiently activate transgene expression during greenhouse growth, while some lines could have higher levels of uninduced basal expression due to position effects. Together, this could lead to minor but cumulative negative effects on final plant characteristics. The observation that rd29A:AtCBF1 and rd29A:AtCBF3 lines exhibit a partial increase in freezing tolerance capacity under warm (non-stress) conditions, as well as detection of minor amounts of transgene under this condition, supports the presence of minor amounts of CBF product during normal growth. However, the observed "partial increase" during controlled growth seemed to be mainly an artificial byproduct of the freezing test component step of the ion leakage assay, so the actual amount of leaky rd29A-based AtCBF activity is likely relatively low. Use of a more tightly regulated *cor* gene promoter, or one that is only responsive to cold treatment, could help to further minimize these slight effects. Overall, this approach demonstrates that use of a stress inducible promoter to drive *CBF* transgene expression can largely alleviate negative effects on tuber formation and yield, while imparting a beneficial increase in freezing tolerance.

Importantly, the inducible nature of the rd29A promoter did not affect the final gain in potato freezing tolerance capacity, as the rd29A:AtCBF lines increased to the same degree after cold treatment as the 35S:AtCBF lines. Interestingly, we observed that the steady state transgene mRNA level of the 35S:AtCBF3 lines was

higher in the cold relative to warm treatment and have observed this trend in independent experiments (data not shown). The *AtCBF3* transcript may therefore either be more stable in the cold or less stable in the warm and this effect is enhanced in the potato background; the *AtCBF3* transcript contains the 5' and 3' untranslated regions, which could be a source of this characteristic. While freezing tolerance was only increased about 2°C in the transgenic potato, this is a significant increase in freezing tolerance for cultivated potato. *S. tuberosum* has a natural freezing tolerance of about -3°C, and as noted above, frost events tend to occur around -3°C to -4°C during the growing season and can cause significant loss in yield (Carrasco et al., 1997; Hijmans et al., 2003). In the Altiplano areas, development of potato cultivars with a freezing tolerance improvement of 1ºC or 2ºC would have a great impact on potato production. Hijmans et al. (2003) have estimated that for the potato production areas of Peru and Bolivia, a freezing tolerance increase of 1ºC would improve potato yield about 26%, while an increase of 2ºC would improve yield nearly 40%. Potatoes which can survive to -5ºC, such as the transgenic potato plants reported in this paper, should be useful in surviving not only spring frosts, but also fall frosts and allow for an extended potato growing season. Additionally, they could be useful in the Mediterranean region, where potato is cultivated year round. Thus, inducible expression of *CBF* genes under a stress inducible promoter is one means to obtain this goal. Determining potato yield, quality, and frost tolerance under field conditions is a logical next step in evaluating the agronomic usefulness of these plants.

S. tuberosum lacks the ability to cold acclimate, i.e., increase its level of freezing tolerance after exposure to low but non-freezing conditions. In the constitutive overexpression lines of *AtCBF1* and *AtCBF3*, two weeks of cold acclimation failed to further increase freezing tolerance of the lines, indicating that excess CBF levels were incapable of conferring this property and the freezing tolerance enhancement was independent of cold stimulus. In the *rd29A* lines, freezing tolerance did increase after cold treatment due to the cold-inducible nature of the promoter, but only to the level seen in the 35S lines. Thus the approximately 2ºC gain in freezing tolerance appears to be the maximum these genes can impart on *S. tuberosum*. We analyzed the expression of potato homologs to two tomato *cor* genes responsive to ectopic *CBF* expression (Zhang et al., 2004). The dehydrin-like *ci18 cor* gene appeared to be only slightly affected by transgene presence whereas the protease inhibitor homolog was a good indicator of *CBF* transgene activity. Expression of the protease inhibitor increased relative to wildtype plants under conditions in which the transgene was expressed, supporting gains in freezing tolerance being associated with capacity to ectopically induce *cor* genes.

In *Arabidopsis*, Gilmour et al. (2004) concluded that the three *Arabidopsis CBF*s had redundant activities when constitutively overexpressed via the 35S promoter – similar plant phenotypes and gene regulons were observed for all three genes. Interestingly, this was not the case for potato. *AtCBF1* and *AtCBF3* behaved the most like expected when driven by the 35S promoter, imparting increased freezing tolerance with associated stunting and delayed flowering, indicating they

specify relatively redundant activities in potato. While the *AtCBF1* growth abnormalities were less pronounced, this is likely a byproduct of the lower transgene expression level of these lines. In contrast, *AtCBF2* did not exhibit a redundant phenotype to *AtCBF1* and *AtCBF3* in potato for freezing tolerance. The 35S:AtCBF2 construct (pMPS11) we employed is the same construct utilized in the Gilmour et al. (2004) study. Potato lines transformed with this construct exhibited the constitutive CBF expression-traits of severe stunting and delayed flowering, and like the AtCBF3 lines, also severely inhibited tuber formation. AtCBF2 lines (35S:AtCBF2 and rd29A:AtCBF2) did not impart a significant increase in freezing tolerance capacity however. The generation of the abnormal growth phenotypes suggests a functional product is expressed. We confirmed via sequencing that no mutations were present in the 35S:AtCBF2 or rd29A:AtCBF2 construct of the respective *A. tumefaciens* cultures used for transformation. The reason for the failure of *AtCBF2* to increase freezing tolerance in potato, in contrast to *Arabidopsis* (Gilmour et al. 2004; Vogel et al. 2005), is unclear. Novillo et al. (2004) proposed *AtCBF2* acts as a negative regulator of *AtCBF1* and *AtCBF3* in *Arabidopsis* by down-regulating their expression. How this role could affect freezing tolerance in potato is unclear. We have found that individual barley *CBF* genes have different binding preferences for CRT motifs based on the sequence flanking the core motif (Skinner et al., 2005). If the AtCBF1-3 products have slight variations in binding preferences, activating similar but not completely identical potato regulons, *AtCBF2* may fail to activate a critical potato *cor* gene(s) necessary

to increase freezing tolerance relative to the AtCBF1- and AtCBF3-specified regulons. *AtCBF1* and *AtCBF3* are more closely related to each other than to *AtCBF2* (Haake et al., 2002), suggesting their binding preferences could be more similar. We have also observed similar effects in poplar, where *AtCBF1* and *AtCBF3* increase freezing tolerance, but *AtCBF2* does not (Meng, 2006).

In conclusion in the current study, we assessed the ability of constitutive and cold-inducible expression of three *Arabidopsis CBF* genes to increase freezing tolerance without negatively impacting plant growth and tuber quality in *S. tuberosum*. Overexpression of *AtCBF1* and *AtCBF3* under the control of an inducible promoter imparted these qualities to potato –freezing tolerance was enhanced to about -5°C with minimal associated negative effects on tuber yield. These results suggest the strategy of cold-inducible expression of a *CBF* transgene is an effective way to increase the freezing tolerance of potato without significantly affecting the agronomic important trait of tuber production.

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Table 3.1 Effects of constitutive 35S:AtCBF transgene expression on potato *S. tuberosum L.* (cv. Umatilla) foliar biomass and tuber yield [1].

35S:AtCBF Transgene	Line	Foliar[2] F.W. (g)	Yield[3] (g/ plant)	Tubers per Plant[3]			
				Total Tubers	% Tubers >75 g	% Tubers 25-75 g	% Tubers <25 g
-----	WT[4]	676.0[a]	627.1[a]	8[a]	45	24	35
AtCBF1	2	511.3[b]	165.1[c]	4[b]	30	35	35
AtCBF1	11	544.9[b]	203.3[b]	8[a]	0	36	64
AtCBF1	15	540.0[b]	121.8[d]	5[b]	0	15	85
AtCBF2	2	376.4[c]	0.0[e]	0[c]	0	0	0
AtCBF2	4	390.2[c]	0.0[e]	0[c]	0	0	0
AtCBF2	7	342.1[c]	7.5[e]	4[b]	0	0	100
AtCBF3	25	388.0[c]	0.0[e]	0[c]	0	0	0
AtCBF3	28	328.0[c]	0.0[e]	0[c]	0	0	0
AtCBF3	40	353.9[c]	0.0[e]	0[c]	0	0	0

[1]Letters after values denote significant differences (p-value<0.0001) between lines based on Duncan's Multiple Range Test

[2]Foliar tissue consisted of leaf and stem tissue from 16 week old plants

[3]Tubers were harvested from 24 week old plants following leaf senescence

[4]WT: wildtype (non-transgenic) control

Table 3.2 Effects of stress inducible rd29A:AtCBF transgene expression on potato *S. tuberosum* foliar biomass and tuber yield [1].

rd29A:AtCBF Transgene[2]	Line	Foliage[3] F.W. (g)	Yield[4] (g/plant)	Tubers per Plant[4]			
				Total Tubers	% Tubers >75 g	% Tubers 75-25 g	% Tubers <25 g
-----	WT[5]	676.1[a]	628.9[a]	8[ab]	44	23	33
AtCBF1	1	640.4[a]	559.6[a]	8[b]	26	27	47
AtCBF1	3	640.0[a]	614.6[a]	7[b]	32	32	36
AtCBF1	6	612.0[a]	309.4[b]	4[c]	0	55	45
AtCBF3	17	632.1[a]	618.5[a]	10[a]	23	38	38
AtCBF3	43	659.0[a]	516.7[a]	7[b]	25	42	34
AtCBF3	48	644.3[a]	537.4[a]	8[ab]	25	42	34

[1]Letters after values denote significant differences (p-value<0.0001) between lines based on Duncan's Multiple Range Test

[2]rd29A:AtCBF2 lines not tested (see text)

[3]Foliar tissue consisted of leaf and stem tissue from 16 week old plants

[4]Tubers were harvested from 24 week old plants following leaf senescence

[5]WT: wildtype (non-transgenic) control

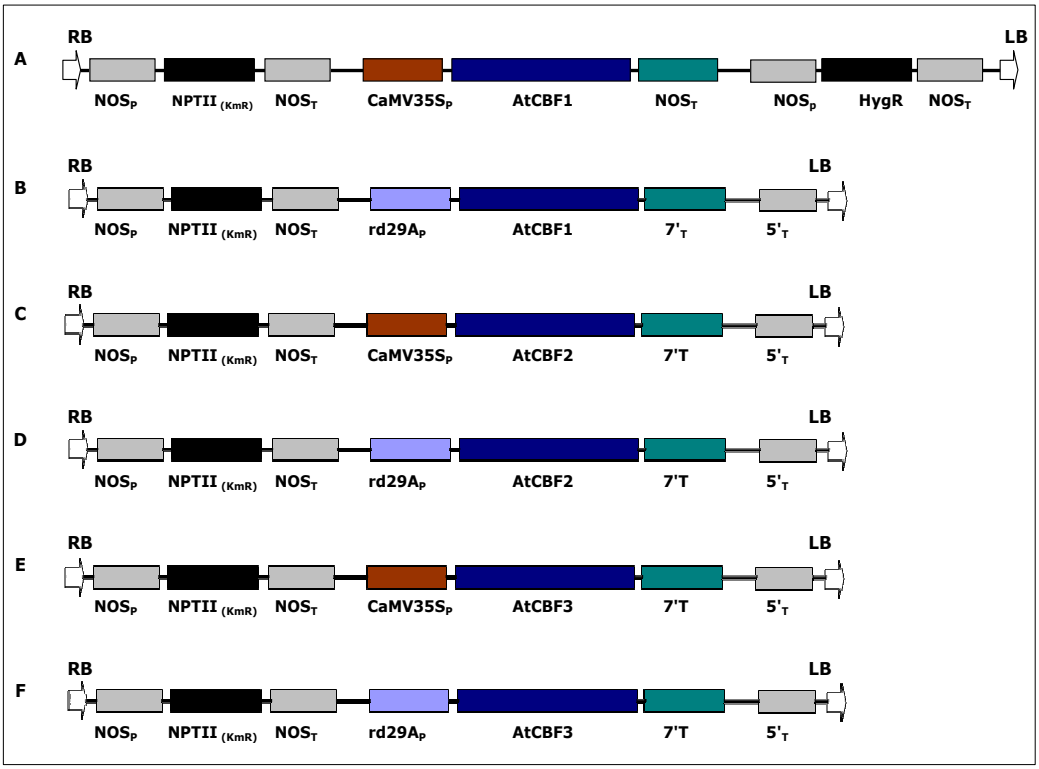

Figure 3.1 T-DNA region of transformation constructs used in *Solanum tuberosum L.*. (cv. Umatilla). **A**, p35S::AtCBF1 **B**, prd29A::AtCBF1 **C**, p35S::AtCBF2 (pMPS11) **D**, prd29A::AtCBF2. **E**, p35S::AtCBF3 (pMPS13) **F**, prd29A::AtCBF3. Genetic elements and hygromycin (Hyg) and kanamycin resistance (NPTII) resistance genes present in each construct are denoted. $_P$: promoter element; $_T$: terminator element.

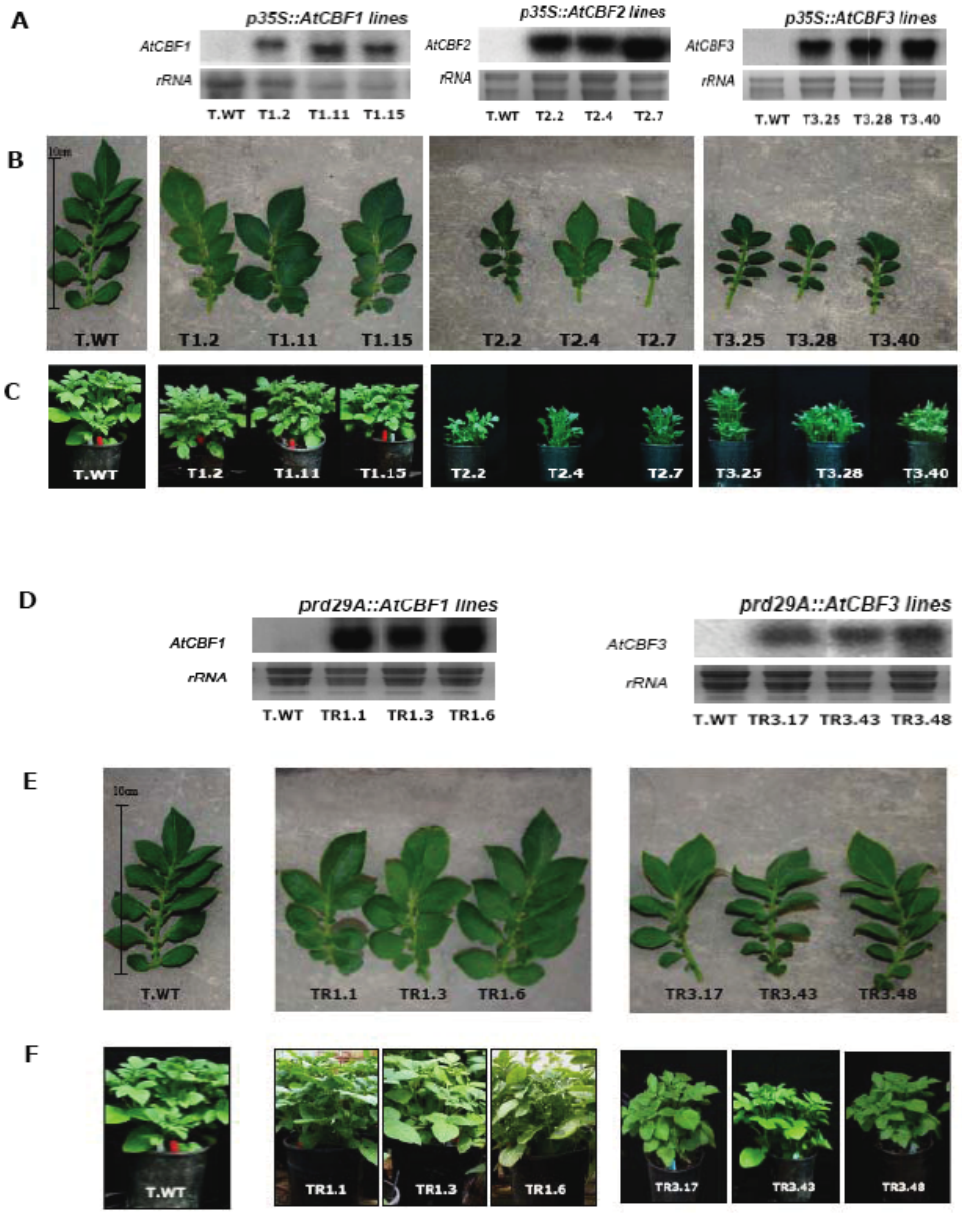

Figure 3.2 Effect of constitutive and stress-inducible overexpression of *AtCBF1-3* genes on growth characteristics of *S. tuberosum*. Effects were analyzed on 16-week old plants of the following types: wildtype (T.WT); 35S:AtCBF1 lines (T1.2, T1.11, T1.15); 35S:AtCBF2 lines (T2.2, T2.4, T2.7); 35S:AtCBF3 lines (T3.25, T3.28, T3.40); rd29A:AtCBF1 lines (TR1.1, TR1.3, TR1.6); and rd29A:AtCBF3 lines (TR3.17, TR3.43, TR3.48). Assessments were conducted on plants growing at 25ºC (Panels A-C, E, F) or after 24 h at 2ºC (Panel D). Analysis of transgene expression of the indicated *CBF* gene (A, D), leaf morphology (B, E), and gross plant phenotype (C, F).

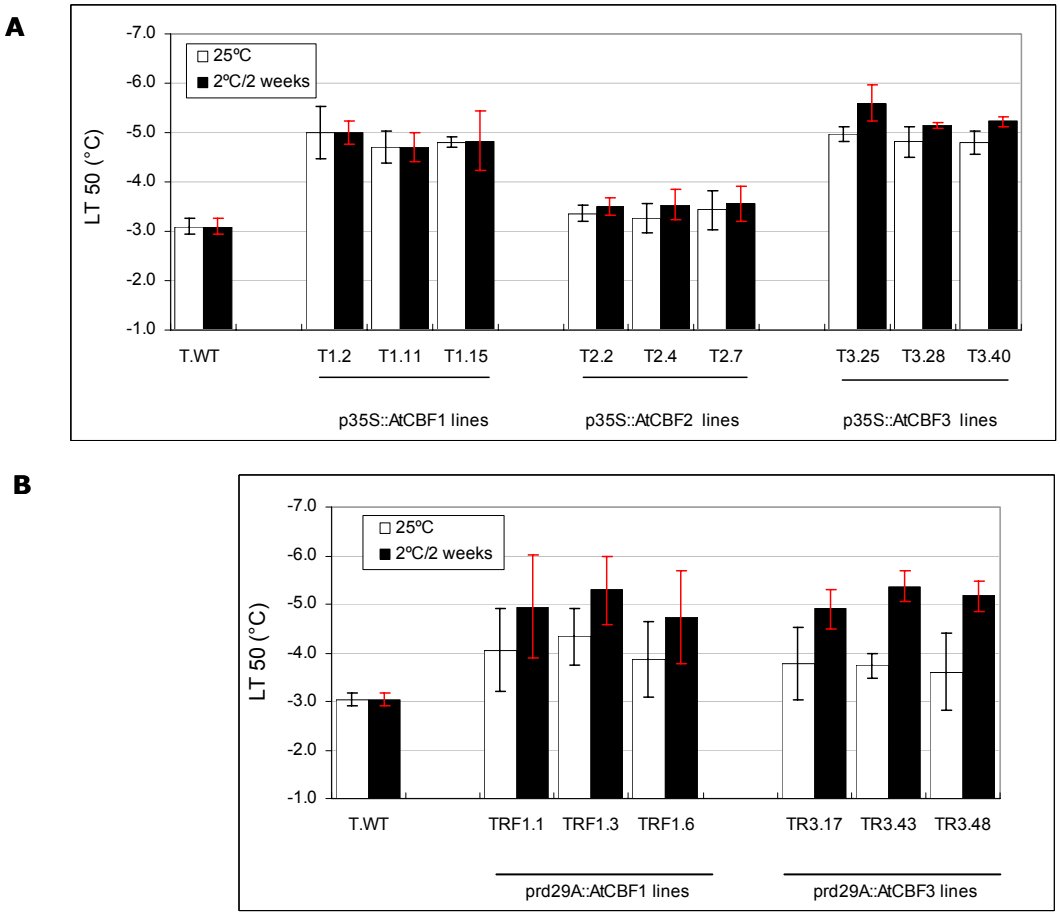

Figure 3.3 Freezing tolerance of wildtype and transgenic *S. tuberosum*. Freezing tolerance is expressed as the LT50 in -ºC for **A,** wildtype (T.WT) and constitutive transgenic lines 35S:AtCBF1 lines 2 (T1.2), 11(T1.11), 15 (T1.15); 35S:AtCBF2 lines 2 (T2.2), 4 (T2.4), 7 (T2.7); 35S:AtCBF3 lines 25 (T3.25), 28 (T3.28), 40 (T3.40). **B,** wildtype (T.WT) and stress-induced transgenic lines rd29A:AtCBF1 lines 1 (TR1.1), 3 (TR1.3), 6 (TR1.6); and rd29A:AtCBF3 lines 17 (TR3.17), 43 (TR3.43), 48 (TR3.48). Plants were either grown at 25ºC (empty bars) or after two weeks of cold-treatment at 2ºC (solid bars). Standard deviation of means is indicated as vertical bars.

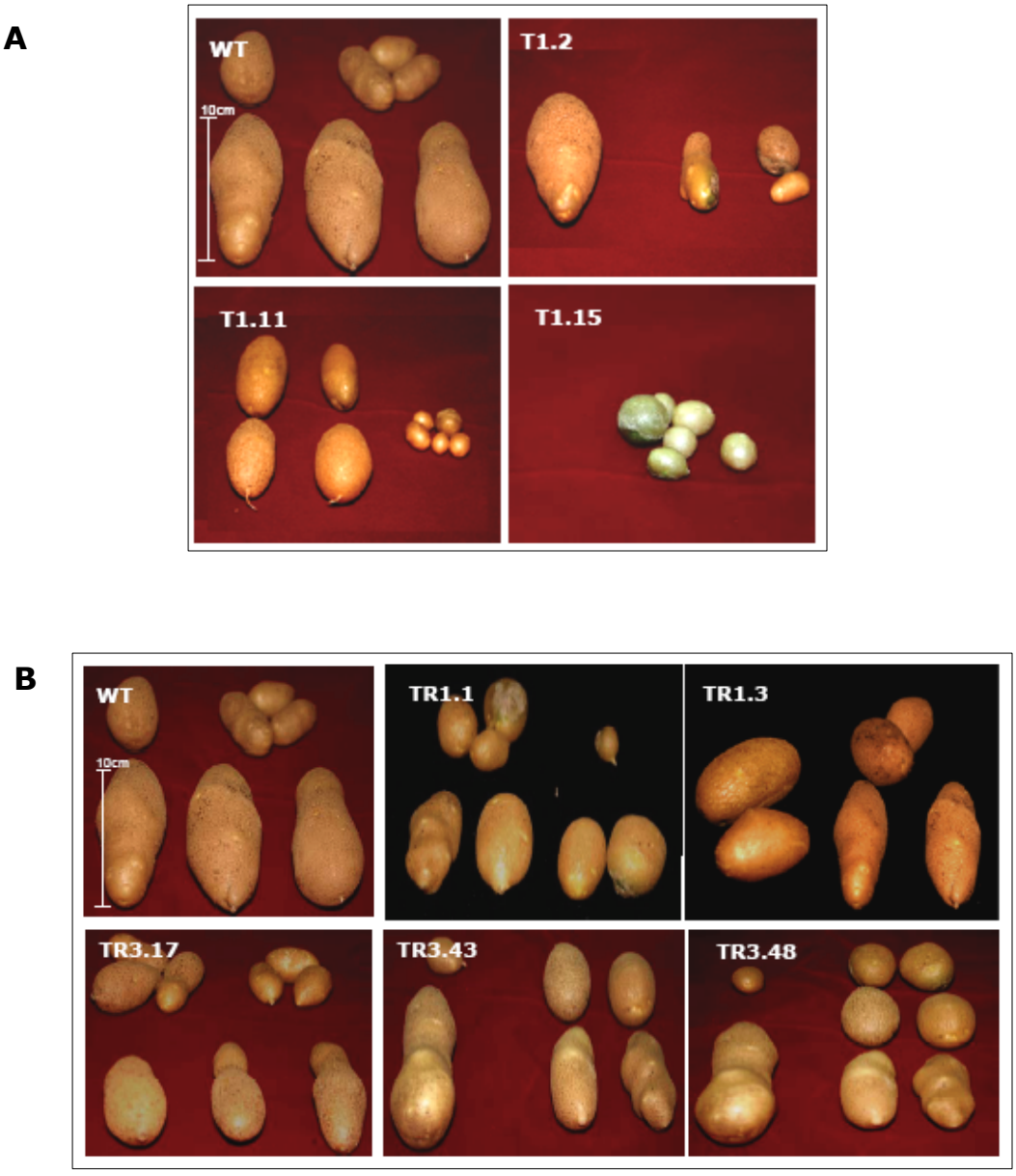

Figure 3.4 Effect of constitutive and stress-inducible *CBF* transgene overexpression on tuber characteristics. Potato tubers of *S. tuberosum* **A,** wild type (T.WT) and transgenic 35S::AtCBF1 lines (T1.2, T1.11, and T1.15) **B,** wild type (T.WT) and transgenic rd29::AtCBF1 lines (TR1.1 and TR1.3), and rd29A:AtCBF3 transgenic lines (TR3.17, TR3.43, and TR3.48). 35S::AtCBF2 and 35S::AtCBF3 overexpression lines failed to produce tubers.

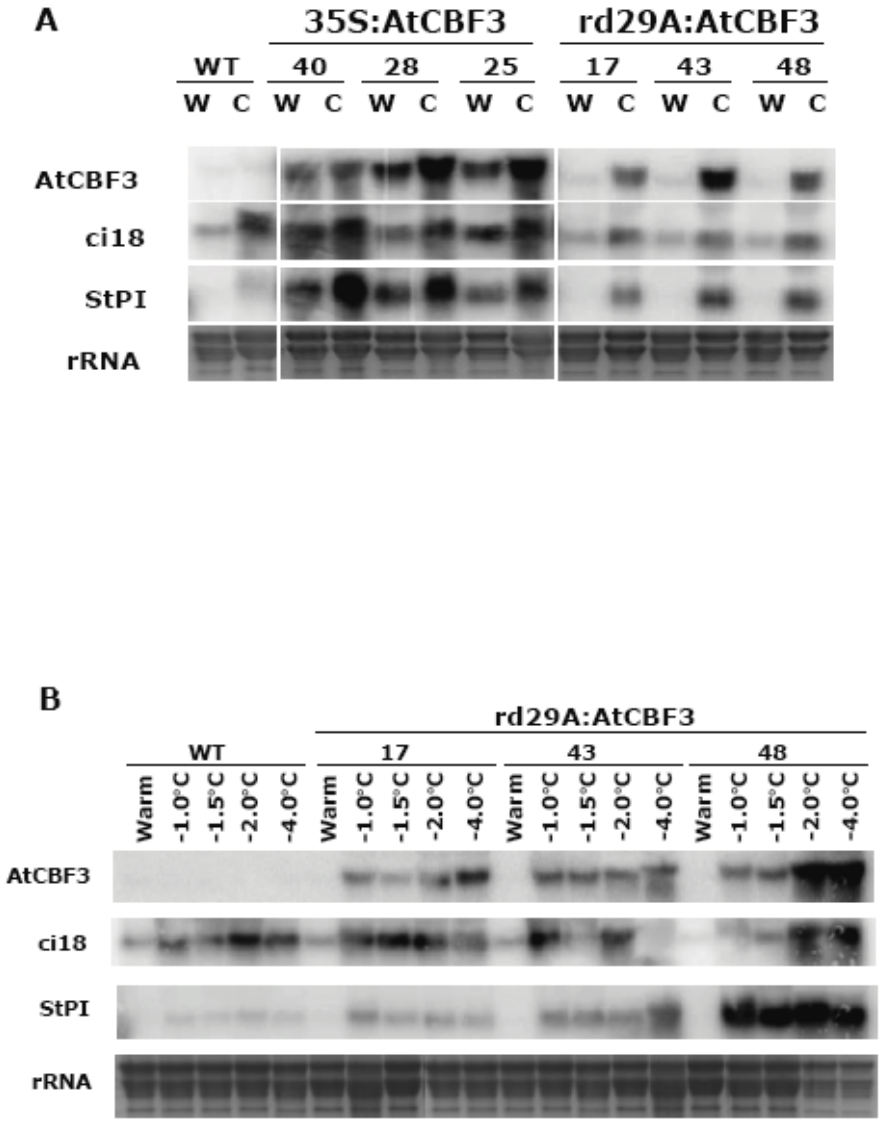

Figure 3.5 Effect of *AtCBF3* transgene expression on two potato *cor* genes. Panel **A,** Expression of the *AtCBF3* transgene and potato *ci18* and *StPI cor* genes during growth of *S. tuberosum* wildtype (WT) and transgenic plants under warm (W) control conditions and after 2 weeks cold (C) treatment. Lines numbers and transgene type are indicated. Panel **B**, Expression of the above genes in *S. tuberosum* WT and rd29A:AtCBF3 transgenic leaf disc samples (lines 17, 43 and 48) during a representative ion leakage assessment assay. The temperatures at which the leaf discs were subjected are indicated. For both panels, ethidium bromide-stained rRNA bands are shown as a loading control.

CHAPTER 4

DIFFERENTIAL EFFECTS OF THE OVEREXPRESSION OF THREE *ARABIDOPSIS CBF*s ON FREEZING TOLERANCE AND COLD ACCLIMATION CAPACITY IN *Solanum commersonii*, A HARDY POTATO SPECIES WHICH CAN BE COLD ACCLIMATED

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Abstract

Solanum commersonii Dun, one of the most cold hardy tuber-bearing potato species, is capable of surviving freezing temperatures of about -5ºC and can increase its freezing tolerance to about -10ºC after becoming cold acclimated. To advance our understanding of the role *CBF* genes may play in potato freezing tolerance, we transformed *S. commersonii* (PI 243503 clone 13) with three *Arabidopsis CBF* genes (*AtCBF1* through *3*) under the control of either the constitutive CaMV*35S* promoter or the stress-inducible *Arabidopsis rd29A* promoter. While constitutive overexpression of all three *AtCBF* genes generated varying degrees of phenotypic alterations, only *AtCBF1* and *AtCBF*3 overexpression led to improved freezing tolerance and cold acclimation capacity in *S. commersonii*. Overexpression of *AtCBF1* and *AtCBF3* under the control of the stress-inducible *rd29A* promoter enhanced freezing tolerance after cold treatment while minimizing negative phenotypic alterations. The AtCBF-based increase in freezing tolerance and cold acclimation capacity was

associated with expression of cold-regulated genes, implying an endogenous CBF cold response pathway exists in *Solanum commersonii* and that CBF factors are likely involved in the cold acclimation process.

Keywords: Potato, *Solanum commersonii*, *AtCBF*s, freezing tolerance, LT_{50}, cold acclimation

Introduction

Many plants from temperate regions are capable of increasing their freezing tolerance when exposed to low but nonfreezing temperatures, a phenomenon known as cold acclimation. Thus, plants capable of cold acclimation possess two levels of freezing tolerance: a pre-acclimation capacity (the intrinsic freezing tolerance level) and a post-acclimation capacity (the cold acclimation level). Plants differ widely in their freezing tolerance and cold acclimation capacity. In tuber-bearing *Solanum* species, the cultivated potato *Solanum tuberosum* L. cannot survive temperatures below approximately -3.0ºC and cannot be cold acclimated, while other tuber-bearing potato species such as *Solanum acaule, S. commersonii, S. boliviense, S. multidissectum,* and *S. sanctae-rosae* are freezing tolerant to about -4 to -5ºC pre-acclimation and can cold acclimate to further increase their freezing tolerance level (Chen and Li, 1980; Barrientos et al., 1994; Vega and Bamberg, 1995). Among them, *S. commersonii*, a diploid tuber-bearing potato species endemic to Argentina, Brazil, Paraguay and Uruguay, is one of the most cold-hardy potato species. *S. commersonii* represents both a potential source of genes for improving *S.*

tuberosum freezing tolerance and a system for studying the pathways involved in superior potato freezing tolerance and cold acclimation capacity. This wild potato species can naturally tolerate freezing temperatures and frosts down to about -5ºC and is capable of increasing its freezing tolerance to as low as -10ºC after becoming cold acclimated (Chen and Li, 1980). This post-cold acclimation increase in freezing tolerance occurs in conjunction with multiple biochemical and physiological alterations that include the synthesis of new proteins (Ryu and Li, 1994), increase in the content of free sugars and starches (Chen and Li, 1980), changes in lipid membrane composition (Palta et al., 1993) and alteration of gene expression patterns (Tseng and Li, 1987, 1990; Zhu et al., 1993, 1996).

Transfer of superior freezing tolerance traits to the cultivated potato *S. tuberosum* has been a major breeding goal in areas with cool climates and frequent occurrence of frost events, such as in Peru and Bolivia where frost can partially or completely destroy potato leaves and lead to substantially decreased photosynthetic capacity and yields. Carrasco et al. (1997) and Hijmans et al. (2003) estimated that improving *S. tuberosum* freezing tolerance by 1ºC to 2ºC in those Andean countries could improve potato yield up to 25% to 40%, respectively. However, the transfer of the superior *S. commersonii* freezing tolerance capacity levels to *S. tuberosum* has not been simple because freezing tolerance and cold acclimation capacity are regulated by different mechanisms (Stone et al., 1993) and involve many genes (Thomashow, 1990; van Buskirk and Thomashow, 2006). Furthermore, our current understanding of the mechanisms and pathways governing freezing tolerance and cold acclimation in *S. commersonii* is very limited. A better understanding of these mechanisms and

pathways that control *S. commersonii* freezing tolerance and cold acclimation capacity would undoubtedly facilitate the development of frost hardy potato varieties.

In *Arabidopsis, CBF* transcription factors (*CBF/DREB*) play a key role in cold acclimation and cold tolerance by regulating the expression of cold-regulated (*COR)* genes (Thomashow, 1990, 1999; van Buskirk and Thomashow, 2006). Constitutive overexpression of *AtCBF1/DREB1b* or *AtCBF3/DREB1a* in transgenic plants increases freezing tolerance, even when grown under warm temperatures, in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999), *Brassica* (Jaglo et al., 2001), tobacco (Kasuga et al., 2004), wheat (Pellegrineschi et al., 2004), and poplar (Benedict et al., 2006). The ectopic overexpression of *AtCBF* genes in *Arabidopsis* results in constitutive expression of downstream *COR* genes and other *CRT/DRE* containing target genes and an associated increase in freezing tolerance (Jaglo- Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000) A study in *Arabidopsis* by Gilmour et al. (2004) using microarrays did not show major differences in freezing tolerance or targeted gene regulons between *AtCBF1, AtCBF2* and *AtCBF3,* suggesting the encoded factors specify redundant activities. Overexpression of *AtCBF2* has not been reported in any species other than *Arabidopsis* to date. In a characterization of a *cbf2* null mutant of *Arabidopsis,* Novillo et al. (2004) proposed that *AtCBF2* plays a crucial role in *Arabidopsis* freezing tolerance by acting as a negative regulator of *CBF1* and *CBF3* to ensure the accurate expression of *CBF1* and *CBF3. CBF2* would be involved in feedback regulation of *CBF1* and *CBF3* expression during cold

acclimation in which *CBF1* and *CBF3* are quickly induced in response to low temperature followed by the induction of *CBF2*, which in turn leads to down-regulation of *CBF1* and *CBF3* expression. The mechanisms by which *CBF2* regulates *CBF1* and *CBF3* expression need to be further investigated as well as the particular role of *CBF1* and *CBF3* in auto-regulation and cold acclimation.

Studies in *Arabidopsis* found *AtCBF3* overexpression induced many biochemical changes that commonly occur during cold acclimation, suggesting the changes were part of a CBF-dependent pathway (Gilmour et al*.,* 2000). Similar results have also been observed in preliminary studies on *S. commersonii*, where constitutive overexpression of *AtCBF1* increased freezing tolerance and induced many biochemical changes under warm growth conditions that are induced during cold acclimation in wildtype plants, suggesting these processes involve the endogenous CBF pathway (Pino et al., 2006). Because *S. commersonii* is a freezing-tolerant and cold-acclimation competent potato species that differs in its cold stress response relative to cultivated potato that seems to have lost the capacity to cold acclimate at some point in its evolution. It is important to know whether a CBF cold response pathway is functioning in *S. commersonii* and whether deficiencies in CBF-dependent processes are a basis for the inferior freezing tolerance of cultivated potato.

In order to investigate the involvement of *CBF* genes in *S. commersonii* freezing tolerance, we transformed *S. commersonii* with the three *Arabidopsis CBF* genes (*AtCBF1* through *3*) and placed each under the control of either the constitutive CaMV *35S* promoter or the stress-inducible *Arabidopsis rd29A*

promoter to characterize the effects on freezing tolerance and cold acclimation capacities of transgenic plants.

Material and Methods

Plant materials, transformation, and identification of transgenic lines

Solanum commersonii (PI 243503 clone 13) was transformed with three *Arabidopsis CBF* genes (*AtCBF1* through *3*) under the control of either the constitutive CaMV *35S* promoter or the stress-inducible *Arabidopsis rd29A* promoter. Binary transformation constructs expressing the *Arabidopsis CBF1-3* genes (*AtCBF1*, *AtCBF2*, *AtCBF3*) driven by either the constitutive *CaMV35S* or abiotic stress-inducible *rd29A* promoter were utilized for transformation of *S. commersonii* (Figure 4.1). Generation of the constitutively expressing *35S:AtCBF1* construct *pGAH-35S::AtCBF1* (Benedict et al., 2006), *35S:AtCBF2* construct pMPS11 (Gilmour et al., 2004), and *35S:AtCBF3* construct pMPS13 (Gilmour et al., 2000) have been previously described. The *rd29A*-based constructs *prd29A::AtCBF1*, *prd29A::AtCBF2*, and *prd29A::AtCBF3* were derived from pMPS8, pMPS11, and pMPS13, respectively, by excising the *35S* promoter region and replacing it with an 1133 bp *rd29A* promoter fragment corresponding to basepairs 4298-5430 of accession D13044; the integrity of the *rd29A*-based derivatives was confirmed by sequencing through the ligation junctions of the *rd29A* promoter. pMPS8, described in Gilmour et al. (2004), contains a 35S::AtCBF1 operon in the same vector backbone as pMPS11 and pMPS13. Constructs were transformed into

either *Agrobacterium tumefaciens* strain EHA105 or GV3101 prior to transformation of *S. commersonii* explants.

Potato plantlets were propagated *in vitro* at 25ºC with constant illumination (95-100μmol $m^{-2}s^{-1}$, cool white fluorescent lights) on hormone-free Murashige and Skoog (MS) medium with sucrose (20 g L^{-1}). Young leaf and stem explants of *S. commersonii* were transformed via *Agrobacterium*-mediated transformation. Specifically, *A. tumefaciens* cultures harboring the construct of interest were grown overnight at 28°C, 240 rpm in liquid YEP medium with 50mg L^{-1} kanamycin to an OD_{600}=0.5–0.7. Cells were collected by centrifugation at 2500 rpm for 10 min and resuspended in liquid MS-2% sucrose (pH 5.2). *S. commersonii* explants that had been pre-cultivated in MS-2% sucrose medium (pH 5.7) with 5 mg L^{-1} 2iP and 2 mg L^{-1} IAA for two days were incubated for 15 min at 50 rpm, RT in the bacterial suspension with 20 mg L^{-1} acetosyringone and then co-cultivated in MS -2% sucrose medium (pH 5.2) with 5mg L^{-1} 2iP, 2mg L^{-1} IAA, and20 μg L^{-1} acetosyringone for 2–3 d at 25°C in the dark. Next, explants were washed three times with a washing solution (MS-2%, pH 5.7 supplemented with 250 mg L^{-1} cefotaxime) and blotted dry on sterile paper towels for 30 s before transfer to a callus induction medium of MS 2% sucrose (pH5.7) with 5mg L^{-1} 2iP and 2mg L^{-1} IAA, 200 mg L^{-1} cefotaxime, and 50 mg L^{-1} kanamycin; explants were transferred to fresh callus induction medium every three weeks. Regenerated shoots were transferred to hormone-free MS 2% sucrose medium containing the same antibiotic concentrations of 200 mg L^{-1} cefotaxime, and 50 mg L^{-1} kanamycin.

Kanamycin-resistant rooted shoots were *in vitro* propagated and leaves of rooted-plantlets were analyzed for transgene integration via PCR using the following primer sets. The *35S* promoter primer 35S-P.001 (5'-cacgtcttcaaagcaagtgg-3') and the *rd29A* promoter primer rd29A.001 (5'-caagccgacacagacacgcg-3') were used as the respective forward primers to verify integration of the three *35S*-based and three *rd29A*-based expression operons. The forward promoter primers were paired with *Arabidopsis CBF* gene reverse primers AtCBF1.002 (5'-ccttcgctctgttccggtgtataaat-3'), AtCBF2.002 (5'-catccccaacatcgcctcttc -3'), or AtCBF3.002 (5' cctccaccaacgtctcctcc3') as appropriate to verify integration of the respective 35S or rd29A AtCBF expression operon.

Plant Growth Conditions

Rooted plants of individual transgenic lines and untransformed controls were transferred to Sunshine SB40 mix (Sun Gro Horticulture Inc., Bellevue, WA) supplemented with the controlled-release fertilizer (Osmocote, The Scotts Company, Marysville, OH) and grown under greenhouse conditions at 25±3ºC on a 16/8 h day/night photoperiod (400-480µmol$m^{-2}s^{-1}$ light intensity) supplemented with 300-400 µmol$m^{-2}s^{-1}$ light supplied via SUN System III lamps (Sunlight Supply, Inc., Vancouver, WA) with weekly fertilization (J.R. Peters, Allentown, PA) prior to transfer to experimental conditioning treatments. Unless noted otherwise, plants used in experimental trials were transferred from the above greenhouse conditions to a Percival Model MB60B growth chamber (16/8h photoperiod, 350µmol $m^{-2}s^{-1}$ PAR at 25ºC) for three days to acclimatize to the controlled environmental conditions before collection

of experimental warm plant material. For cold-treated plants, following the three day controlled environmental conditioning, plants were transferred to an environmentally-controlled cold room maintained at 2°C (16h photoperiod; Very High Output Phillips CW/VHO fluorescent bulbs, 75µmol $m^{-2}s^{-1}$ light intensity) for two weeks, unless specified otherwise, before harvesting of plant material.

Gene expression analysis

Leaf tissue was used as the RNA source for all expression analyses. Total RNA was extracted from leaf tissue using RNeasy Plant Mini kits (Qiagen, Valencia, CA) and 20 µg total RNA was electrophoretically separated per sample and transferred to nylon membrane as previously described (Skinner et al., 2005). Blots were probed in Ultrahyb solution (Ambion Inc., Austin, TX) and washed following the manufacturer's guidelines; labeled probes were generated using a High Prime Labeling Kit (Roche Biochemicals, Indianapolis, IN). Probe fragments to each of the three *AtCBF* transgenes were prepared that excluded the conserved AP2 domain and consisted of only the C-terminal domain and 3' UTR to minimize cross hybridization to endogenous potato *CBF*s. A fragment to a cold-responsive potato protease inhibitor-like gene, designated *StPI*, was prepared by excising the 509 bp cDNA insert of EST clone CK854013 and used as a probe. A 496 bp fragment to the cold-responsive potato *ci18 cor* gene was PCR amplified and cloned based on the TIGR Potato Gene TC103027 sequence and used as a probe. Probed blots were exposed and scanned using an MD-SI PhosphorImager system (Amersham Biosciences, Piscataway, NJ).

Controlled freeze tests

Freezing tolerance of wild type and transgenic plants was determined via controlled freezing tests (Sukumaran and Weiser, 1972) on leaf tissue of warm and two week cold-treated plants. For each sample and temperature point evaluated, three independent experiments were conducted using three replicate samples per experiment; tubes for each experimental set were arranged in a randomized design. Briefly, three 10 mm leaf discs were collected from fully expanded third and fourth leaves per sample assayed and placed in 16×120 mm test tube. Tubes were incubated at -1ºC in a low temperature NESLAB bath (Model LT-50DD, Newington, NH) for 1 h, then ice nucleation was initiated by adding an ice chip to each tube, samples were maintained at -1.5 ºC for an additional 1 h, and then the temperature was lowered 1ºC/h. Sample tubes were removed at -2, -4, -6, -8, -10, -12, -14 and -16ºC and slow-thawed overnight at 2°C. Freezing injury of thawed leaf samples was assessed by determining electrolyte leakage using a YSI Model 35 conductance meter (Yellow Springs, OH). Following conductivity measurements, all samples were frozen at -20°C for 24 h, thawed at room temperature, and total conductivity determined. LT_{50} values (temperature causing 50% electrolyte leakage) were plotted as a function of freezing temperature. For the time course study, LT_{50} values were determined as above on samples following 0, 1, 2, 4, 7, 14, 21 and 28 days cold-treatment.

Plant morphology analysis

Wildtype and transgenic plants used for leaf morphology and foliar fresh weight analyses were *in vitro* propagated, transferred to soil (one plant per 1.5 L pot), and grown in a greenhouse in Santiago, Chile (33º 27′ S.L.) under natural

photoperiod at 25±3ºC from October to February for 16 weeks total; plants were fertilized weekly with foliar fertilizer (J.R. Peters, Allentown, PA). Following 16 weeks of greenhouse growth, total above-ground foliar biomass (leaf and stem tissue) was collected and fresh weight determined prior to analysis and documentation of leaf morphology. Five plants per genotype for each transgenic line and wildtype plant were evaluated per experiment, and each experiment was repeated three times.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) and the differences among means were compared using Duncan's Multiple Range test. All statistical analyses were performed using the SAS statistical program (SAS Inc., 2000).

Results

Production of transgenic *S. commersonii* plants over-expressing three *AtCBF* genes driven by the CaMV *35S* or the *rd29A* gene promoter

Stem explants of *S. commersonii* were infected with *Agrobacterium* strains harboring six recombinant plasmids (Figure 4.1). Kanamycin-resistant transgenic lines were screened by PCR to confirm the presence of the respective *AtCBF* transgene in each line's genome. Northern blot analysis was then conducted to evaluate the presence and level of *AtCBF* transgene expression in each PCR-positive line (see Experimental procedures for details). For each construct, up to 15 independent transgenic lines were obtained with detectable levels of *CBF* transgene expression when grown under either warm (*35S*-based

series) or after 24 h cold treatment at 2°C (*rd29*-based series) and three expressing lines per construct were selected for further analysis.

Growth retardation and phenotypic alterations in *S. commersonii* generated by constitutive overexpression of three *AtCBF* genes

As shown in Figure 4.2A, all three lines of each of the three transgenic plant construct classes accumulated different levels of the respective *AtCBF* transgene transcript at warm temperatures when driven by the constitutive *CaMV35S* promoter. Among the three construct types, AtCBF1 transgenic line C1.4 and AtCBF2 transgenic line C2.40 displayed the lowest level of AtCBF transcript accumulation while AtCBF2 transgenic lines C2.45 and C2.62 accumulated the highest level of AtCBF transcript. The leaf morphological shape and size characteristics were altered to varying degrees in all three transgenic plant types. Major differences were most evident in the *AtCBF2* overexpression lines C2.45 and C2.62, in which leaves were not only noticeably smaller (Figure. 4.2B), but also thicker (data not shown).

In *S. commersonii* high levels of *AtCBF2* overexpression as determined by steady state transcript levels such as those of the C2.45 and C2.62 lines were always associated with severe plant phenotypic alterations that included a dwarf phenotype, reduction in leaf area, and a prostrate growth habit. In contrast, transgenic lines with lower levels of *AtCBF2* overexpression such as line C2.40 showed the least amount of growth retardation, suggesting the level of *AtCBF2* transgene expression is directly related to the degree of phenotypic modifications. The transgenic 35S::AtCBF1 and 35S::AtCBF3 lines also exhibited some degree of growth retardation at early growth stages, but

recovered over time and grew to almost the size as wildtype plants (Figure. 4.2A and Figure. 4.2C). Plant size was quantified by determining total foliar fresh weight (Table 4.1). In wildtype plants the foliar fresh weight was 676.1 g/plant, while in the majority of all three transgenic plant types the foliar fresh weight was significantly reduced to between 143 to 575 g/ plant, in agreement with the visibly stunted phenotypes. AtCBF2 transgenic lines displayed the most severe growth retardation and had lowest foliar fresh weights of 143 g/plant and 190.9 g/plant for C2.45 and C2.62, respectively (Table 4.1). While lines of the AtCBF1 and AtCBF3 plants grew out of the stunted phenotype and grew similar to wildtype plants thereafter, the delay in normal growth was sufficient to significantly reduce the total foliar biomass produced with foliar fresh weight between to between 420 g/plant and 575 g/plant. In addition, high *AtCBF* overexpression was associated with the delays and/or lack of flowering and tuberization.

Constitutive overexpression of the three *AtCBF* genes results in differential effects on freezing tolerance and COR gene expression

Constitutive overexpression of *AtCBF1* significantly enhanced freezing tolerance in transgenic *S. commersonii* plants growing under both warm conditions and after two weeks of cold treatment at 2ºC (Figure. 4.3A). In AtCBF1 overexpressing lines, there was about 3 - 4.5°C increase in freezing tolerance when plants were grown under warm conditions. Whereas the LT_{50} was about -5.5ºC in non-cold acclimated wildtype plants and about -8.5ºC post-cold acclimation, the LT_{50} of transgenic plants was increased under both conditions. For example, the transgenic line C1.7 had a freezing tolerance of

about -10°C under warm growth conditions and about -13.0°C post-cold acclimation. Of the three AtCBF2 overexpressing lines tested, only line C2.62 showed an increased freezing tolerance when grown under warm conditions which was minor and only about 1°C, whereas the freezing tolerance of the other two lines did not differ from wildtype plants, and after two weeks cold acclimation, the freezing tolerance was basically the same as that of wildtype plants. For the AtCBF3 overexpressing lines, there was about 1–2°C increase in freezing tolerance under warm conditions and about 2–4°C after cold-treatment. For example, the LT_{50} of transgenic line C3.19 increased from -7.5ºC in non-cold acclimated plants to about -12.0ºC. These results indicate that transgenic overexpression of *AtCBF1* and *AtCBF3* in *S. commersonii* is effective at improving freezing tolerance and cold acclimation capacity, while *AtCBF2*, despite conferring the most severe negative growth effects, did not improve *S. commersonii* freezing tolerance and cold acclimation capacity.

The expression of two *cor* genes, *ci18* and *StPI*, whose orthologs are responsive to ectopic CBF activity in the related Solanaceous plant tomato (Zhang et al., 2004), was examined in *S. commersonii*. Transgenic AtCBF lines displayed increased and enhanced expression of these two cold-regulated *COR* genes under both warm and cold conditions relative to wildtype plants (Figure 4.3B), suggesting ectopic overexpression of CBF activity can induce the expression of *cor* genes in the absence of a cold stimulus and imply that an endogenous CBF-based cold response pathway is present in *S. commersonii* and is involved in the cold acclimation process of this potato species.

Control *AtCBF* overexpression via an abiotic stress-inducible promoter enhances freezing tolerance without imparting significant phenotypic growth effects on transgenic *S. commersonii* potato plants

The above results demonstrated that while *AtCBF1* and *AtCBF3* can enhance *S. commersonii* freezing tolerance, the constitutive overexpression of CBF activity also causes undesirable levels of growth retardation in the transgenic plants. We therefore generated and transformed *S. commersonii* with the same three *AtCBF* genes driven by the abiotic stress-inducible *rd29A* promoter, generating the rd29A:construct vector, we obtained at least 15 transgenic lines each that displayed varying levels of *AtCBF* transcript accumulation. As for the 35S::AtCBF construct sets, three overexpressing lines were selected per construct for further analysis. Preliminary freezing tolerance assays established that *AtCBF2* driven by a stress-inducible promoter did not increase freezing tolerance in any of the transgenic lines tested (data not shown). We therefore excluded them from further analysis, focusing analysis on the rd29A::AtCBF1 and rd29A::CBF3 transgenic lines.

For the rd29A::AtCBF1 and rd29A::AtCBF3 transgenic lines, following 24 h of cold treatment at 2°C, the presence of *AtCBF1* and *AtCBF3* transcripts was readily detectable in all transgenic lines (Figure. 4.4A). No major differences in leaf morphology (Figure. 4.4B) or plant height (Figure. 4.45C) were visible between wildtype and transgenic plants. Few transgenic lines initially displayed slight growth retardation and minor variations in leaf size during the first stages of plant growth in tissue culture, but recovered a wildtype-like leaf and growth habit by three weeks of growth under greenhouse

conditions. This observation is corroborated by the foliar fresh weight measurements, which indicates the minor early growth phase differences did not lead to a collective loss of foliar biomass production relative to wildtype plants (Table 4.2). The flowering capacity, flowering time, or tuber production capacity of rd29A::AtCBF1 and rd29A::AtCBF3 transgenic plants were not significantly affected, indicating the probable minor levels of AtCBF activity occurring under greenhouse conditions were insufficient to affect these traits in contrast to the 35S::AtCBF plants.

Transgenic plants of both the rd29A::AtCBF1 and rd29A::AtCBF3 lines showed a significant increase in freezing tolerance in relation to wildtype plants (Figure 4.5A). While cold-acclimated transgenic plants showed an increase in freezing tolerance relative to wildtype, transgenic plants that were not cold treated (i.e., growing under warm conditions) also displayed a slight increase in freezing tolerance of about 0.5 - 1ºC, further supporting the possibility of the presence of minor levels of AtCBF activities under control conditions. In agreement with the cold inducible nature of the *rd29A* promoter controlling *AtCBF* transgene expression, cold treatment resulted in a further gain in freezing tolerance. As noted above, the observed increase in freezing tolerance of non-cold treated rd29A::AtCBF transgenic plants suggested either leaky *AtCBF* expression and/or *AtCBF* expression was being triggered at some point during the controlled freezing test. We thus studied whether the controlled freezing test itself was an induction source of *AtCBF* transgene expression in non-cold treated transgenic plants using the rd29A::AtCBF3 lines. Leaf discs were prepared from wildtype and transgenic plants growing at 25°C and

subjected to freezing test temperatures (Figure 4.5B). Northern analysis of RNA prepared from these leaf discs revealed that *AtCBF3* transcript was readily detectable at all the assessed cold temperature points after 1 h of cold treatment (Figure 4.5B), indicating the controlled freezing test conditions themselves were capable of inducing transgene expression. To address whether this experimental-based induction of transgene expression led to production of translated product and impartation of ectopic CBF activity, we assessed whether the two CBF-responsive cor genes had also been induced. Expression of both cor genes was induced under the assessed cold treatment and correlated with *AtCBF* transgene presence, indicating functional AtCBF activity was most likely also present. These results suggest transgene-based AtCBF activity is produced during the controlled freezing test assay and that the observed minor increase in warm grown transgenic plant freezing tolerance relative to wildtype plants is a likely byproduct of the assay.

Analysis of cold acclimation and its relationship to final freezing tolerance capacity during extended cold treatment (Figure 4.6A and 4.6B) showed that constitutive *AtCBF1* and *AtCBF3* overexpression resulted in a freezing tolerance gain and an increase in cold acclimation capacity during the four weeks of cold treatment. In both wildtype and the transgenic plants, a rapid gain in the majority of the post-cold acclimation freezing tolerance capacity was obtained within the first four days of cold treatment, with a minor but insignificant subsequent increase sometimes occurring over the prolonged cold treatment for lines C1.6 and C1.7. Importantly, this analysis demonstrates that the gain in freezing tolerance under warm conditions is not simply a process of

"turning on" the cold acclimation pathway in the absence of a cold stimulus. For both 35S::AtCBF1 and 35S::AtCBF3 lines, the freezing tolerance of the plants showed further gains in freezing tolerance during cold acclimation as is observed for wildtype plants. This suggests that in addition to the AtCBF transgene-imparted gain, additional increases in *S. commersonii* freezing tolerance are being induced by the cold treatment as an additive effect. Thus, overexpression of *AtCBF1* and *AtCBF3* increased both the intrinsic freezing tolerance capacity (pre-acclimation level) and the cold acclimation capacity (post-cold acclimation level) of *S. commersonii*. The analysis of freezing tolerance over four weeks of cold treatment revealed that the cold-treatment based increase in freezing tolerance gain of the transgenic rd29A::AtCBF1 and rd29A::AtCBF3 lines primarily occurred during the first two days of the cold treatment, while a gradual additional gain occurred over the remainder of the treatment (Figure 4. 6C and 4. 6D). For the rd29A::AtCBF3 lines, the maximum level of gained freezing tolerance was equivalent to that obtained in the 35S::AtCBF3 lines (Figure. 4.6B vs. 4.64D). In contrast, the maximum level of gained freezing tolerance for the rd29A::AtCBF1 lines was less than that of the 35S::AtCBF1 lines (Figure. 4.6C vs. 4.6A).

Discussion and Conclusions

Similar to previous studies of constitutive *AtCBF* gene overexpression in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004), tomato (Hsieh et al., 2002a, b), tobacco (Kasuga et al., 2004) and poplar (Benedict et al., 2006), the ectopic overexpression in *S.*

commersonii of *Arabidopsis AtCBF* genes was associated with phenotypic alterations in transgenic plants. However, not all studies in which ectopic overexpression of *AtCBF* genes have been reported have observed phenotypic alterations. A study in *Brassica napus* on overexpression of *AtCBF1*, *AtCBF2* and *AtCBF3* did not report significant phenotypic alterations of the transgenic plants under controlled growth conditions (Jaglo et al., 2001). Likewise, constitutive overexpression of *Arabidopsis AtCBF3* in transgenic rice increased abiotic stress tolerance without stunting growth (Oh et al., 2005). Here, we observed that *S. commersonii* plants constitutively overexpressing *AtCBF2* showed the most severe growth retardation relative to the AtCBF1 or AtCBF3 lines. While *AtCBF2* overexpression resulted in severe growth retardation and lack of flowering, *AtCBF1* and *AtCBF3* overexpression lines primarily displayed growth retardation only during the first stages of plant growth, although delayed flowering was also observed. Gilmour et al. (2004) reported that *Arabidopsis* plants constitutively overexpressing *AtCBF1* and *AtCBF2* were delayed in flowering and displayed morphological abnormalities, with the growth of AtCBF2 transgenic lines being more severely affected than AtCBF1 lines. The differences in severity on resultant *S. commersonii* plant phenotype abnormalities between the *AtCBF* genes could be explained by the expression level of each gene. The 35S::AtCBF2 overexpressing lines C2.45 and C2.62, which showed the highest levels of transcript accumulation, were also the most severe in growth retardation. All of these negative effects of constitutive *AtCBF1-3* overexpression were minimized by using the abiotic stress-inducible *rd29A* promoter. Our results were similar to those previously reported in

Arabidopsis and tobacco where this regulatory element was used to control *CBF* transgene expression (Kasuga et al., 1999, 2004). Similarly, in the closely related Solanaceous plant tomato, control of the expression of *AtCBF1* by an ABA/stress inducible promoter conferred enhanced stress tolerance without affecting yield (Lee et al., 2003). We also observed that lines with higher levels *rd29A*-based *AtCBF* expression, such as CR1.21 and CR3.3, exhibited minor levels of observable growth retardation during tissue culture propagation and the initial phases of growth under greenhouse conditions. However, all of these lines recovered completely after a couple additional weeks of greenhouse growth, and the transitory phenotype may be a byproduct of the tissue culture conditions. Similar results were also reported in transgenic tobacco plants (Kasuga et al., 2004). Compared with the similar work in *S. tuberosum,* this initial growth retardation in the transgenic rd29A::AtCBF plants was more severe in for *S. commersonii* than for *S. tuberosum.*

Our finding that the overexpression of three *AtCBF* genes imparted differential levels of freezing tolerance in transgenic *S. commersonii* plants wasunexpected. While *AtCBF1* and *AtCBF3* increased both freezing tolerance and cold acclimation capacity in *S. commersonii* when grown under warm conditions, in contrast *AtCBF2* failed to cause any significant increase in these traits. This result is quite distinct from that found in *Arabidopsis* and *B. napus*, in which the over-expression of those three *AtCBF* genes had similar effects on freezing tolerance (Jaglo et al., 2001; Gilmour et al., 2004). Gilmour et al. (2004) concluded from their studies that *AtCBF1* through *3* encode for redundant functional properties. We observed similar findings for the

relationship of *AtCBF1* and *AtCBF3* relative to *AtCBF2* in transgenic lines in *S. tuberosum* (Pino et al, in press) and Meng (2006) observed similar effects in transgenic poplar. Preliminary analysis of six independent transgenic 35S::AtCBF2 lines showed a similar lack of increased freezing tolerance over wildtype and the AtCBF2 operon portion of the 35S- and rd29A-transformation constructs were resequenced to confirm no mutations were present in the transgene. The observed severe growth retardation and phenotypic alterations caused by overexpression of *AtCBF2* in *S. commersonii* confirmed a functional activity was likely being specified (Figure 4.2 and Table 4.1), and the expression of the two assessed *cor* genes were also affected in a manner consistent with the presence of functional AtCBF2 activity (Figure 4.3). The basis of why *AtCBF2* can impart many CBF-related properties but fail to increase freezing tolerance in *S. commersonii* is currently not known. Chinnusamy et al. (2003) suggested that there are differences in the regulations of the three transcription factors in *Arabidopsis* and found that the *ice1* (inducer of CBF expression 1) mutation blocks the expression of *CBF3* and decreases the expression of many genes downstream of CBFs, which leads to a significant reduction in chilling and freezing tolerance. Novillo et al. (2004) propose that *AtCBF2* acts as a negative regulator of *AtCBF1* and *AtCBF3* in *Arabidopsis*, suggesting that *AtCBF1* and *AtCBF3* are rapidly induced by cold stimulus and followed by the induction of At*CBF2,* which in turn leads to the suppression of At*CBF1* and At*CBF3* expression. As these effects occur on the endogenous promoters of *AtCBF1-3* and our constructs place these *CBFs* under the control of alternative promoters; these scenarios are unlikely to account for the

observed lack of AtCBF2-based freezing tolerance gain. While Gilmour et al., (2004) concluded that the three *Arabidopsis CBFs* have essentially redundant activities when constitutively overexpressed via the 35S promoter as similar plant phenotypes and gene regulons were observed for all three genes, there were minor differences that could be important but were not prominent in *Arabidopsis*. Individual barley *CBFs* display differential binding preferences for *cor* gene promoter motifs that differ only in the sequence flanking the core motif (Skinner et al., 2005). If the *AtCBF1-3* products have slight variations in binding preferences and activate similar but not completely identical regulons in *S. commersonii*, it is possible that *AtCBF2* fails to activate one or more critical potato *cor* genes necessary to increase freezing tolerance relative to the *AtCBF1*- and *AtCBF3*-specified regulons.

Ectopic overexpression of *Arabidopsis CBF* genes in other freezing tolerant plants such as *Arabidopsis* and *B. napus* results in a further gain in freezing tolerance following cold treatment to acclimate the plants and induces biochemical changes without a cold stimulus that are commonly associated with the cold-acclimation process (Gilmour et al., 2000, 2004; Jaglo et al., 2001). Ectopic overexpression of the three *AtCBF* genes in *Arabidopsis* not only increased freezing tolerance to similar levels, but also caused similar biochemical modifications commonly associated with cold-acclimation, such as increases in the levels of proline and sugars (Gilmour et al., 2004). In a preliminary study, we found that *AtCBF1* overexpression in *S. commersonii* induced biochemical changes in the absence of cold treatment, such as increased levels of proline and sugars (Pino et al., 2006). Similar results were also found

for transgenic *S. commersonii* plants overexpressing *AtCBF3* gene (data not published).

The overexpression of the three *AtCBF* genes in *Arabidopsis* induces the expression of similar *cor* gene regulons and microarray analysis did not find major differences in the genes targeted by *AtCBF1*, *AtCBF2* and *AtCBF3* (Gilmour et al., 2004). Likewise, in *S. commersonii AtCBF1* and *AtCBF3* overexpression increased freezing tolerance both before and after cold acclimation and induced the expression of two *cor* genes. We observed that rd29::AtCBF1 and rd29::AtCBF3 plants were slightly more freezing tolerant than wildtype plants when grown under warm conditions, even though only negligible levels of *AtCBF* transcript were present in those plants (Figure 4.6) and the plants did not display significant negative effects on plant growth like the 35S-based versions. We confirmed that the controlled freeze test assay itself was a source of induction of the rd29::*AtCBF3* transgene operon (and presumably the rd29::*AtCBF1* operon also) and the correlated effect on the assessed *cor* genes indicates that the transgene expression is likely resulting in synthesis of resultant *CBF* transgene activity. Thus, the observed increased freezing tolerance capacity of the rd29::AtCBF1 and rd29::AtCBF3 plants under warm conditions may be artifactual.

Taken together, our results suggest that the introduction of transgenic CBF activity is able to override and supplement the endogenous cold response pathways of *S. commersonii*. Similarly, the results imply that an endogenous CBF-based cold response pathway is present in *S. commersonii*. The observation of a further gain in freezing tolerance during cold treatment of the

transgenic *S. commersonii* plants suggests that in additions to an endogenous CBF-based cold response pathway, CBF-independent pathways are also present that are not stimulated by the *CBF* transgene activity and are still dependent on activation by cold stimulus.

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Table 4.1. Effect of constitutive overexpression of three different *AtCBF* genes on *S. commersonii* Dun (PI 243503 clone 13) growth characteristics[1]

Line[2]	Transgene	Growth Retardation[3]	Foliar F.W.[4]	Flowering Capacity and Timing[5]	Tuber Production[6]
C.WT	None	None	627.1 g[a]	Yes, Normal	Yes
C1.4	AtCBF1	Yes, (Variable)	424.0 g[d]	Yes, Delayed (Variable)	No
C1.6	AtCBF1	Yes, (Initial)	460.2 g[cd]	Yes, Delayed	Yes
C1.7	AtCBF1	Yes, (Initial)	574.6 g[ab]	Yes, Delayed	Yes
C2.40	AtCBF2	Yes, (Initial)	334.4 g[e]	Yes, Delayed	Yes
C2.45	AtCBF2	Yes (Sustained)	143.0 g[f]	None	No
C2.62	AtCBF2	Yes (Sustained)	190.9 g[f]	None	No
C3.12	AtCBF3	Yes, (Initial)	450.6 g[cd]	Yes, Normal	Yes
C3.19	AtCBF3	Yes, (Initial)	418.7 g[d]	Yes, Delayed	Yes
C3.23	AtCBF3	Yes, (Initial)	516.3 g[bc]	Yes, Delayed	No

[1]Measurements were conducted on plants after 16 weeks of growth in soil
[2]See Figure 2 Legend for line abbreviation codes
[3]Variable: Phenotype occurrence varied between line replicates; Initial: Plants resumed normal growth after transfer to soil; Sustained: Plants retained stunted phenotype after transfer to soil
[4]Superscripted letters indicate significant differences (p-value<0.0001) according to Duncan's Multiple Range Test
[5]Foliar Normal: Flowered at same time as wildtype plants; Delayed: Flowered later than wildtype plants; Variable: Phenotype occurrence varied between line replicates
[6]Tubers were examined after 24 weeks of growth in soil

Table 4.2 Effect of abiotic stress-inducible AtCBF1 and AtCBF3 activity on *S. commersonii* growth characteristics[1]

Line[2]	Transgene	Growth retardation[3]	Foliar F.W.[4]	Flowering Capacity and Timing[5]	Tuber Production[6]
C.WT		None	627.1 g[a]	Yes, Normal	Yes
CR1.10	AtCBF1	None	618.0 g[a]	Yes, Normal	Yes
CR1.11	AtCBF1	None	609.4 g[a]	Yes, Normal	Yes
CR1.21	AtCBF1	Yes, (Initial)	552.9 g[a]	Yes, Normal	Yes
CR3.3	AtCBF3	Yes, (Initial)	618.4 g[a]	Yes, Normal	Yes
CR3.33	AtCBF3	Yes, (Initial)	593.4 g[a]	Yes, Normal	Yes
CR3.35	AtCBF3	Yes, (Initial)	615.7 g[a]	Yes, Normal	Yes

[1]Measurements were conducted on plants after 16 weeks of growth in soil
[2]See Figure 5 Legend for line abbreviation codes
[3]Initial: Plants resumed normal growth after transfer to soil
[4]Non significant differences were detected (p-value=0.7523) according to Duncan's Multiple Range Test
[5]Foliar Normal: Flowered at same time as wildtype plants
[6] Tubers were examined after 24 weeks of growth in soil

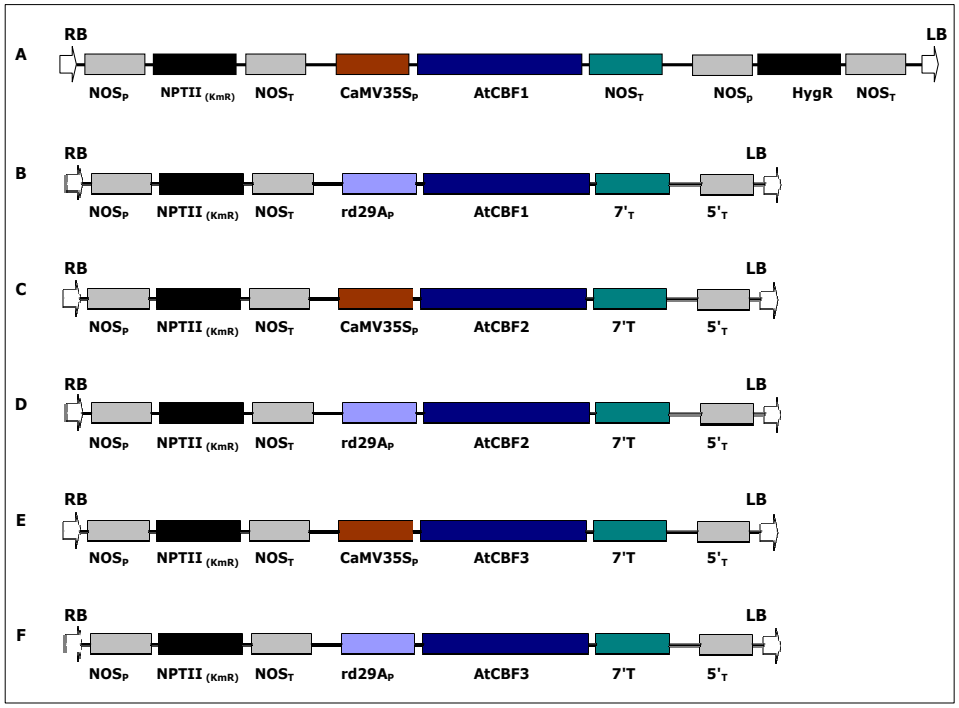

Figure 4.1 T-DNA region of transformation constructs used in *Solanum commersonii* Dun (PI 243503 clone 13). **A**, p35S::AtCBF1 **B**, prd29A::AtCBF1 **C**, p35S::AtCBF2 (pMPS11) **D**, prd29A::AtCBF2 **E**, p35S::AtCBF3 (pMPS13) **F**, prd29A::AtCBF3. Genetic elements and hygromycin (Hyg) and kanamycin resistance (NPTII) resistance genes present in each construct are denoted. $_P$: promoter element; $_T$: terminator element.

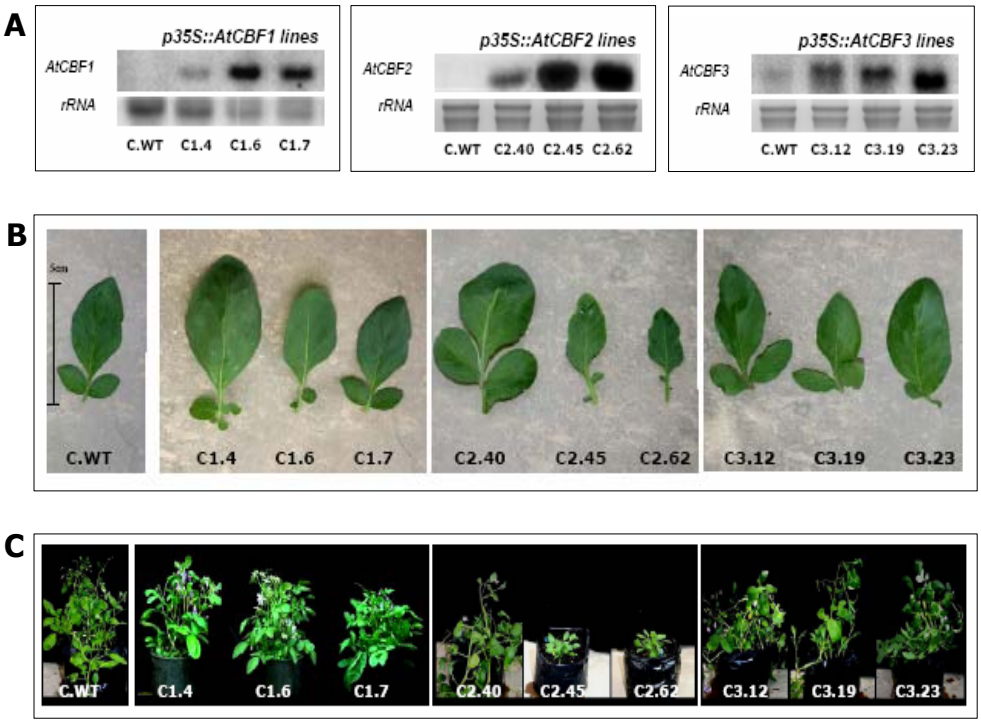

Figure 4.2 Effect of constitutive overexpression of *AtCBF1-3* genes on growth characteristics of *S. commersonii*. Effects were analyzed on 16-week old plants of the following types: wildtype (C.WT), 35S::AtCBF1 overexpressing lines (C1.4, C1.6, and C1.7), 35S::AtCBF2 overexpressing lines (C2.40, C2.45, and C2.62), and 35S::AtCBF3 overexpressing lines (C3.12, C3.19, and C3.23). Assessments were conducted on plants growing at 25ºC (Panels A, B, C). **A,** Analysis of transgene expression of the indicated *CBF* gene **B,** leaf morphology **C,** gross plant phenotype.

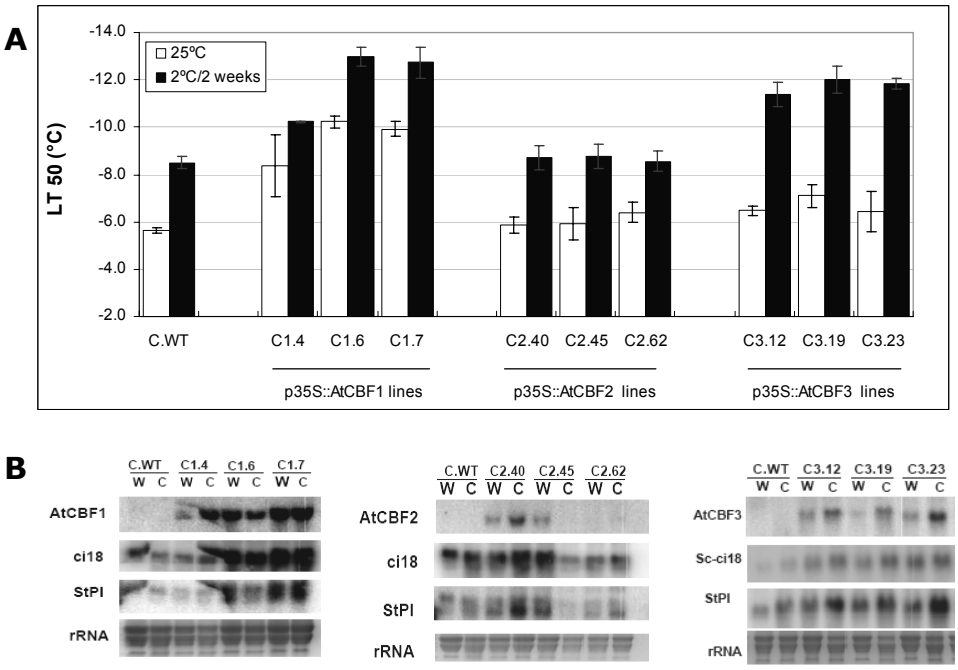

Figure 4.3 Effect of *AtCBF1-3* transgene expression on freezing tolerance of *S. commersonii* and on two potato *cor* genes. **A,** Freezing tolerance is expressed as the LT50 in -ºC for wildtype (C.WT) and transgenic lines 35S::AtCBF1 (C1.4, C1.6, and C1.7), 35S::AtCBF2 (C2.40, C2.45, and C2.62), and 35S::AtCBF3 (C3.12, C3.19, and C3.23). Plants were either grown at 25ºC (empty bars) or after two weeks of cold-treatment at 2ºC (solid bars). Standard deviation of means is indicated as vertical bars. **B,** Expression of the *AtCBF1-3* transgene and potato *ci18* and *StPI cor* genes during growth of *S. commersonii* wildtype (WT) and transgenic plants under warm (W) control conditions and after 2 weeks cold (C) treatment. Lines numbers and transgene type are indicated.

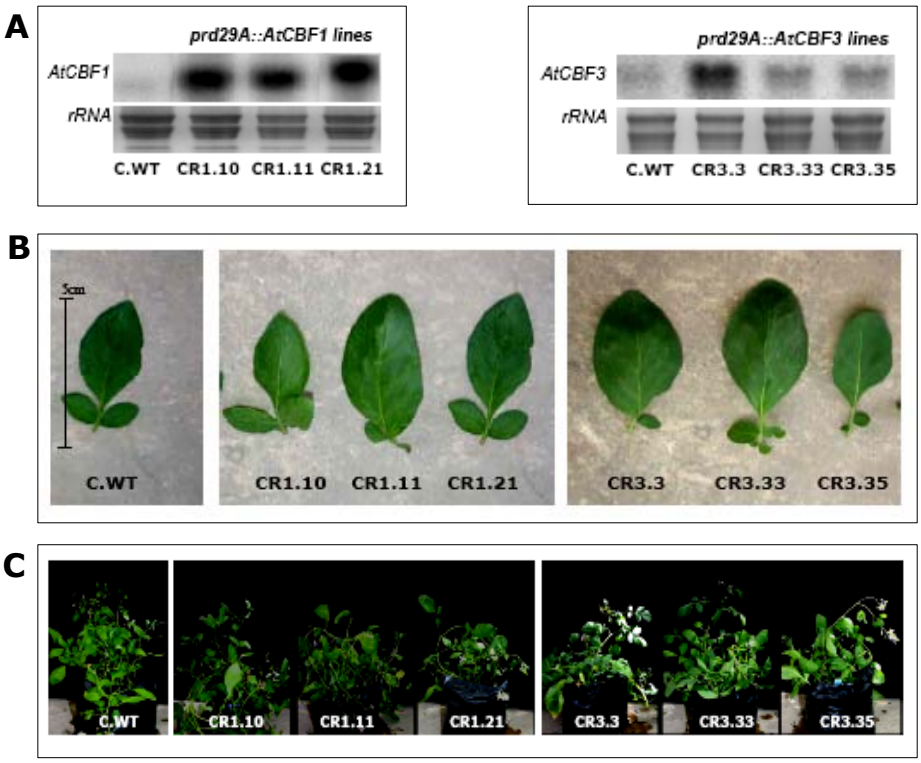

Figure 4.4 Effect of stress-inducible overexpression of *AtCBF1 and AtCBF3* genes on growth characteristics of *S. commersonii*. Effect of stress-inducible overexpression of *AtCBF1 and AtCBF3* genes on growth characteristics of *S. commersonii* Dun (PI 243503 clone 13). Effects were analyzed on 16-week old plants of the following types: wildtype (C.WT) and transgenic plants from rd29A::AtCBF1 lines (CR1.10, CR1.11, and CR1.21) and rd29A::AtCBF3 lines (CR3.3, CR3.33, and CR3.35). Assessments were conducted on plants growing at 25ºC (Panels A, B, C). **A,** Analysis of transgene expression of the indicated *CBF* gene **B,** leaf morphology **C,** gross plant phenotype.

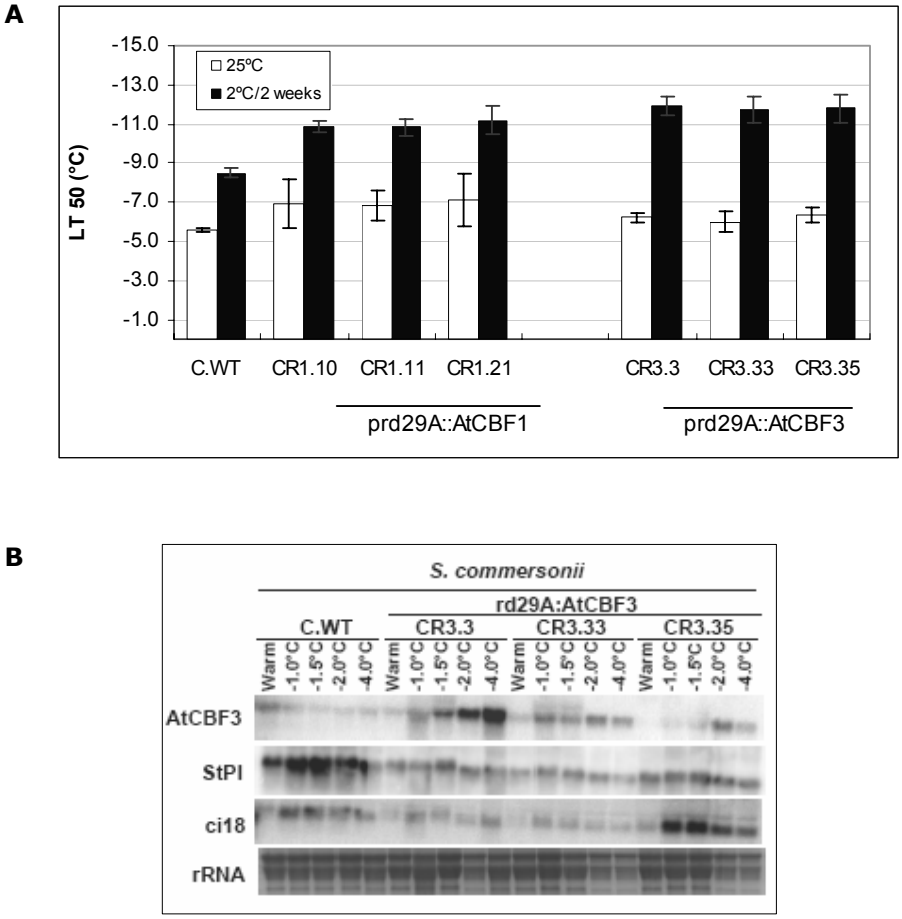

Figure 4.5 Effect of stress-inducible overexpression of *AtCBF1 and AtCBF3* genes on freezing tolerance and during ion leakage test **A,** freezing tolerance (LT50) of *S. commersonii* wild type (C.WT) and transgenic plants from rd29A::AtCBF1 lines (CR1.10, CR1.11, and CR1.21) and rd29A::AtCBF3 lines (CR3.17, CR3.43, and CR3.48) when growing at 25ºC or following two weeks cold treatment at 2ºC. **B,** Expression of the above genes in *S.* commersonii wildtype (C.WT) and rd29A:AtCBF3 transgenic leaf disc samples (lines CR3.3, CR3.33, CR3.33) during a representative ion leakage assessment assay. Leaf discs were treated at the indicated temperature for 1h under controlled freezing test conditions (temperatures at which the leaf discs were subjected are indicated.); "warm" samples were incubated at 25ºC. Ethidium bromide-stained rRNA bands are shown as a loading control.

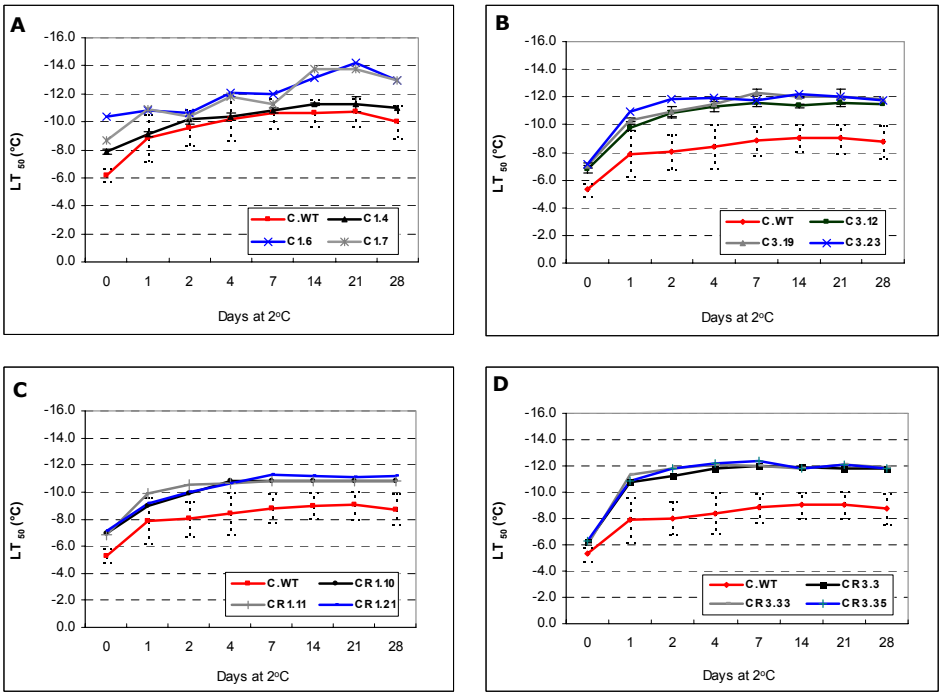

Figure 4. 6 Effect of *AtCBF1* and *AtCBF3* overexpression under control either the constitutive promoter *CaMV35S* or stress inducible promoter *rd29A* on time course freezing tolerance of transgenic *S. commersonii* plants. Time course was measured as LT_{50} (-°C) for wildtype plants (C.WT) and **A,** *p35S::CBF1*-transgenic plants (C1.4, C1.6, and C1.7) **B,** *p35S::CBF3* transgenic plants (C3.12, C3.19, and C3.21) **C,** *prd29A::CBF1*-transgenic plants (CR1.10, CR1.11, and CR1.21) and **D,** *prd29A::CBF1*-transgenic plants (CR3.3, CR3.33, and CR3.12) after 0, 1, 2, 4, 7, 14, 21 and 28 days in cold acclimation at 2ºC.

CHAPTER 5

GENERAL CONCLUSIONS

To advance our understanding of the role *CBF* genes play in cold tolerance of *Solanum* species and whether their manipulation could improve this trait, we transformed the two potato species *S. tuberosum L.* (cv. Umatilla) and *S. commersonii* Dun (PI 243503 clone 13), which differ in their degree of frost tolerance and cold acclimation capacity, with the three *Arabidopsis AtCBF* genes driven by either the constitutive *CaMV35S* promoter or the stress-inducible *Arabidopsis rd29A* gene promoter. Transgenic plants were evaluated relative to alterations in morphology, freezing tolerance both before and after cold acclimation, and for other modifications that are known to occur during the cold acclimation process. The general conclusions of this research are:

1 The effects of ectopic *AtCBF1* overexpression on freezing tolerance and other physiological responses were compared between freezing-sensitive *S. tuberosum* and freezing-tolerant *S. commersonii* both before and after cold acclimation. Relative to wildtype, constitutive *AtCBF1* overexpression yielded a significant freezing tolerance gain of 2°C for *S. tuberosum* and up to 4°C for *S. commersonii*. Cold acclimation capacity was improved for *S. commersonii*, but was remained absent from *S. tuberosum*. During cold treatment, leaves of wildtype *S. commersonii*, but not *S. tuberosum*, showed a significant thickening due to palisade cell lengthening and enlargement of intercellular spaces. Ectopic *AtCBF1* activity mimicked cold acclimation by increasing proline and total sugar content in *S.*

commersonii in the absence of cold. Additionally, transgenic *S. commersonii* displayed leaves with increased chlorophyll content that coincided with a net gain in photosynthetic capacity that was maintained after cold acclimation, suggesting the plants could exhibit higher potential productivity under cold stress conditions.

2 To investigate the effects of *AtCBF* overexpression on frost tolerance capacity and tuber production and whether cold-inducible regulation of *AtCBF* transgene expression would reduce negative phenotypic effects, the cultivated potato *S. tuberosum* cv. Umatilla was transformed with the *AtCBF1-3* genes under the control of either the constitutive *35S* promoter or the stress-inducible *rd29A* promoter. *AtCBF1* and *AtCBF3* overexpression enhanced freezing tolerance by about 2°C, while *AtCBF2* overexpression failed to increase freezing tolerance. For all three *AtCBF* genes, constitutive expression resulted in negative phenotypic alterations that included smaller leaves, shorter plants, delayed flowering and reduction or elimination of tuber production, thus limiting the agronomic application of these genes for potato improvement. Use of the stress inducible *rd29A* promoter to direct *AtCBF* gene expression improved freezing tolerance to the same level while minimizing the negative effects on tuber production. This suggests that overexpression of *AtCBF* genes under the control of a stress inducible promoter may be a practical approach towards improving potato frost tolerance.

3 To investigate the role *CBF* genes may play in *Solanum* species with high levels of frost tolerance, we transformed *S. commersonii* with the *AtCBF1-3* genes under the control of either the constitutive *35S* promoter or the stress-inducible *rd29A* promoter. As in *S. tuberosum*, *AtCBF1* and *AtCBF*3 overexpression effectively improved freezing tolerance and cold acclimation capacity in *S. commersonii*, while *AtCBF2* did not. While constitutive overexpression of all three *AtCBF* genes resulted in varying degrees of negative phenotypic alterations, *AtCBF1* and *AtCBF3* overexpression under the control of the stress inducible *rd29A* promoter enhanced freezing tolerance after cold treatment without any significant phenotypic alterations. The increase in freezing tolerance and cold acclimation capacity imparted by ectopic *AtCBF* expression was associated with activation of cold-regulated gene expression. The results from this study indicate the CBF cold response pathway is an active component of *S. commersonii* freezing tolerance and endogenous *CBF*(s) are likely involved in the cold acclimation process.

Taken together, these results suggest that the endogenous CBF pathway of potato is a component of the final frost tolerance capacity in these two species and that in *S. commersonii*, the CBF pathway is also involved in the cold acclimation process. Manipulation of this pathway by ectopic overexpression of a *CBF* gene provides a means to overcome the limitations encountered to date through traditional breeding at improving the frost tolerance of cultivated potato. Finally, ectopic overexpression of a *CBF* gene under the control of a cold inducible

promoter is a viable means to improve the frost tolerance capacity of cultivated potato while minimizing detrimental effects on tuber production.

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APPENDIX

TableA.1 ANOVA for leaf thickness and leaf palisade length in *S. commersonii* and *S. tuberosum* (see Table 2.1).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Leaf thickness (µm): Effects of cold treatment and *AtCBF1*-overexpression on *S. commersonii* leaf thickness	Treatment:	9	8342.955	*
	WT vs Trangenics	3	18928.163	*
	Warm vs cold	1	11085.402	*
	CBF*Condition	3	2001.885	*
	Blocks	2	605.524	NS
	Error	14	234.648	
	Corrected total	23		
Palisade length (µm): Effects of cold treatment and *AtCBF1*-overexpression on *S. commersonii* palisade cell length	Treatment:	9	828.910	*
	WT vs Trangenics	3	1837.032	*
	Warm vs cold	1	1181.607	*
	CBF*Condition	3	209.808	*
	Blocks	2	69.033	NS
	Error	14	62.223	
	Corrected total	23		
Leaf thickness (µm): Effects of cold treatment and *AtCBF1*-overexpression on *S. tuberosum* leaf thickness	Treatment:	9	1385.297	*
	WT vs Trangenics	3	4015.802	*
	Warm vs cold	1	162.760	NS
	CBF*Condition	3	46.086	NS
	Blocks	2	59.627	NS
	Error	14	169.047	
	Corrected total	23		
Palisade length (µm): Effects of cold treatment and *AtCBF1*-overexpression on *S. tuberosum* palisade cell length	Treatment:	9	380.686	*
	WT vs Trangenics	3	1128.941	*
	Warm vs cold	1	13.500	NS
	CBF*Condition	3	1.930	NS
	Blocks	2	10.030	NS
	Error	14	31.840	
	Corrected total	23		

(*) significant differences were observed
(NS) No significant differences

TableA.2 ANOVA for the effects of cold treatment and *AtCBF1*-overexpression proline and total sugar content in *S. commersonii* and *S. tuberosum* (see Table 2.2).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Proline (mg/g DW): Effects of cold treatment and *AtCBF1*-overexpression on proline content in *S. commersonii*	Treatment:	9	1.683	*
	WT vs Trangenics	3	2.619	*
	Warm vs cold	1	6.542	*
	CBF*Condition	3	0.181	NS
	Blocks	2	0.105	NS
	Error	14	0.055	
	Corrected total	23		
Sugar (mg/g DW): Effects of cold treatment and *AtCBF1*-overexpression on total sugar content in *S. commersonii*	Treatment:	9	4568.673	*
	WT vs Trangenics	3	9393.531	*
	Warm vs cold	1	11634.487	*
	CBF*Condition	3	289.396	NS
	Blocks	2	217.395	NS
	Error	14	428.973	
	Corrected total	23		
Proline (mg/g DW): Effects of cold treatment and *AtCBF1*-overexpression on proline content in *S. tuberosum*	Treatment:	9	0.302	NS
	WT vs Trangenics	3	0.613	NS
	Warm vs cold	1	0.508	NS
	CBF*Condition	3	0.022	NS
	Blocks	2	0.154	NS
	Error	14	0.213	
	Corrected total	23		
Sugar (mg/g DW): Effects of cold treatment and *AtCBF1*-overexpression on total sugar content in *S. tuberosum*	Treatment:	9	836.723	NS
	WT vs Trangenics	3	585.154	NS
	Warm vs cold	1	1868.782	NS
	CBF*Condition	3	12.752	NS
	Blocks	2	1934.002	*
	Error	14	488.305	
	Corrected total	23		

Table A.3 ANOVA for the effects of cold treatment and AtCBF1-overexpression on *S. commersonii* pigment content (see Table 2.3).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Chlorophyll a (µg/g FW): Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Chlorophyll a	Treatment:	9	144305.211	*
	WT vs Trangenics	3	387617.047	*
	Warm vs cold	1	10929.068	NS
	CBF*Condition	3	278.994	NS
	Blocks	2	62064.854	NS
	Error	14	23575.787	
	Corrected total	23		
Chlorophyll b(µg/g FW): Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Chlorophyll b.	Treatment:	9	67450.039	*
	WT vs Trangenics	3	76565.997	*
	Warm vs cold	1	289511.880	*
	CBF*Condition	3	20043.150	NS
	Blocks	2	13855.516	NS
	Error	14	11366.558	
	Corrected total	23		
Chlorophyll a+b(µg/g FW): Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Chlorophyll a+b	Treatment:	9	287582.226	*
	WT vs Trangenics	3	763936.444	*
	Warm vs cold	1	187943.911	*
	CBF*Condition	3	23446.987	NS
	Blocks	2	19072.913	NS
	Error	14	38106.904	
	Corrected total	23		
Chlorophyll a:b ratio: Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Chlorophyll a:b ratio	Treatment:	9	1.330	*
	WT vs Trangenics	3	0.573	NS
	Warm vs cold	1	8.568	*
	CBF*Condition	3	0.148	NS
	Blocks	2	0.621	NS
	Error	14	0.192	
	Corrected total	23		

TableA.3 ANOVA for the effects of cold treatment and AtCBF1-overexpression on *S. commersonii* pigment content (see Table 2.3). (Continued)

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Carotenoids(µg/g FW): Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Carotenoids	Treatment:	9	14375.015	*
	WT vs Trangenics	3	23804.937	*
	Warm vs cold	1	22899.993	*
	CBF*Condition	3	4818.785	NS
	Blocks	2	10301.9884	NS
	Error	14	3769.323	
	Corrected total	23		
Anthocyanins (µg/g FW): Effects of cold treatment and AtCBF1-overexpression on *S. commersonii* leaf Anthocyanins.	Treatment:	9	107.693	*
	WT vs Trangenics	3	275.273	*
	Warm vs cold	1	129.410	*
	CBF*Condition	3	4.457	NS
	Blocks	2	0.318	NS
	Error	14	9.923	
	Corrected total	23		

Table A.4 ANOVA for the effects of cold treatment and AtCBF1-overexpression on *S. commersonii* photosynthetic parameters (see Table 2.4).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Fv/Fm:	Treatment:	12	0.006	*
Effects of cold treatment and	WT vs Trangenics	3	0.001	NS
AtCBF1-overexpression on *S.*	Warm vs cold	1	0.059	*
commersonii Fv/Fm	CBF*Condition	3	0.000	NS
	Blocks	5	0.001	NS
	Error	33	0.001	
	Corrected total	45		
Photosynthesis Rate (A)	Treatment:	12	43.130	*
µmol $m^{-2}s^{-1}$	WT vs Trangenics	3	45.328	*
Effects of cold treatment and	Warm vs cold	1	330.353	*
AtCBF1-overexpression on *S.*	CBF*Condition	3	4.534	NS
commersonii photosynthesis	Blocks	5	7.524	NS
rate	Error	33	2.741	
	Corrected total	45		
Transpiration Rate (EVAP)	Treatment:	12	5.986	*
mol $m^{-2}s^{-1}$	WT vs Trangenics	3	5.579	*
Effects of cold treatment and	Warm vs cold	1	49.007	*
AtCBF1-overexpression on *S.*	CBF*Condition	3	0.000	NS
commersonii transpiration rate	Blocks	5	1.401	NS
	Error	33	0.948	
	Corrected total	45		
Stomatal Conductance (GS)	Treatment:	12	9564.841	*
mol $m^{-2}s^{-1}$	WT vs Trangenics	3	20475.555	*
Effects of cold treatment and	Warm vs cold	1	35384.876	*
AtCBF1-overexpression on *S.*	CBF*Condition	3	4590.300	NS
commersonii stomatal	Blocks	5	839.131	NS
conductance	Error	33	1811.732	
	Corrected total	45		
Internal CO_2 Concentration	Treatment:	12	3862.805	*
(C_i) ppm	WT vs Trangenics	3	10365.408	*
Effects of cold treatment and	Warm vs cold	1	6828.182	*
AtCBF1-overexpression on *S.*	CBF*Condition	3	1683.891	NS
commersonii internal CO_2	Blocks	5	675.516	NS
Concentration	Error	33	1427.023	
	Corrected total	45		

Table A.5 ANOVA for the Effect of *AtCBF1* transgene overexpression on freezing tolerance as LT50 of *S. tuberosum* plants (see Figure 2.2B).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	9	2.038	*
Effect of *AtCBF1* transgene	WT vs Trangenics	3	4.745	*
overexpression on freezing	Warm vs cold	1	0.002	NS
tolerance as LT50 of *S.*	CBF*Condition	3	0.009	NS
tuberosum plants.	Blocks	2	0.250	NS
	Error	14	0.117	
	Corrected total	23		

Table A.6 ANOVA for the effect of *AtCBF1* overexpression on freezing tolerance of *S. commersonii* plants as LT50 (see Figure 2.4A).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	23	6.794	*
Effect of *AtCBF1* transgene	WT vs Trangenics	10	6.524	*
overexpression on freezing	Warm vs cold	1	72.555	*
tolerance as LT50 of *S.*	CBF*Condition	10	1.795	*
commersonii plants.	Blocks	2	0.253	NS
	Error	42	0.411	
	Corrected total	65		

Table A.7 ANOVA for the effects of constitutive AtCBF1-3 over expression on *S. tuberosum* foliar biomass and tuber yield (See Table 3.1)

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Foliar F.W. (g)	Treatment:	11	48704.790	*
Effects of constitutive	Wt vs Transgenics	9	40114.150	*
AtCBF1-3 over expression on	Blocks	2	87362.670	*
S. tuberosum foliar biomass	Error	18	589.681	
	Corrected total	29		
Yield (g/ plant)	Treatment:	11	96014.421	*
Effects of constitutive	Wt vs Transgenics	9	116726.780	*
AtCBF1-3 over expression on	Blocks	2	2808.809	NS
S. tuberosum yield	Error	18	729.845	
	Corrected total	29		
Total tubers per Plant	Treatment:	11	32.013	*
Effects of constitutive	Wt vs Transgenics	9	37.445	*
AtCBF1-3 over expression on	Blocks	2	7.569	NS
S. tuberosum total tuber per	Error	18	1.646	
plant.	Corrected total	29		

Table A.8 ANOVA for the effect of stress inducible rd29A:AtCBF1 and 3 transgene expression on *S. tuberosum* foliar biomass and tuber yield (See Table 3.2)

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Foliar F.W. (g)	Treatment:	8	1223.834	NS
Effects of stress inducible	Wt vs Transgenics	6	1223.834	NS
AtCBF1-3 over expression	Blocks	2	10663.386	*
on *S. tuberosum* foliar	Error	12	1771.923	
biomass	Corrected total	20		
Yield (g/ plant)	Treatment:	8	42694.2893	*
Effects of stress inducible	Wt vs Transgenics	6	36858.3287	*
AtCBF1-3 over expression	Blocks	2	60202.1710	*
on *S. tuberosum* yield	Error	12	1252.5392	
	Corrected total	20		
Total tubers per plant	Treatment:	8	10.945	*
Effects of stress inducible	Wt vs Transgenics	6	9.504	*
AtCBF1-3 over expression	Blocks	2	15.274	*
on *S. tuberosum* total tuber	Error	12	0.141	
per plant.	Corrected total	20		

Table A.9 ANOVA for the effect of constitutive *AtCBF1* to *3* overexpression on *S. tuberosum* freezing tolerance (see Figure3.3A).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	21	1.945	*
Effect of constitutive	WT vs Trangenics	9	4.383	*
AtCBF1 to *3* overexpression	Warm vs cold	1	0.486	*
on *S. tuberosum* freezing	CBF*Condition	9	0.075	NS
tolerance as LT50.	Blocks	2	0.122	NS
	Error	38	0.087	
	Corrected total	59		

Table A.10 ANOVA for the effect of stress inducible *AtCBF1* and *AtCBF3* overexpression on *S. tuberosum* freezing tolerance (see Figure3.3B).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	15	1.852	*
Effect of constitutive *AtCBF1*	WT vs Trangenics	6	1.885	*
to *3* overexpression on *S.*	Warm vs cold	1	10.600	*
tuberosum freezing tolerance	CBF*Condition	6	0.416	NS
as LT50.	Blocks	2	1.685	*
	Error	26	0.322	
	Corrected total	41		

Table A.11 ANOVA for the effects of constitutive AtCBF1-3 over expression on *S. commersonii* foliar biomass (See Table 4.1)

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Foliar F.W. (g)	Treatment:	11	70550.856	*
Effects of constitutive	Wt vs Transgenics	9	71591.167	*
AtCBF1-3 over expression on	Blocks	2	65869.456	*
S. commersonii foliar biomass	Error	18	1241.287	*
	Corrected total	29		

Table A.12 ANOVA for the effects of stress induced AtCBF1 and 3 over expression on *S. commersonii* foliar biomass (See Table 4.2)

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
Foliar F.W. (g)	Treatment:	8	7751.425	*
Effects of constitutive AtCBF1	Wt vs Transgenics	6	1909.814	NS
and 3 over expression on *S.*	Blocks	2	25276.258	*
commersonii foliar biomass	Error	12	1335.949	
	Corrected total	20		

Table A.13 ANOVA for the effect of constitutive *AtCBF1* to *3* overexpression on *S. commersonii* freezing tolerance (see Figure 4.3A).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	21	15.927	*
Effect of constitutive *AtCBF1*	WT vs Trangenics	9	16.134	*
to *3* overexpression on *S.*	Warm vs cold	1	168.003	*
commersonii freezing	CBF*Condition	9	2.330	*
tolerance as LT50.	Blocks	2	0.140	NS
	Error	38	0.285	
	Corrected total	59		

Table A.14 ANOVA for the effect of stress inducible *AtCBF1* and *AtCBF3* overexpression on *S. tuberosum* freezing tolerance (see Figure 4.5A).

Dependent Variable and description	**Source**	**df**	**Mean Square**	**Pr>F**
LT50 (ºC):	Treatment:	15	16.552	*
Effect of stress inducible	WT vs Trangenics	6	3.287	*
AtCBF1 and *AtCBF3 on S.*	Warm vs cold	1	217.604	*
commersonii freezing	CBF*Condition	6	1.810	*
tolerance as LT50.	Blocks	2	0.047	NS
	Error	26	0.495	
	Corrected total	41		