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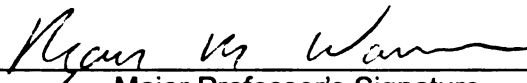
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**ANALYSIS OF COLD ACCLIMATION ABILITY AND DROUGHT
TOLERANCE OF *PETUNIA* SPP.**

By

Aaron Emery Walworth

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

ANALYSIS OF COLD ACCLIMATION ABILITY AND DROUGHT TOLERANCE OF *PETUNIA* SPP.

By

Aaron Emery Walworth

Freezing tolerance is a dynamic characteristic, and many plant species increase in freezing tolerance following exposure to low non-freezing temperatures, a process referred to as cold acclimation. The genus *Petunia* is composed of diverse species, with the capacity for cold acclimation already known to exist in at least one species, *P. hybrida*. In this study, *P. exserta*, *P. integrifolia*, and two accessions of *P. axillaris* were also found to cold acclimate. All *Petunia* species had similar basal freezing tolerance of $EL_{50} = -2\text{ }^{\circ}\text{C}$, but freezing tolerance varied significantly among species following cold acclimation. *Petunia axillaris* (accession 28548) showed the greatest acclimated freezing tolerance with an EL_{50} temperature of $-8\text{ }^{\circ}\text{C}$, compared to only $-5\text{ }^{\circ}\text{C}$ for *P. exserta*. Temperature, but not photoperiod, was critical for induction of cold acclimation in *P. hybrida*. Cold acclimation of *Arabidopsis* is largely controlled by genetic factors in the CBF cold-response pathway. High levels of constitutive heterologous *AtCBF3* expression in *P. hybrida* 'Mitchell' resulted in increased basal freezing tolerance of one transgenic line by ca. $2.5\text{ }^{\circ}\text{C}$, while expression of *LeCBF1* (a CBF homolog from tomato) had no effect on freezing tolerance. However, high expression of *AtCBF3* resulted in phenotypic changes including delayed flowering. Heterologous CBF expression did not enhance drought tolerance. Expression of putative endogenous CBF transcription factors, *petCBF1-4*, was induced at $3\text{ }^{\circ}\text{C}$ and two putative downstream genes of the petunia CBF pathway whose expression was induced by cold and CBF overexpression were identified.

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Floriculture in the United States is a major industry worth over \$4.1 billion in wholesale value in 2007. Within the floriculture industry, bedding/garden plants make up the largest portion of sales at over \$1.76 billion (USDA-NASS 2008). The majority of bedding plant sales occur during a short period in the spring and early summer months. However, a few of the more cold tolerant species such as pansy and ornamental kale have the ability to withstand multiple frosts, allowing them to be sold earlier in the spring and later into the fall. Consumers wishing to add color to their spring and late fall landscapes have traditionally been limited to these and a few other species because few herbaceous plants tolerate the environmental conditions at this time of year. Additionally, greenhouse growers have been very dependent upon springtime sales to provide the majority of their annual income (Kessler 2004). Increasing the cold tolerance of other bedding plants would add to the number of species offered for sale in the cooler months. Developing additional cold tolerant cultivars of already-popular bedding plants would also allow growers to keep their greenhouses productive during summer months by producing plants for sale in the fall.

Garden petunias (*Petunia hybrida*) are already a very popular bedding plant, ranking first in sales among bedding plants in 2007, with a wholesale value of over \$111 million (USDA-NASS 2008). The current popularity of petunias can be partially

attributed to recent breeding efforts which have combined novel characteristics, such as prostrate growth habits, with increased environmental stress tolerances (Griesbach 2007). *Petunia* is therefore a prime target for efforts aimed at further increasing the cold and drought tolerance of bedding plants.

Understanding the genetics controlling abiotic stress tolerance traits is critical for trait improvement through biotechnology. Biotechnological approaches, such as transgenic manipulation of gene expression, require identification of genes which increase stress tolerance when up- or down-regulated. Traditional breeding methods require the identification of germplasm with elite qualities that can be used for crosses. This present study adds to our understanding of cold and drought tolerance traits in *Petunia* spp. and can serve as a starting point for future efforts to create more stress tolerant cultivars.

LITERATURE REVIEW

The genus *Petunia*

There is disagreement among taxonomists about how many species belong in the genus *Petunia*, but numbers generally range from 11 (Kulcheski *et al.* 2006; Lorenze-Lemke 2006) to 16 species (Griesbach 2007). The genus *Petunia* can be further divided into two subgenera, *Pseudonicotiana* and *Eupetunia*. Plants of *Pseudonicotiana* have a salver-shaped corolla with filaments attached to the middle of the corolla tube. Plants in *Eupetunia* have a funnel-shaped corolla and filaments which attach below the middle of the corolla tube (Griesbach 2007). Regardless of the number of species in the genus, all species originate in South America (Kulcheski 2006). The southeastern Sierra region of

Brazil has been identified as one of the centers of diversity (Lorenz-Lemke 2006). All *Petunia* species have $2n=14$ chromosomes; the feature distinguishing them from the closely related genus, *Calibrachoa*, which has $2n=18$ (Griesbach 2007). While origin and chromosome numbers are similar for all *Petunia* species, there is considerable diversity within the genus in terms of flower color, morphology, and pollination syndrome. The three wild species chosen for these studies, *P. axillaris* (Lamarck) Britton, Sterns & Poggenburg, *P. integrifolia* (Hooker) Schinz & Thellung, and *P. exserta* Stehmann, span the range of variation for these traits within the genus.

Petunia axillaris is a white flowered species in the subgenus *Pseudonicotiana* (Griesbach 2007). The flowers are strongly fragrant, especially at night, and are pollinated by the nocturnal hawkmoth (*Manduca* spp.) (Ando *et al.* 2001). This species can be further divided into three subspecies, *P. a. ssp. axillaris*, *P. a. ssp. parodii* (Steere) Cabrera, and *P. a. ssp. subandina* Ando (Ando *et al.* 2001).

Petunia exserta also belongs to the subgenus *Pseudonicotiana* and possesses several characteristics unique among other wild petunias. *Petunia exserta* is the only ornithologically pollinated petunia and is also the only red flowered species within the genus (Griesbach *et al.* 1999). *Petunia exserta* is very rare, being found growing in shady cracks on only four sandstone towers in the Southeastern Sierra region of Brazil (Lorenz-Lemke 2006). The red color of *P. exserta* is the result of a mixture of several anthocyanin pigments which are distinct from those present in modern red cultivars of *P. hybrida* (Ando *et al.* 2000). It has been speculated that the genetic differences between *P. exserta* and *P. hybrida* will be useful in the breeding of new red-flowered cultivars (Griesbach *et al.* 1999; Ando *et al.* 2000).

Petunia integrifolia is a scentless, purple flowered species in the subgenus *Eupetunia*, that is pollinated by bees (Ando *et al.* 2001). This species exhibits a high degree of self-incompatibility (Ando *et al.* 2001; personal observation), making visits from pollinating bees very important for reproduction. Similar to *P. axillaris*, *P. integrifolia* can be further divided into several subspecies (Griesbach 2007). *Petunia integrifolia* seems to be gaining popularity among home gardeners and is often marketed as “wild petunia.”

Petunia hybrida ‘Mitchell’ is a doubled-haploid hybrid petunia. This particular hybrid arose from the anther culture of a plant resulting from the crossing of *P. axillaris* x (*P. axillaris* x *P. hybrida* ‘Rose du Ciel’). This plant is particularly useful in genetic studies because the doubled-haploid nature of the cultivar results in homozygosity at all alleles (Griesbach 2007).

Cold tolerance of plants

Cold temperatures are among the many abiotic stresses that plants must endure. Plant species can be grouped according to their ability to tolerate cold temperatures. The least cold tolerant species are referred to as chilling sensitive and suffer damage when temperatures are cool but remain above freezing. Freezing sensitive species tolerate cool temperatures, but are damaged when temperatures fall below freezing. The hardiest species are freezing tolerant and able to withstand temperatures below freezing (Chen and Li 1980).

Cold tolerance is a dynamic characteristic, changing as environmental conditions change. When grown in warm temperatures, even cold hardy species have low tolerance

to freezing. Exposure to low non-freezing temperatures brings about an increase in cold tolerance by a process called cold acclimation. This acclimation process allows plants to survive future temperatures that are much lower than would be tolerated without acclimation (Thomashow 1999).

Genetics of cold tolerance

Gene expression (Guy *et al.* 1985) and cellular metabolite profile (Cook *et al.* 2004) changes occur during cold acclimation. Expression of hundreds of genes is altered following exposure to low temperatures. Vogel *et al.* (2005) defined a set of low temperature-responsive genes, termed the COS (COld Standard) set, that are reliably up- or down-regulated in response to low temperature whether plants are grown in soil or on agar plates. This COS set includes more than 300 genes that are up-regulated in *Arabidopsis* in response to low temperature and 212 genes that are downregulated.

The COS set includes members of the previously identified COR (COld-Responsive) family of cold-induced genes in *Arabidopsis* (Baker *et al.* 1994; Thomashow 1999). These COR genes are regulated by both an ABA-dependent and an ABA-independent pathway (Gilmour and Thomashow 1991). COR genes such as *COR15a* encode a variety of polypeptides which act to increase freezing tolerance (Steponkus *et al.* 1998; Thomashow 1999). Other cold-regulated genes encode antifreeze proteins, signal transduction proteins, and transcription factors (Maruyama *et al.* 2004, Vogel *et al.* 2005).

The promoter regions of the cold-induced COR genes contain a *cis*-acting element called the C-repeat/Dehydration responsive element (CRT/DRE) (Gilmour *et al.* 1998)

that is necessary for maintaining the cold-responsiveness of the genes (Baker *et al.* 1994). This CCGAC sequence is also present in the promoter region of *Bn115*, a cold-regulated gene in *Brassica napus* (Jiang *et al.* 1996), as well as some cold-regulated tomato genes (Zhang *et al.* 2004). The cold-induced transcriptional activator *CBF1* (C-repeat/Dehydration responsive element binding factor 1) binds to the CRT/DRE sequence to induce transcription of other cold-responsive genes in *Arabidopsis*. This transcriptional activator protein has a molecular mass of 24kDa and contains a nuclear localization sequence, an acid activation domain, and an AP2 DNA binding domain of 60 amino acids (Stockinger *et al.* 1997).

CBF1, along with two other genes, *CBF2* and *CBF3*, comprise a small family of transcriptional activators in *Arabidopsis* (Gilmour *et al.* 2004). All three proteins are 88% identical at the amino acid level and are located in tandem array on chromosome IV. As with *CBF1*, both *CBF2* and *CBF3* bind to CRT/DRE sequences in the promoter regions of cold regulated genes (Gilmour *et al.* 1998; Medina *et al.* 1999) and all three are functionally redundant (Gilmour *et al.* 2004). Simultaneous to the discovery of the *CBF1-3* genes by the Thomashow lab, the same genes were discovered by the Shinozaki and Yamaguchi-Shinozaki lab which named them *DREB1b*, *DREB1c*, and *DREB1a*, respectively (Liu *et al.* 1998).

The level of CBF transcripts increases within 15 minutes of exposing the plant to 2.5°C. These levels continue to increase for two more hours and then begin to slowly fall. Transcript levels remain higher than in non-chilled plants for at least 24 hours. After two hours of cold exposure, transcripts for cold induced genes *COR15a* and *COR78* also begin to accumulate (Gilmour *et al.* 1998).

CBF transcript accumulation is not controlled by a simple on/off mechanism. Transcript levels do increase in response to a sudden cold shock, but they also increase in response to gradual cooling (Zarka *et al.* 2003). Colder temperatures result in greater transcript accumulation and slightly warmer temperatures result in less transcript accumulation. The temperature sensing mechanism can become desensitized after a long period of exposure to a given cold temperature, at which point, CBF expression decreases. The cold sensing mechanism becomes resensitized after exposure to warm temperatures for a period of time ranging from 8 to 24 hours. While in the desensitized state, CBF expression will continue to increase if the temperature is decreased even further (Zarka *et al.* 2003).

Constitutive expression of *CBF1* in *Arabidopsis* induces COR gene expression and increases freezing tolerance of nonacclimated plants (Jaglo-Ottosen *et al.* 1998). Likewise, transgenic *Arabidopsis* overexpressing *CBF3* are more tolerant of salt, drought, and cold stresses compared to control plants (Kasuga *et al.* 1999). Negative phenotypic effects have been observed when *CBF3* is expressed in *Arabidopsis* behind the strong constitutive cauliflower mosaic virus (*CaMV*) 35S promoter. Transgenic plants are delayed in flowering, smaller in overall size, more prostrate in growth form, and have shorter petioles (Kasuga *et al.* 1999; Gilmour *et al.* 2000). The use of the stress-inducible *rd29A* (*Cor78*) promoter to drive expression of *CBF3* minimizes these negative effects while still increasing stress tolerance of transgenic plants following cold acclimation (Kasuga *et al.* 1999).

Many biochemical changes occur in the *CBF3* overexpressing plants which are similar to the changes that occur when wild type plants are cold acclimated. For

example, overexpression of *CBF3* results in increased proline levels in non-acclimated plants and increased transcript levels for *P5CS*, an enzyme involved in proline biosynthesis in plants (Gilmour *et al.* 2000).

While all three CBF genes appear to be functionally redundant within their roles of cold-responsive gene regulation, freezing tolerance, and plant development (Gilmour *et al.* 2004), *CBF2* seems to also play a role in the negative regulation of *CBF1* and *CBF3* (Novillo *et al.* 2004). The *cbf2* mutant has increased tolerance to salt, drought, and freezing stress. The *cbf2* plants are actually more freezing tolerant than wild type plants with or without acclimation. Basal expression of *CBF1* and *CBF3* is increased in the *cbf2* mutant and high expression levels are sustained longer in response to cold temperatures. In wild type plants, upon exposure to stress, *CBF1* and *CBF3* transcripts accumulate sooner than *CBF2* transcripts. Taken together, these data suggest that *CBF2* is a negative regulator of *CBF1* and *CBF3* expression (Novillo *et al.* 2004). ZAT12, another cold-responsive transcription factor which is induced parallel to *CBF1-3*, has also been shown to be involved in the negative regulation of *CBF1* and *CBF3* (Vogel *et al.* 2005).

Control of CBF expression is not accomplished through autoregulation since the promoter regions lack the CCGAC sequence to which CBF binds during the induction of other cold-regulated genes (Gilmour *et al.* 1998). CBF expression is regulated by an upstream transcription factor, ICE1 (Inducer of CBF Expression), which binds to promoter regions of the CBF genes. An *Arabidopsis* mutant defective in ICE1 production, *ice1*, shows reduced *CBF3* expression, lowered COR gene expression when exposed to cold temperatures, and lower freezing tolerance compared to wild-type plants,

indicating that ICE1 positively regulates CBF gene expression. The *ICE1* gene is constitutively expressed in the nucleus yet the CBF genes require a cold treatment for expression. Therefore, a cold-induced modification such as phosphorylation/dephosphorylation likely occurs to activate the ICE1 protein upon exposure to cold temperature. This change allows ICE1 to bind to the promoters of the CBF genes and induces their expression (Chinnusamy *et al.* 2003).

As opposed to *CBF3*, *CBF1* and *CBF2* are not as strongly affected by the *ice1* mutation, indicating that there may be multiple mechanisms regulating expression of the three CBF genes (Lee *et al.* 2005; Chinnusamy *et al.* 2003). The promoter of *CBF2* contains two regions which are sufficient for cold-responsiveness. These two regions, ICeR1 and ICeR2 (Induction of CBF Expression regions 1 and 2), act together to stimulate *CBF2* transcription in response to cold and are also involved in gene expression in response to mechanical agitation. However, it is still unclear which proteins bind to the ICeR1 and ICeR2 regions (Zarka *et al.* 2003).

It has been suggested that CBF proteins need to be activated before they can function in the induction of cold inducible genes. The *sfr6* mutant in *Arabidopsis* accumulates normal levels of CBF transcripts following cold exposure but produces low levels of the cold-induced genes targeted by the CBF proteins, suggesting a role for *sfr6* in the activation of CBF proteins (Knight *et al.* 1999).

The expression of the CBF genes is partially dependent upon the time of day during which the low temperature stress is present. If the cold stress is present earlier in the day, then CBF expression will be greater than if the stress is present later in the day. The highest levels of expression occur when *Arabidopsis* plants are exposed to low

temperatures 4 hours after dawn and the lowest levels occur at 16 hours after dawn. This difference is regulated at the transcriptional level (Fowler *et al.* 2005).

Of the 514 COS genes that have been identified, 93 are in the CBF regulon. Of these 93 genes in the regulon, 85 are upregulated and 8 are downregulated by low temperatures. The upregulated genes have a CRT/DRE element where CBF transcription factors may bind. The downregulated COS genes do not have CRT/DRE elements so they are most likely not directly targeted by CBF transcription factors (Vogel *et al.* 2005). Within the CBF regulon, two other transcription factors, RAP2.1 and RAP2.6, are activated by CBF in response to cold temperature. These RAP transcription factors may control subregulons of the CBF regulon (Fowler and Thomashow 2002).

Changes in the metabolome of cold acclimated *Arabidopsis* plants are largely, but not entirely, controlled by the action of the CBF genes. In one study, 325 metabolites were found to increase in response to low temperatures in wild type plants. The metabolome of nonacclimated *CBF3* overexpressing plants also had increased levels of 256 (79%) of these metabolites. Therefore, a majority of the metabolome profile changes are under the control of the CBF regulon (Cook *et al.* 2004).

However, there is evidence for the presence of freezing tolerance genes which are not controlled by the CBF regulon. Warm-grown plants overexpressing *CBF3* with a constitutive promoter are more freezing tolerant than non-acclimated control plants, but they become tolerant to even lower temperatures following cold exposure. This provides evidence for freezing tolerance genes which are not controlled by *CBF3* but which are activated at low temperatures (Gilmour *et al.* 2000).

The CBF genes are also induced in response to stresses other than cold temperatures. *CBF1* and *CBF2* transcripts accumulate in response to mechanical agitation while *CBF3* does not (Gilmour *et al.* 1998). *CBF1-3* are not responsive to dehydration stress (Medina *et al.* 1999) while *CBF4*, a fourth member of the CBF family, is highly induced in response to drought stress (Haake *et al.* 2002).

Conservation of CBF in other species

CBF appears to be well conserved among the plant species, including monocots and herbaceous and woody dicots. CBF orthologs have been identified in many species including *Brassica napus* L. cv. Jet neuf (Gao *et al.* 2002), *B. napus* L. cv. Westar, *Solanum esculentum* L., *Triticum aestivum* L., *Secale cereale* L. (Jaglo *et al.* 2001), *Fragaria ×ananassa* Duchesne, *Prunus cerasus* L. (Owens *et al.* 2002), *P. avium* L. (Kitashiba *et al.* 2004), *Eucalyptus gunnii* Hook. (Kayal *et al.* 2006), *Hordeum vulgare* L. (Choi *et al.* 2002), *Festuca arundinacea* Schreb (Tang *et al.* 2005), and *Zea mays* L. (Qin *et al.* 2004). As with *Arabidopsis* CBF proteins, the CBF protein orthologs contain the AP2/EREBP DNA-binding domain and are upregulated in response to cold temperatures. The orthologs in *B. napus*, *T. aestivum*, *S. cereale*, *H. vulgare*, *F. arundinacea*, and *E. gunnii* also contain the PKK/RPAGRxKFXETRHP and DSAWR CBF signature sequences immediately upstream and downstream, respectively, from the AP2/EREBP domain (Jaglo *et al.* 2001; Choi *et al.* 2002; Tang *et al.* 2005; Kayal *et al.* 2006).

Ectopic expression of several of these orthologs increases the freezing tolerance of *Arabidopsis*. Constitutive expression of *ZmDREB1A* (a CBF ortholog in maize) in *Arabidopsis* induces expression of CBF target genes and results in increased tolerance to

drought and freezing stress in non-acclimated plants (Qin *et al.* 2004). Likewise, transformation of *CIG-B* (a CBF ortholog from sweet cherry) into *Arabidopsis* with a constitutive promoter induces *COR15a* expression without stress treatment. These transgenic plants are more cold and salt tolerant than wild type plants and exhibit stunted growth (Kitashiba *et al.* 2004).

Additionally, expressing *Arabidopsis* CBF genes in several of the previously mentioned species enhances expression of cold-response genes and increases freezing tolerance. Constitutive expression of *Arabidopsis* CBF genes in *B. napus* cv. Westar stimulates *Bn115* and *Bn28* (orthologs of *Arabidopsis* *COR15a* and *COR6.6*, respectively) expression in transgenic plants without cold acclimation and increases plant freezing tolerance (Jaglo *et al.* 2001). Similarly, the freezing tolerance of nonacclimated leaves from transgenic strawberry plants, which overexpress *AtCBF1* under control of the CaMV 35S promoter, is increased compared to nonacclimated wild type plants. However, the tolerance of the strawberry plant receptacles to freezing remains unchanged (Owens *et al.* 2002).

In the Solanaceae family, freezing-sensitive tomato plants have been shown by Zhang *et al.* (2004) to contain three CBF genes (*LeCBF1-3*). These three CBF genes are located in tandem array on chromosome III in the tomato genome. All three *LeCBF* genes are induced by mechanical agitation but not by ABA, salinity, or drought. *LeCBF1* is the only one induced by low temperatures and this induction varies depending on the photoperiod in which the plant is grown. When grown under constant light, peak levels of transcript accumulation are reached after 2 hours of cold and return to normal levels after 24 hours. When plants are grown in a 16 hour photoperiod, transcript levels remain

elevated for 16 hours and decrease to slightly above normal levels by 24 hours (Zhang *et al.* 2004).

When *LeCBF1* is overexpressed in *Arabidopsis*, COR genes are induced and freezing tolerance is increased. Freezing tolerance does not increase when *LeCBF1* or *AtCBF1* is overexpressed in tomato plants (Zhang *et al.* 2004), but drought stress tolerance increases with overexpression of *AtCBF1* (Hsieh *et al.* 2002). Only four tomato genes are induced by the overexpression of either CBF gene. Therefore, tomato plants do have a CBF regulon, but it is smaller than the CBF regulon present in *Arabidopsis* (Zhang *et al.* 2004).

Freezing-sensitive *Solanum tuberosum* (potato) and freezing-tolerant *S. commersonii* (wild potato) also respond to CBF overexpression by exhibiting increased cold tolerance (Pino *et al.* 2008). *Solanum tuberosum* normally has a freezing tolerance of -3°C before cold acclimation and does not display any increased tolerance following acclimation. However, when *AtCBF1* or *AtCBF3* are expressed behind a constitutive CaMV 35S promoter, freezing tolerance of warm grown plants increases to -5°C (Pino *et al.* 2007; Pino *et al.* 2008). Additionally, expression of these same genes behind a cold-inducible *rd29A* promoter does not alter freezing tolerance of warm grown plants, but increases the freezing tolerance of cold acclimated plants to -5°C following a two-week treatment at 2°C (Pino *et al.* 2007).

S. commersonii is a wild relative of the cultivated potato, but unlike *S. tuberosum*, this plant is frost tolerant and able to cold acclimate. Prior to acclimation it can survive temperatures down to -5°C, and following acclimation it can tolerate temperatures as low as -12°C (Chen and Li 1980). Heterologous expression of *AtCBF1* with a *CaMV 35S*

promoter in *S. commersonii* results in an increase in the freezing tolerance of warm grown plants by 2 to 4 °C. Cold acclimation of these transgenic lines results in further increases in freezing tolerance by anywhere from 1 to 4 °C (Pino *et al.* 2008).

Cold acclimation and CBF in petunia

Petunia hybrida is Solanaceous species capable of cold acclimation (Yelenosky and Guy 1989; Pennycooke *et al.* 2003). The freezing tolerance of petunia has been successfully increased by creating transgenic plants with modified expression of α -Galactosidase (α -Gal) (Pennycooke *et al.* 2003). α -Gal is responsible for breaking down raffinose oligosaccharides and the activity of α -Gal increases during deacclimation of petunia resulting in lower raffinose content (Pennycooke *et al.* 2004). Transgenic petunias created with reduced α -Gal transcript levels leads to increased raffinose content. These plants display an increased ability to cold acclimate and are able to increase in freezing tolerance by 3 to 6°C. On the other hand, plants with increased α -Gal gene expression have lower raffinose content and lower freezing tolerance (Pennycooke *et al.* 2003).

Our present research furthers our understanding of the genetic mechanisms controlling cold tolerance in *Petunia* spp. and identifies possible methods for increasing the cold tolerance of garden petunias.

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CHAPTER 2

CHARACTERIZING COLD ACCLIMATION ABILITY OF *PETUNIA* SPP.

ABSTRACT

Freezing tolerance of many plant species increases following exposure to low, non-freezing temperatures, a process termed cold acclimation. In some species, shortened photoperiods also bring about an increase in freezing tolerance. Within the plant family Solanaceae, species vary widely in cold acclimation ability. The objectives of this work were to examine the roles of low temperature and photoperiod in cold acclimation of *Petunia hybrida* and to evaluate cold acclimation of several wild *Petunia* species. Temperature, but not photoperiod, influenced cold acclimation of *P. hybrida*. Whether grown under long days or short days, nonacclimated plants had an EL₅₀ value (temperature at which 50% of cellular electrolytes are lost) of ca. -2 °C. Plants acclimated by gradual cooling at temperatures of 15 °C, 10 °C and 3 °C for 7 days each, reached an EL₅₀ of ca. -5 °C, regardless of photoperiod. Exposure to 3 °C under short days for one or three weeks resulted in EL₅₀ temperatures of -3.9 °C and -4.9 °C, respectively. Freezing tolerance of petunia species *P. exserta*, *P. integrifolia*, *P. axillaris* (USDA accessions 28546 and 28548), and *P. hybrida* 'Mitchell' was similar prior to cold acclimation, but varied from -5 °C for *P. exserta* to -8 °C for *P. axillaris* (accession 28548) following cold acclimation.

INTRODUCTION

Plants endure many biotic and abiotic stresses throughout their lives including exposure to cold temperatures. Tremendous damage to plant cells can result from freezing temperatures, primarily to the cellular membranes (Thomashow 1999). Many plants have adapted to life in cooler climates by developing mechanisms to tolerate low temperatures, but cold tolerance is a dynamic characteristic. When grown in warm temperatures, even cold hardy plants have low tolerance to freezing. Exposure to low non-freezing temperatures, and in some species shortened photoperiods, brings about an increase in cold tolerance through cold acclimation. Acclimation processes allow plants to survive temperatures that are much lower than would be tolerated without acclimation (Thomashow 1999).

Cold acclimation brings about changes in gene expression (Guy *et al.* 1985) and cellular metabolite profiles (Cook *et al.* 2004). Of the changes that occur, those regulated by the CBF cold response pathway are the most thoroughly characterized. In *Arabidopsis thaliana* L., this pathway consists of three, functionally redundant transcription factors, *AtCBF1-3* (Gilmour *et al.* 2004). Downstream components of the pathway such as the COR (COld-Responsive) family of genes encode a variety of polypeptides which act to increase freezing tolerance. For example, *COR15a* is involved in the stabilization of membranes during cold temperature exposures (Steponkus *et al.* 1998; Thomashow 1999). Promoter regions of these downstream genes contain a *cis*-acting element called the C-repeat/Dehydration responsive element (CRT/DRE) to which CBF binds (Stockinger *et al.* 1997; Gilmour *et al.* 1998). Expression of the *A. thaliana* genes *AtCBF1-3* increases in response to low temperatures thereby inducing expression of the

downstream components and ultimately increasing tolerance to freezing temperatures (Gilmour *et al.* 1998; Jaglo-Ottosen *et al.* 1998).

In addition to exposure to low non-freezing temperatures, shortened photoperiods induce cold acclimation of many woody plant species (Howell and Weiser 1970; Li *et al.* 2002). Acclimation of *Pyrus malus* L. occurs in two stages. Shortened photoperiods induce the first stage and result in an increase in bark hardness (defined as the highest temperature at which bark from 1-year-old branches is killed) from -10 °C in nonacclimated plants down to -25 °C. Frost induces the second stage and results in acclimation down to -55 °C (Howell and Weiser 1970). In *Betula pendula* Roth., both shortened photoperiod (12 h) and low temperatures (4 °C) result in acclimation, but low temperatures produce a stronger response. An additive response is seen when low temperatures and short days are experienced at the same time (Li *et al.* 2002).

Freezing tolerance of some herbaceous plant species such as *Hordeum vulgare* L. cv. Dicktoo is also influenced by photoperiod and plants attain greater freezing tolerance when exposed to 4 °C under short photoperiods (8 h) compared to long photoperiods (20 h). Plants grown under short days gradually increase in freezing tolerance and reach LT₅₀ (temperature at which 50 percent of frozen plant crowns survive) of -12 °C after 56 d at 4 °C, while plants grown under long days peak at LT₅₀ of -8 °C after 7 and then slowly become less freezing tolerant. In this species, the increased freezing tolerance is associated with a short-day induced delay in the transition from vegetative to reproductive stage (Fowler *et al.* 2001). The roles that photoperiod and temperature play in the acclimation of petunia (*Petunia hybrida* Vilm.) are currently unknown.

Electrolyte leakage assays are useful for comparing *in vitro* freezing tolerance between different plant types or plants exposed to different environmental conditions (Jaglo-Ottosen *et al.* 1998; Thapa *et al.* 2008; Pino *et al.* 2007; Pino *et al.* 2008; Pennycooke *et al.* 2003). This method provides a means of estimating cellular membrane damage by determining the percentage of total electrolytes lost due to freeze induced membrane damage at various sub-zero temperatures. Typically, the temperature at which 50% of total electrolytes escape (EL₅₀) is used for comparisons. Lower EL₅₀ temperatures indicate a greater tolerance to freezing.

Garden petunias (*P. hybrida*) are very popular bedding plants, ranking first in sales among bedding plants in 2007, with a wholesale value of over \$111 million (USDA-NASS 2008). Estimates for the number of species belonging to the genus *Petunia* vary, but numbers generally range from 11 (Kulcheski *et al.* 2006; Lorenz-Lemke 2006) to 16 species (Griesbach 2007). All *Petunia* spp. originate from South America (Kulcheski 2006) with the southeastern Sierra region of Brazil being one of the centers of diversity (Lorenz-Lemke 2006). While the exact origin of *P. hybrida* is unclear, it is believed to be the result of hybridization between two species, *P. axillaris* Lam. and *P. integrifolia* Hook. (Sink 1984). *Petunia hybrida* is capable of cold acclimation (Yelenosky and Guy 1989; Pennycooke *et al.* 2003).

Within the Solanaceae family, the genus *Solanum* encompasses species that vary significantly in their ability to cold acclimate. The chilling-sensitive *S. lycopersicum* L. (tomato) (Hsieh *et al.* 2002) and frost-sensitive *S. tuberosum* L. (potato) are species that do not cold acclimate, while *S. commersonii* Dun. (a wild potato relative) is frost-tolerant and capable of acclimation (Chen and Li 1980). Although *S. tuberosum* does not

normally cold acclimate in response to cool temperatures, heterologous expression of *AtCBF1* or *AtCBF3* under a constitutive cauliflower mosaic virus (*CaMV*) 35S promoter enhances the EL₅₀ value of non-acclimated plants by about 2°C, from -3 °C to -5 °C. When *AtCBF1* or *AtCBF3* are expressed behind a cold-inducible *rd29A* (*Cor78*) promoter, non-acclimated plants modestly increase in freezing tolerance by 0.5-1 °C, and following acclimation at 2 °C for two weeks these lines show the same 2 °C increase in freezing tolerance seen in the constitutively expressing lines (Pino *et al.* 2007). In the cold-acclimating *S. commersonii*, ectopic expression of *AtCBF1* behind a constitutive promoter also increases freezing tolerance of non-acclimated plants by 4 °C. Unlike in *S. tuberosum*, transgenic *S. commersonii* further increases in freezing tolerance following two weeks at 2 °C (Pino *et al.* 2008). Heterologous expression of either *AtCBF3* or *LeCBF1* (a CBF homolog from tomato) in *S. lycopersicum* does not result in a change in freezing tolerance (Zhang *et al.* 2004), but *AtCBF1* does confer an increase in tolerance to low non-freezing temperatures (Hsieh *et al.* 2002).

During the processes of domestication and breeding, the genetic diversity of a plant species tends to decrease dramatically (Tanksley and McCouch 1997). Strong selection imposed by plant breeders, along with crossing between closely related individuals, leads to a narrowing of the genetic diversity in future generations. For example, soybean (*Glycine max* L.) is a crop with a very narrow range of genetic diversity, and pedigree analysis has shown that just 35 genotypes contributed to 95% of the germplasm base in modern cultivars (Gizlice *et al.* 1994).

Utilizing wild germplasm for crop improvement has proven to be an effective method for increasing genetic diversity of crops. For example, quantitative trait locus

(QTL)-alleles transferred from the wild tomato species, *Solanum habrochaites* Knapp & Spooner and *S. pimpinellifolium* L., to the cultivated tomato, *S. lycopersicum* L. by advanced backcross methods improved horticultural traits in 22 out of 25 cases (Bernacchi *et al.* 1998).

In the genus *Petunia*, interspecific hybridization is relatively easy to accomplish (Ando *et al.* 2001; Griesbach 2007; personal experience), making utilization of wild germplasm possible. Griesbach *et al.* (1999) found that the red color of *P. exserta* results from genetic factors distinct from those in current red colored *P. hybrida* cultivars. Interspecific hybridization using *P. exserta* is adding to the genetic diversity of *P. hybrida* (Griesbach *et al.* 1999). The simplest method of introducing wild germplasm into a cultivated plant is via interspecific hybridization between a wild species and the cultivated species. Hybrids from a segregating population exhibiting desirable traits are then backcrossed using the cultivated species as the recurrent parent.

Improving cold tolerance of petunia would benefit both consumers and commercial growers. The selection of bedding plants suited for growth in early spring and late fall, when cold night temperatures and frosts still limit the survival of most bedding plants, is limited. The objectives of the current study are 1) to examine the roles of low temperature and photoperiod in cold acclimation of petunia and 2) to evaluate cold acclimation of *P. axillaris*, *P. integrifolia*, *P. exserta*, and *P. hybrida* ‘Mitchell’, in an effort to identify genetic material that may be useful in breeding more stress tolerant cultivars.

MATERIALS AND METHODS:

Environmental cues influencing acclimation of *P. hybrida* ‘Mitchell’

Plant growth and acclimation treatments

Seeds of *P. hybrida* ‘Mitchell’ were surface planted onto 288-cell plug trays filled with 70% peat moss, 21% perlite, 9% vermiculite (v/v) (Suremix, Michigan Grower Products Inc., Galesburg MI, USA). Trays were placed under intermittent overhead mist irrigation until the development of at least two true leaves. Plants were then transferred to a greenhouse at 22 °C with a 16 h photoperiod (ambient plus supplemental high-pressure sodium lighting from 0600 to 2200 HR) until three weeks post planting. Seedlings were then transplanted to 50-cell trays containing the same soilless media and moved to a controlled environment growth chamber at 22 °C under a 16 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent plus incandescent lighting) for one week. Fifty plants were then subjected to each of seven different treatments shown in table 2.1.

Cold tolerance determination by electrolyte leakage assay

Following temperature/photoperiod treatments, leaf discs from the upper portion of the plant were collected using a 0.6 cm cork borer. The leaves chosen for sampling were the youngest leaves that could be punched to obtain complete 0.6 cm diameter discs without cutting into the midrib. Discs were immediately transferred to plastic weigh boats filled with deionized water and stirred gently. Approximately 120 punches were taken from a population of 50 plants for each treatment. Three discs were then transferred to each of 30 (16x100mm) borosilicate glass culture tubes for each treatment and placed on ice. When all discs were transferred, tubes were placed in a -1 °C

controlled temperature antifreeze bath (master bath) for 60 min. Three tubes of each treatment were left on ice as controls. After 60 min, a small amount of ice was added to each tube to nucleate extracellular ice formation. Tubes were plugged with foam and kept at -1 °C for an additional 60 min, after which three tubes of each treatment were moved to a second antifreeze bath at -1 °C, kept there for 40 min, and removed to ice. Meanwhile, the temperature of the master bath was lowered to -2 °C. After 20 min, three tubes of each treatment were moved from the master bath to another antifreeze bath at -2 °C for 40 min and then removed to ice (a total of 60 min at the test temperature). This process continued for all temperatures tested (-1 °C to -9 °C). Tubes were then placed in racks on top of ice and placed at 2.5°C to thaw slowly overnight.

The following day, 6 ml of dH₂O was added to each tube followed by gentle shaking for 3 h at room temperature to allow released electrolytes to diffuse into the water. The water was then transferred to a new culture tube and electrical conductivity (L_1) was measured using a CON 110 conductivity meter (Oakton Instruments, Vernon Hills IL, USA). Plant discs remained in the original tube and were frozen to -80 °C overnight to release all electrolytes. The next day, the water from the previously measured tubes was poured back into the corresponding plant disc tube, followed by shaking for 3 h at room temperature. Conductivity of the water was again measured to obtain the reading for maximum electrolyte leakage (L_2). Percent of total electrolyte leakage at each test temperature was calculated by $(L_1 \div L_2) * 100$. Data analysis was carried out using Sigmaplot (SPSS Inc., USA) and SAS (SAS Institute Inc., USA) software. A sigmoidal curve was fitted to the leakage data for each treatment according to the equation: $y = a1 + (a2 \div (1 + \exp(a3 - a4 * T)))$, where y is the average percent

electrolyte leakage of the three tubes at each temperature T , using the curve fitting function of Sigmaplot. The initial parameters were specified as $a_1=0.1$, $a_2=99.9$, $a_3=0.1$ and $a_4=0.1$ with constraints imposed such that $a_1>0$ and $0<a_2<100$. The temperature at which 50% of total electrolytes were released (EL_{50}) was calculated by solving the equation $EL_{50} = ((a_3 - \log((a_2 \div (50 - a_1)) - 1)) \div a_4)$ where the parameters a_1 , a_2 , a_3 and a_4 are given by the equation of the fitted curve. At least two EL_{50} values were calculated for each treatment from two separate replications of the experiment. ANOVA analysis and mean separations using Fisher's LSD ($\alpha=0.05$) were conducted on the EL_{50} values using PROC GLM to compare treatment effects.

Assay of wild *Petunia* detached leaf freezing tolerance

Plant growth and acclimation of wild *Petunia* spp.

Sowing and germination of *P. integrifolia*, *P. exserta*, *P. axillaris* (USDA Accession 28546), *P. axillaris* (USDA Accession 28548) and *P. hybrida* 'Mitchell' were as described above. When seedlings had two true leaves, plants were transferred to a greenhouse at 20 °C with a 16 h photoperiod for four weeks. Seedlings were then transplanted into 50-cell trays and moved to a controlled environment growth chamber at 22 °C, 16 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light). After two weeks, plants of each species were divided into two groups; a non-acclimated group remaining at 22 °C for an additional week before testing, and an acclimated group subjected to a gradual cooling process. The acclimated group was moved to 15 °C, 9 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) for 1 week followed by 10 °C, 9 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) for 1 week, and finally 3 °C, 9 h photoperiod (30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) for an additional week. At

the time of testing, non-acclimated plants were 7 weeks old and acclimated plants were 9 weeks old. However, plants in both groups had developed approximately the same number of nodes at the time of sampling. Electrolyte leakage assays were performed as described above.

RESULTS

Influences of environmental cues on cold acclimation of *P. hybrida* 'Mitchell'

Temperature regime, but not photoperiod, influenced cold acclimation of *P. hybrida* 'Mitchell' (Fig. 2.1 and 2.2; Table 2.2 and 2.3). When grown at 22 °C and 16 h long days (NONLD), *P. hybrida* 'Mitchell' EL₅₀ value was -1.9 °C (Fig. 2.1A and 2.2). Exposing plants to three weeks of short days alone (NONSD) did not increase freezing tolerance and the EL₅₀ temperature remained at -2.0 °C (Fig. 2.1B and 2.2). Both NONLD and NONSD reached maximum electrolyte leakage at -4 °C (Fig. 2.1A and B). Gradual cooling, whereby plants were grown under 16 h photoperiod for 7 d at each temperature of 15 °C, 10 °C and 3 °C (RampdownLD) resulted in significant cold acclimation, as the EL₅₀ temperature decreased to -5.1 °C (Fig. 2.1G and 2.2). The same gradual cooling experienced under 9 h days (RampdownSD) also caused significant acclimation but did not increase the acclimation response compared with RampdownLD and the EL₅₀ temperature remained at -5.0 °C (Fig. 2.1F and 2.2).

Moving plants directly from long days at 22 °C to short days at 3 °C for just one week (CA1) increased freezing tolerance slightly (EL₅₀ = -3.9°C) compared to NONLD and NONSD (Fig. 2.1C and 2.2). Prolonging time of exposure to 3 °C with short days to 3 weeks (CA3) caused a more pronounced increase in freezing tolerance (EL₅₀ = -4.9 °C)

compared to the CA1 treatment (Fig. 2.1E and 2.2). Maximum leakage for CA1 occurred at -7 °C compared with -8 °C for CA3 (Fig. 2.1C and E). Exposing plants to short days at 22 °C for two weeks prior to exposure to 3 °C with short days for another week (CA2), gave an EL₅₀ value of -4.0 °C and maximum leakage at -7 °C (Fig. 2.1D and 2.2), which was similar to CA1 (Fig. 2.2).

Exposure to three weeks of gradual cooling in both the RampdownSD and RampdownLD treatments resulted in the same degree of acclimation as the CA3 treatment with three weeks at 3 °C (Fig. 2.2). However, the RampdownSD and RampdownLD treatments were more effective than the CA1 treatment with just one week at 3 °C (Fig. 2.2).

Freezing tolerance of wild species

All species tested had similar constitutive freezing tolerance (Fig. 2.3 and 2.4; Table 2.4A) with an EL₅₀ temperature of ca. -2 °C without cold acclimation. Acclimated EL₅₀ temperatures varied across species (Fig. 2.4; Table 2.4B and 2.5). The most cold-tolerant species was *P. axillaris* (USDA accession 28548) with an acclimated EL₅₀ temperature of -7.8 °C, while *P. exserta* was the least cold-tolerant with an EL₅₀ temperature of -4.9 °C. In addition to the observed interspecific variation, there was also significant variation in the acclimated freezing tolerance between the two *P. axillaris* accessions. The acclimated EL₅₀ temperatures for *P. hybrida*, *P. axillaris* (USDA accession 28546) and *P. integrifolia* are -6.0 °C, -6.1 °C and -6.6 °C, respectively. While there was no statistical difference in the acclimated cold tolerance between these three species, they were all significantly less cold tolerant than *P. axillaris* (accession 28548).

and all except *P. axillaris* (accession 28546) were significantly more cold tolerant than *P. exserta*.

DISCUSSION AND CONCLUSIONS

Petunia hybrida is capable of cold acclimation (Yelenosky and Guy 1989; Pennycooke *et al.* 2003), however, the environmental changes that signal the plant to undergo acclimation have not previously been determined. The impacts of photoperiod and cool temperatures were examined in this study. Previous work indicated that reduced photoperiod and low temperatures both play a role in cold acclimation of woody trees such as *Pyrus malus* (Howell and Weiser 1970) and *Betula pendula* (Li *et al.* 2002), and in the acclimation of some non-woody plants such as *Hordeum vulgare* cv. Dicktoo (Fowler *et al.* 2001). Our results indicate that cool temperatures, but not reduced photoperiods, influence cold acclimation of *P. hybrida* 'Mitchell' (Fig. 2.2). Plants grown under long day conditions (NONLD) showed similar basal freezing tolerance levels as those exposed to a reduced photoperiod without a reduction in temperature (NONSD), indicating that in the absence of cooling, short days alone are insufficient to induce cold acclimation. *Petunia hybrida* 'Mitchell' did cold acclimate when exposed to 3 °C for one week along with a reduction in photoperiod from 16 h of light to 9 h (CA1), increasing in freezing tolerance by ca. 2 °C. However, freezing tolerance did not further increase with the addition of two weeks of exposure to short days prior to cold treatment (CA2) (Fig. 2.2). Also, subjecting plants to gradual cooling had the same affect on cold acclimation whether under long days (Rampdown LD) or short days (Rampdown SD). The EL₅₀ value was ca. -4.9 °C following either treatment.

Duration at 3 °C influenced cold acclimation. One week at 3 °C (CA1) resulted in an acclimated EL₅₀ temperature of -3.9 °C while 3 weeks (CA3) resulted in an acclimated freezing tolerance of -4.9 °C. Similar results were reported for *S. commersonii* where longer exposures to cool temperatures (2 °C) result in increased acclimation. In *S. commersonii*, freezing tolerance gradually increases with duration of exposure until a maximum is reached after 7 days (Pino *et al.* 2008). Likewise, in *H. vulgare* cv Dicktoo maximum freezing tolerance is not reached until 56 days of acclimation at 4 °C under short days (Fowler *et al.* 2001). The mechanisms involved in cold acclimation of petunia are not fully understood and it would be of interest to determine the physiological and molecular adaptations occurring during prolonged cooling.

One week at 3 °C preceded by a week at 15 °C and a week at 10 °C (RampdownSD and RampdownLD) induced similar freezing tolerance as 3 weeks at 3 °C (CA3). In *A. thaliana*, the level of CBF transcription is dependent upon the magnitude of temperature change, with shifts to lower temperatures causing higher accumulation, but sudden changes as slight as a drop from 20 °C to 10 °C are sufficient to induce CBF transcription. With gradual cooling at a rate of 2 °C h⁻¹, CBF transcripts become detectable in *A. thaliana* at temperatures as high as 14 °C and continued cooling causes even higher levels of transcript accumulation (Zarka *et al.* 2003). The initial weeks at 15 °C and 10 °C during the RampdownSD and RampdownLD regimes appear to be sufficiently cool to induce petunia cold-acclimation pathways, allowing plants to eventually reach the same level of freezing tolerance during the final week at 3 °C as was reached following three weeks at 3 °C (CA3).

We examined the freezing tolerance of several wild *Petunia* species to identify genetic sources of cold tolerance for use in future breeding. All of the tested species showed similar freezing tolerance in the absence of cold acclimation, with an EL₅₀ value near -2 °C (Fig. 2.4). These results are consistent those of Pennycooke *et al.* (2003) who reported an EL₅₀ value of ca. -2.5 °C on nonacclimated *P. hybrida* 'Mitchell' using a slightly different electrolyte leakage assay procedure. The nonacclimated freezing tolerance of *P. hybrida* 'Mitchell' is similar to *S. lycopersicum* with an EL₅₀ temperature of -2 °C (Zhang *et al.* 2004), and *S. tuberosum* with an EL₅₀ of -3 °C (Pino *et al.* 2007). The frost-resistant species *S. commersonii* and *A. thaliana* ecotype RLD have nonacclimated EL₅₀ temperatures of -5 °C and -4 °C, respectively (Pino *et al.* 2008; Jaglo-Ottosen *et al.* 1998). These comparisons suggest that *Petunia* is relatively sensitive to freezing prior to acclimation.

Although all *Petunia* species showed similar nonacclimated freezing tolerance, significant variation was seen following acclimation. The extent of acclimation ranged from only a 3.0 °C increase in freezing tolerance for *P. exserta* to 5.8 °C for *P. axillaris* (accession 28548). It is interesting to note that although *P. axillaris* (accession 28548) was the most cold-tolerant plant that we tested, our other accession of the same species, *P. axillaris* (accession 28546), was not statistically different than *P. exserta* and increased in freezing tolerance by 4.3 °C. These two accessions of *P. axillaris* showed significant morphological differences as well, with accession 28548 having a much longer corolla tube and leaves than accession 28546 (data not shown). Although the exact collection site of these two accessions is unknown, they are likely different ecotypes. Accession 28546 may have come from a warmer environment than accession 28548, and therefore

environmental pressures have resulted in the selection of more cold tolerant individuals in accession 28548.

Three of the species, *P. integrifolia*, *P. hybrida* and *P. axillaris* (accession 28546), had freezing tolerances that are not statistically different following acclimation, with an EL₅₀ near -6 °C. The modern garden petunia, *P. hybrida*, is believed to have arisen from the hybridization of *P. integrifolia* and *P. axillaris*. We also know that the *P. hybrida* ‘Mitchell’ cultivar used in these tests arose from the crossing of *P. axillaris* × (*P. axillaris* × *P. hybrida* ‘Rose du Ciel’) (Griesbach 2007). Therefore, it is not surprising that *P. hybrida* ‘Mitchell’ has cold tolerance characteristics that are so similar to these two putative parental species.

As a point of comparison between petunia and other species, neither *S. lycopersicum* (Zhang *et al.* 2004) nor *S. tuberosum* (Pino *et al.* 2007) increase in freezing tolerance following cold acclimation. On the other hand, freezing-tolerant *S. commersonii* (Pino *et al.* 2008) and *A. thaliana* ecotype RLD (Jaglo-Ottosen *et al.* 1998) both increase in freezing tolerance by ca. 4 °C to reach acclimated EL₅₀ temperatures of -9 °C and -8 °C, respectively. Therefore, we conclude that *Petunia*, and especially *P. axillaris* (accession 28548) with an acclimated EL₅₀ temperature near -8 °C (an increase in tolerance of 5.8 °C over nonacclimated plants), has the ability to cold acclimate by a significant amount for an herbaceous species.

The results of these experiments show that there is significant variation in the freezing tolerance of *Petunia* spp. following a period of cold acclimation. This variation could be utilized when breeding new *Petunia* cultivars by incorporating germplasm from the more cold tolerant wild species, *P. axillaris* (accession 28548), into the breeding

program. Further work is necessary to identify genetic differences controlling this variation in acclimation ability. Cold tolerance is a quantitative trait expected to be controlled by several genetic factors. Identification of QTL underlying cold acclimation ability in *Petunia* would be useful for implementing marker assisted breeding programs aimed at efficiently transferring cold tolerance traits from the wild species to the cultivated plants.

Table 2.1. Growth conditions (temperature, photoperiod, photosynthetic photon flux (PPF) and duration) of *P. hybrida* 'Mitchell' plants prior to electrolyte leakage assays.

Growth regimes	Temperature (°C)	Photoperiod (h)	PPF ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Duration (weeks)
NONLD	22	16	100-130	6
NONSD	22	16	100-130	4
	22	9	100-130	3
CA1	22	16	100-130	6
	3	9	30	1
CA2	22	16	100-130	4
	22	9	100-130	2
	3	9	30	1
CA3	22	16	100-130	5
	3	9	30	3
RampdownSD	22	16	100-130	5
	15	9	100-130	1
	10	9	100-130	1
	3	9	30	1
RampdownLD	22	16	100-130	5
	15	16	100-130	1
	10	16	100-130	1
	3	16	30	1

Table 2.2. ANOVA for effect of acclimation regime on freezing tolerance of *P. hybrida* 'Mitchell' as measured by electrolyte leakage assays.

Dependent variable:		Sum of	Mean	F	Pr > F
EL ₅₀ temperature	Source	DF	Squares	Square	
	Model	6	22.4	3.73	21.3
	Error	7	1.23	0.18	0.0004
	Total	13	23.6		

Table 2.3. Pairwise comparisons with Fisher's LSD between EL₅₀ values of *P. hybrida* 'Mitchell' grown under different acclimation regimes. Shown are p-values for each comparison.

	NONLD	NONSD	CA1	CA2	CA3	RampdownSD
NONSD	0.810					
CA1	0.002	0.003				
CA2	0.002	0.002	0.721			
CA3	0.000	0.000	0.036	0.062		
RampdownSD	0.000	0.000	0.035	0.060	0.978	
RampdownLD	0.000	0.000	0.022	0.038	0.744	0.765

Table 2.4. ANOVA for effect of species on nonacclimated (A) and acclimated (B) EL₅₀ temperature as determined by electrolyte leakage assay.

A. Dependent variable:						
EL ₅₀ temperature	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	4	0.12	0.03	0.07	0.99
	Error	10	4.47	0.45		
	Total	14	4.59			
B. Dependent variable:						
EL ₅₀ temperature	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	4	15.6	3.89	9.63	0.002
	Error	10	4.04	0.40		
	Total	14	19.6			

Table 2.5. Pairwise comparisons with Fisher's LSD between acclimated EL₅₀ values of different *Petunia* species. Shown are p-values for each comparison.

	<i>P. axillaris</i> (28546)	<i>P. axillaris</i> (28548)	<i>P. exserta</i>	<i>P. integrifolia</i>
<i>P. axillaris</i> (28548)	0.009			
<i>P. exserta</i>	0.080	0.000		
<i>P. integrifolia</i>	0.439	0.044	0.018	
<i>P. hybrida</i> 'Mitchell'	0.967	0.003	0.046	0.353

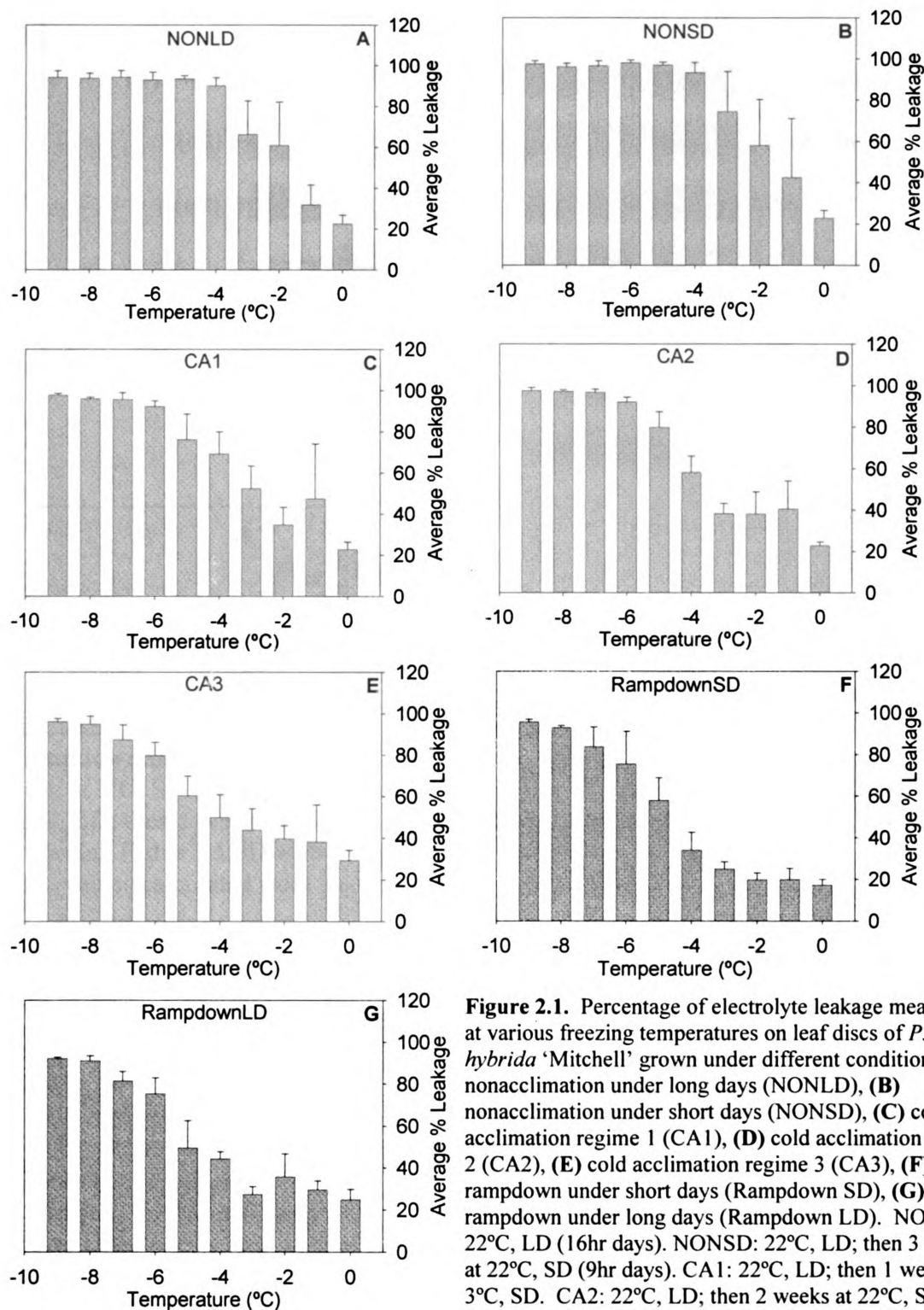


Figure 2.1. Percentage of electrolyte leakage measured at various freezing temperatures on leaf discs of *P. hybrida* 'Mitchell' grown under different conditions. (A) nonacclimation under long days (NONLD), (B) nonacclimation under short days (NONSD), (C) cold acclimation regime 1 (CA1), (D) cold acclimation regime 2 (CA2), (E) cold acclimation regime 3 (CA3), (F) rampdown under short days (RampdownSD), (G) rampdown under long days (RampdownLD). NONLD: 22°C, LD (16hr days). NONSD: 22°C, LD; then 3 weeks at 22°C, SD (9hr days). CA1: 22°C, LD; then 1 week at 3°C, SD. CA2: 22°C, LD; then 2 weeks at 22°C, SD; then 1 week at 3°C, SD. CA3: 22°C, LD; then 3 weeks at 3°C, SD. RampdownSD: 22°C, LD; then 1 week at 15°C, SD; then 1 week at 10°C, SD; then 1 week at 3°C, SD. RampdownLD: 22°C, LD; then 1 week at 15°C, LD; then 1 week at 10°C, LD; then 1 week at 3°C, LD. Error bars represent standard deviation of six measurements.

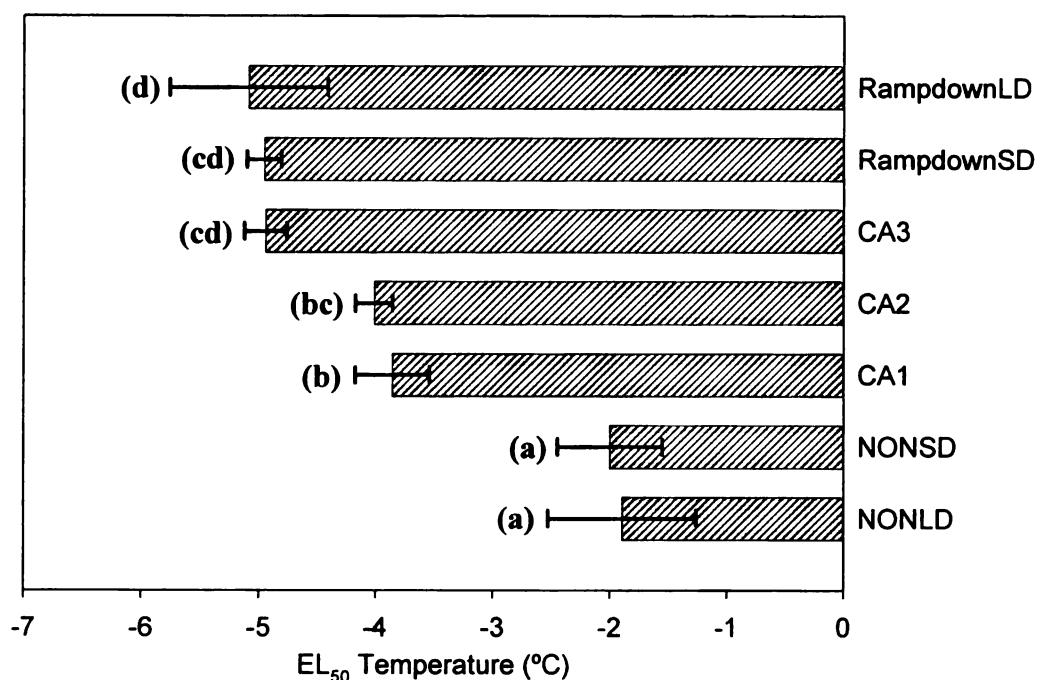


Figure 2.2. EL₅₀ values for *P. hybrida* 'Mitchell' after various cold acclimation regimes. **NONLD:** 22°C, LD (16hr long days). **NONSD:** 22°C, LD; then 3 weeks at 22°C, SD (9hr short days). **CA1:** 22°C, LD; then 1 week at 3°C, SD. **CA2:** 22°C, LD; then 2 weeks at 22°C, SD; then 1 week at 3°C, SD. **CA3:** 22°C, LD; then 3 weeks at 3°C, SD. **RampdownSD:** 22°C, LD; then 1 week at 15°C, SD; then 1 week at 10°C, SD; then 1 week at 3°C, SD. **RampdownLD:** 22°C, LD; then 1 week at 15°C, LD; then 1 week at 10°C, LD; then 1 week at 3°C, LD. Bars with the same letter are not statistically different according Fisher's LSD with $\alpha = 0.05$. Error bars represent standard deviation of the two EL₅₀ values calculated from two replications for each treatment.

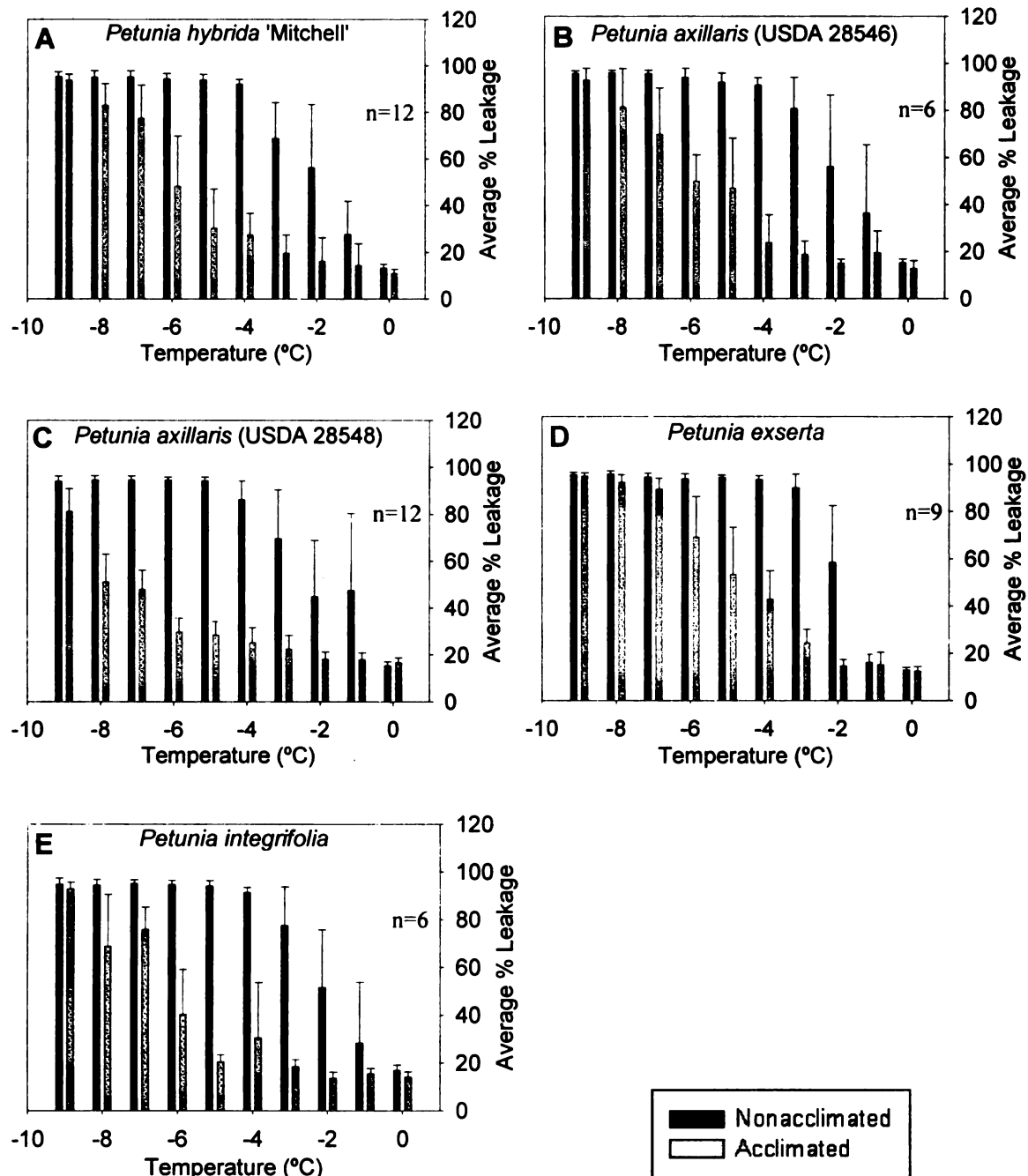


Figure 2.3. Average percent electrolyte leakage at each temperature tested for *Petunia hybrida* (A) and wild *Petunia* species (B-E). Nonacclimated plants were grown at 22°C LD and acclimation was accomplished by exposing plants to 15°C SD for 1 week, 10°C SD for 1 week, then 3°C SD for 1 week. Leakage data at each temperature point is averaged over *n* measurements. Standard deviation shown by error bars.

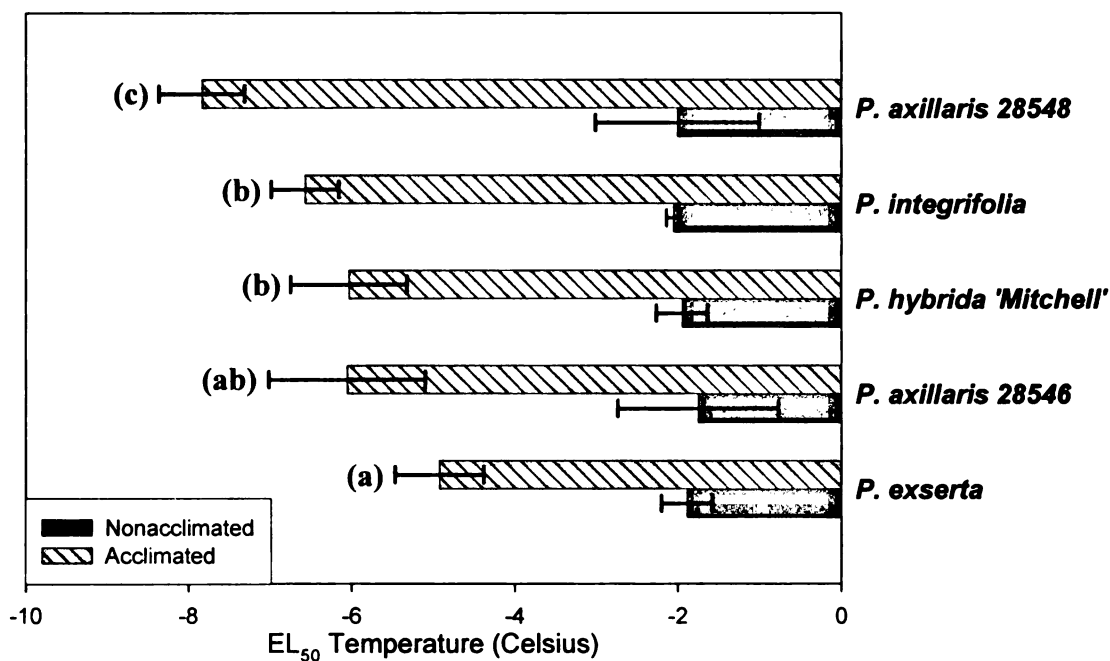


Figure 2.4. EL₅₀ temperatures for nonacclimated (7 weeks at 22°C LD) and acclimated (6 weeks at 22°C LD, 1 week at 15°C SD, 1 week at 10°C SD, and 1 week at 3°C SD) wild *Petunia* species. Bars depict EL₅₀ temperatures averaged over at least 2 replications for each species. Bars with the same letter are not statistically different according Fisher's LSD with $\alpha = 0.05$. Standard deviation shown by error bars.

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CHAPTER 3

EVIDENCE FOR CONSERVATION OF THE CBF COLD-RESPONSE PATHWAY AND ITS ROLE IN COLD ACCLIMATION OF *PETUNIA HYBRIDA* 'MITCHELL'

ABSTRACT

Freezing tolerance of many plant species increases following exposure to low, non-freezing temperatures, a process termed cold acclimation. In the model plant species *Arabidopsis thaliana*, the CBF family of transcriptional activators plays an important role in cold acclimation. The CBF-regulon is conserved in a number of species including members of the family Solanaceae. The objectives of this work were to determine whether heterologous expression of CBF genes improves freezing tolerance of *Petunia hybrida* and whether a functional CBF regulon is present in petunia. *Petunia hybrida* 'Mitchell' plants were transformed with either *AtCBF3* from *Arabidopsis* or *LeCBF1* from *Solanum lycopersicum*, both under the strong constitutive *CaMV 35S* promoter. A single transgenic line with high expression levels of *AtCBF3* showed an increase in constitutive freezing tolerance, while acclimated tolerance was similar to wild-type. None of the *LeCBF1*-expressing lines showed any significant improvements in constitutive or acclimated freezing tolerance. Expression of putative petunia CBF transcription factors (*petCBF1-4*) was induced in response to chilling at 3 °C, and two putative downstream genes of the petunia CBF pathway exhibiting increased expression in response to both cold treatment and CBF over-expression were identified.

INTRODUCTION

Freezing temperatures can cause tremendous damage to plant cells, primarily to the cellular membranes (Thomashow 1999). Plants vary greatly in their ability to tolerate cold temperatures and are categorized as chilling sensitive, freezing sensitive, or freezing tolerant. Chilling sensitive species suffer damage when exposed to cool, nonfreezing temperatures. Freezing sensitive species suffer damage when temperatures drop below freezing. Freezing tolerant species are able to tolerate temperatures below 0 °C.

Cold tolerance is a dynamic characteristic controlled by genetic and environmental factors. When exposed to low non-freezing temperatures, many plant species undergo a process termed cold acclimation which allows them to transiently survive previously lethal low temperatures. During the process of cold acclimation, changes occur in gene expression (Guy *et al.* 1985) and the cellular metabolite profile (Cook *et al.* 2004). Some of the genes that are upregulated during cold acclimation serve to stabilize cellular membranes (Steponkus *et al.* 1998). Other cold-regulated genes encode antifreeze proteins, signal transduction proteins, and transcription factors (Maruyama *et al.* 2004, Vogel *et al.* 2005), or act to increase concentrations of compatible solutes such as proline and sugars in the cytoplasm (Gilmour *et al.* 2000).

In *Arabidopsis thaliana* (L.) Heynh., the cold-induced transcriptional activator *CBF1* (C-repeat/Dehydration responsive element binding factor 1) is largely responsible for changes that occur during cold acclimation (Fowler and Thomashow 2002). *CBF1* belongs to a small family of transcriptional activators in *Arabidopsis* consisting of two other apparently functionally redundant genes, *CBF2* and *CBF3* (Gilmour *et al.* 1998, 2004) located in tandem array on chromosome IV. The CBF proteins bind to CRT/DRE

(C-repeat/Dehydration responsive element) sequences within the promoter regions of cold regulated genes (Stockinger *et al.* 1997; Gilmour *et al.* 1998). CBF1, CBF2, and CBF3 proteins all have a mass of 24kDa, and are 88% identical at the amino acid level. CBF transcripts begin to accumulate within 15 minutes of exposure to 2.5 °C. Transcript levels continue to increase for two hours and then begin to slowly decline (Gilmour *et al.* 1998).

A robust set of low temperature-responsive genes, termed the COS (COld Standard) set, that are reliably up- or down-regulated in response to low temperature, have been identified in *Arabidopsis* (Vogel *et al.* 2005). Of the 513 genes comprising the COS set, 93 are under CBF control (Fowler and Thomashow 2002). Among the COS genes are members of the COR (COld-Responsive) family of genes that are induced in *Arabidopsis* in response to cold temperatures (Baker *et al.* 1994; Thomashow 1999). *COR15a* is involved in the stabilization of membranes during cold temperatures (Steponkus *et al.* 1998; Thomashow 1999) and transcripts for *COR15a* along with *COR78* begin to accumulate after two hours of cold exposure (Gilmour *et al.* 1998).

Constitutive expression of *CBF1* in *Arabidopsis* also induces COR gene expression without a low temperature exposure. This increase in COR expression results in an increase in the freezing tolerance of nonacclimated plants (Jaglo-Ottosen *et al.* 1998). Overexpression of *CBF3* in transgenic *Arabidopsis* results in plants that are more salinity, drought, and cold tolerant compared to wild-type plants (Kasuga *et al.* 1999). However, there are often undesirable phenotypic effects from placing CBF genes under the control of the constitutive *CaMV 35S* promoter, including stunted growth, a more prostrate growth form, shorter petioles, a bluish tint, and delayed flowering (Kasuga *et al.*

1999; Gilmour *et al.* 2000). When the *CBF3* gene was expressed under the control of the stress inducible *rd29A* (*Cor78*) promoter, plants exhibit some stunting but not to the extent present in the *CaMV 35S* plants. Transgenic plants in which the inducible promoter is used are still more cold tolerant than control plants following cold acclimation (Kasuga *et al.* 1999).

Functional CBF genes have been identified in numerous species. A CBF ortholog isolated from *Zea mays* L. (*ZmDREB1A*) induces expression of CBF target genes and increases the freezing tolerance of nonacclimated *Arabidopsis* when expressed behind the constitutive *CaMV 35S* promoter (Qin *et al.* 2004). Constitutive expression of the CBF ortholog *CIG-B* from *Prunus avium* L. induces expression of *COR15a* when expressed in *Arabidopsis*. These transgenic plants are more cold and salt tolerant than wild type plants (Kitashiba *et al.* 2004). The freezing tolerance of several crop species, including *Brassica napus* L. (Jaglo *et al.* 2001), *Fragaria ×ananassa* Duchesne (Owens *et al.* 2002) and *Solanum tuberosum* (Pino *et al.* 2007; Pino *et al.* 2008), has been enhanced by heterologous expression of *Arabidopsis* CBF genes.

The Solanaceae family comprises members that span the spectrum of cold tolerance (Chen and Li 1980). Tomato (*S. lycopersicum* L.) is a chilling sensitive member of the family that is incapable of cold acclimation (Hsieh *et al.* 2002a). Potato (*S. tuberosum* L.) is a freezing sensitive species lacking the ability to cold acclimate (Chen and Li 1980; Pino *et al.* 2007). A wild potato relative, *S. commersonii* Dun., is a frost resistant species that does cold acclimate (Chen and Li 1980). Garden petunias (*Petunia hybrida* Vilm.) are a moderately cold tolerant species with the capacity to cold acclimate (Yelenosky and Guy 1989; Pennycooke *et al.* 2003).

Tomato encodes three CBF homologs (*LeCBF1-3*) (Zhang *et al.* 2004). All three *LeCBF* genes are induced by mechanical agitation but not by ABA, salinity, or drought. *LeCBF1* is the only one of the three induced by low temperatures (Zhang *et al.* 2004). While CBF genes are present in tomato, plants do not cold acclimate. Freezing tolerance does not increase when *LeCBF1* or *AtCBF1* is overexpressed in tomato (Zhang *et al.* 2004), but chilling and drought tolerance increases with *AtCBF1* overexpression (Hsieh *et al.* 2002a,b). Overexpression of *LeCBF1* in *Arabidopsis* leads to COR gene induction and increased freezing tolerance. However, in tomato, only four genes are substantially induced by overexpression of either CBF gene (Zhang *et al.* 2004). Therefore, tomato does encode a CBF regulon, but it is insufficient for cold acclimation.

Both freezing-sensitive *S. tuberosum* and freezing-tolerant *S. commersonii* are responsive to heterologous expression of *Arabidopsis* CBF genes (Pino *et al.* 2007; Pino *et al.* 2008). Constitutive expression of *AtCBF1* or *AtCBF3* in potato increases freezing tolerance from -3 °C to -5 °C without a cold treatment. Additionally, expression of these genes behind a cold-inducible *rd29A* promoter increases the freezing tolerance to -5°C following a two-week treatment at 2 °C (Pino *et al.* 2007). When *AtCBF3* is constitutively expressed in *S. commersonii*, freezing tolerance of warm grown plants increases by 2 to 4 °C. Cold acclimation of these transgenic lines results in further increases in freezing tolerance by anywhere from 1 to 4 °C (Pino *et al.* 2008).

Together, these studies show that the CBF pathway is highly conserved in the family Solanaceae, yet cold tolerance varies widely. Although endogenous CBF transcription factors are present in tomato, the downstream components required for cold acclimation seem to be lacking. In potato, CBF overexpression enhances freezing

tolerance, indicating that downstream components of the regulon are present and functional. In *S. commersonii*, CBF overexpression enhances freezing tolerance, but the plants are able to acclimate further following cold treatment, suggesting that other pathways independent of CBF are also responsible for the cold tolerance of this species. The conservation of the CBF regulon and the role it may play in the cold tolerance of *P. hybrida* is not yet known. While the ability to cold acclimate demonstrates that the cellular machinery for acclimation exists in petunia, the genetics controlling this ability remain to be determined.

The objective of the present study was to determine whether *Petunia hybrida* has a functioning CBF cold-response pathway. This was accomplished by determining whether heterologous expression of CBF genes from *Arabidopsis* or *S. lycopersicum* confers an increase in freezing tolerance when expressed behind a constitutive *CaMV 35S* or a cold-inducible *AtCor15a* promoter in petunia.

MATERIALS AND METHODS

Plant transformation and characterization

Transformation of *Petunia hybrida* ‘Mitchell’

Genetic transformation of *Petunia hybrida* ‘Mitchell’ was carried out by the Plant Biotechnology Resource and Outreach Center at Michigan State University.

Agrobacterium-mediated transformation on leaf explants was used to integrate one of four CBF-containing constructs into petunia. Empty vector and non-transformed explants were also carried through tissue culture as negative controls. See Figure 3.1 for an explanation of constructs used in transformation. The pMPS13 (Gilmour *et al.* 2000)

and pXIN1 (Zhang *et al.* 2004) constructs were kindly provided by Dr. Mike Thomashow (Michigan State University) and the pSPUD constructs were kindly provided by Dr. Dave Douches (Michigan State University). Two separate rounds of transformation were performed.

PCR of putative transgenic plants for transgene integration

DNA from all putative T₀ generation transgenic plants was collected onto FTA PlantSaver Cards (Whatman, United Kingdom) for use in PCR. A young expanding leaf was detached from the plant and placed with the abaxial surface facing the card. A small piece of plastic film was placed over the sample while it was pressed onto the card with a pestle. The cards were air-dried overnight before use in a PCR reaction. For each reaction, a 1 mm diameter punch was removed from the FTA card and placed into a thin-walled PCR tube. The disc was washed twice for 5 min with 200 µl FTA Purification Reagent (Whatman, United Kingdom) followed by two washes with 200 µl TE_{0.1} (10 mM tris-hydrochloride (Tris), 0.1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0). Discs were dried for 20 min at 56 °C and cooled to room temperature prior to the addition of a PCR master mix (1X Taq buffer, 0.2 mM dNTPs, 1 µM forward primer, 1 µM reverse primer, 2.5 mM magnesium chloride (MgCl₂), 1 unit GoTaq Flexi DNA polymerase (Promega, Madison WI, USA)) in 50 µl reactions. Twenty microliters of the reaction product was separated on a 1% agarose gel for visualization. Table 3.1 lists primer sequences and PCR reaction conditions.

Southern hybridization analysis

Southern hybridization was performed to determine transgene copy number and to confirm PCR results for presence of the transgenic construct. DNA was obtained using a

cetyl trimethylammonium bromide (CTAB) extraction method. Fresh (~2 g) or freeze dried (~300 mg) plant tissue was ground in the presence of ~10 mg of polyvinyl pyrrolidone (PVP mw 40,000) and transferred to a 15 ml conical tube with 5 ml CTAB extraction buffer (2% CTAB, 100 mM Tris pH 8.0, 1.4 M (sodium chloride) NaCl, 20 mM EDTA pH 8.0, 1% β -mercaptoethanol (BME)). Samples were incubated at 65 °C for 1 h with intermittent agitation. Samples were then extracted twice with an equal volume chloroform:iso-amyl alcohol (24:1) and precipitated with 1/10 volume 3 M sodium acetate and either 2 volumes ethanol or 1 volume isopropanol at -20 °C. DNA was pelleted, washed once with 70% ethanol, dried, resuspended in 500 μ l TE and incubated with 5 μ l DNase-free RNase (Roche Applied Science, Germany) for 1 h at 37 °C. DNA was again precipitated with 1/10 volume 3 M sodium acetate and 2 volumes ethanol at -20 °C. DNA was pelleted, washed twice with 70% ethanol, dried and resuspended in sterile deionized water. Quantification was carried out using a BioSpec-mini UV-Visible spectrophotometer (Shimadzu Corporation, Japan).

Total DNA of all transgenic lines was digested with either PstI or EcoRI (New England Biolabs, Beverly MA, USA) restriction enzymes according to manufacturer's recommendations. A second blot of most transgenic lines was performed using a double digest with EcoRI (New England Biolabs, Beverly MA, USA) and HindIII (Invitrogen Corp., Carlsbad CA, USA) restriction enzymes to verify the results. Digested DNA (10 to 15 μ g) was separated on a 1% agarose gel at 70 volts for 4 h. DNA was transferred to a Hybond N+ (GE Healthcare, Buckinghamshire, United Kingdom) membrane by overnight capillary transfer with 10X SSC (1.5 M NaCl, 0.15 M sodium citrate). DNA was affixed to the membrane by UV crosslinking (Stratalinker).

The membrane was prehybridized at 45 °C for 2 h in 15 ml prehybridization solution made using DIG Easy Hyb Granules (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche Applied Science, Germany). A DIG-labeled 364 bp fragment of the *NPTII* gene was created using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. Primers used for probe synthesis were DIGNPTII For: 5'-TGCTCCTGCCGAGGAACTAT-3' and DIGNPTII Rev: 5'-AATATCACGGGTAGCCAACG-3'. PCR reaction conditions consisted of 40 cycles of 94 °C, 30 s; 57 °C, 1 min; 72 °C, 2.5 min; plus a final extension of 72 °C, 10 min using 10 pg of pSPUD74 plasmid as template.

Twelve microliters of the labeled probe was mixed with 6 ml DIG Easy Hyb solution and hybridized to the membrane at 45 °C overnight. Detection of the probe was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) according to the manufacturer's instructions using a CDP-Star substrate and was visualized by autoradiography.

Gene expression analysis by RT-PCR

Transgenic and wild-type control plants were the same as those used for electrolyte leakage analysis. RNA from lines containing constitutive promoters was collected from plants growing at 22 °C. RNA from lines with cold-inducible promoters was harvested from warm grown plants and following various exposures to 3 °C (15 min, 2 h, 24 h and following the ramp-down acclimation regime). Approximately 100 mg of plant tissue from upper leaves of the same size as those sampled for electrolyte leakage assay were harvested directly into liquid nitrogen and ground to a fine powder. Total RNA was extracted using RNeasy Plant Mini Kits (Qiagen Inc., Valencia CA, USA) and

genomic DNA was removed by on-column DNase treatment with a RNase-free DNase set (Qiagen Inc.). Two micrograms of total RNA were reverse transcribed using Superscript II (Invitrogen Corp., Carlsbad CA, USA) with oligo d(T)₁₂₋₁₈ primers. PCR reactions were carried out using 2 µl of the cDNA product as template and gene specific primers for *AtCBF1*, *AtCBF3* and *LeCBF1*. Actin primers designed for the *Tom51* gene (GenBank accession no. U60481) from tomato were used as a loading control (Jones *et al.* 2005). Table 3.2 lists reaction conditions and primer sequences. Control reactions were carried out using only the extracted RNA as template to verify that genomic DNA contamination was not present following extraction.

Assay of detached leaf freezing tolerance

Plant growth of transgenic *Petunia hybrida* lines

Seeds from transgenic lines (Table 3.3), empty vector control, and wild type were surface sterilized by soaking for 10 to 15 min in a 50% bleach, 0.1% Triton-X-100 solution with gentle agitation. Seeds were rinsed with sterile deionized water 3 to 4 times to remove all traces of bleach and then suspended in a 0.1% sterile agar solution to facilitate pipetting. Seeds from transgenic lines were pipetted onto 100 x 15 mm disposable Petri plates containing Gamborg's B5 medium (minus sucrose, kinetin and 2,4-D; plus 0.7% agar) supplemented with 100 µg/ml kanamycin. Seeds from wild-type control plants were pipetted onto identical plates without the addition of kanamycin. Seeds were plated at a density of ~60 seeds per plate. Plates were placed in a 22 °C, 16 h photoperiod (100-130 µmol m⁻² s⁻¹ light) chamber for three weeks. After three weeks, kanamycin resistant transgenic plants and wild type control plants were transplanted to 50-cell trays containing 70% peat moss, 21% perlite, 9% vermiculite (Suremix, Michigan

Grower Products Inc., Galesburg MI, USA). Trays were placed in a 22 °C, 16 h photoperiod ($100\text{-}130\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ light) chamber and covered with humidity domes for several days. Three weeks after transplant, plants for each line were divided into two groups; a non-acclimated group remaining at 22 °C for an additional week before testing, and an acclimated group subjected to a gradual cooling process. The acclimated group was moved to 15 °C, 9 h photoperiod ($100\text{-}130\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ light) for 1 week followed by 10 °C, 9 h photoperiod ($100\text{-}130\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ light) for 1 week, and finally moved to 3 °C, 9 h photoperiod ($30\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ light) for an additional week. At the time of testing, non-acclimated plants were 7 weeks old and acclimated plants were 9 weeks old. Plants in both groups were approximately the same size and had similar node number at the time of sampling.

Electrolyte Leakage Assay

Leaf discs from the upper portion of the plant were harvested using a 0.6 cm cork borer. The youngest leaves that could be punched to obtain complete 0.6 cm diameter discs without cutting into the midrib were chosen for sampling. Discs were immediately placed in deionized water and stirred gently. Approximately 120 punches were taken from a population of 50 plants for each transgenic line. Three discs were then transferred to each of 30 (16 x 100 mm) borosilicate culture tubes and placed on ice. When all discs had been transferred, tubes were placed in a -1 °C controlled temperature antifreeze bath (master bath) for 60 min. Three tubes of each line were left on ice as controls. After 60 min, a small amount of ice was added to each tube to nucleate extracellular ice formation. Tubes were then plugged with foam and kept at -1 °C for an additional 60 min. After 60 min, three tubes of each line were moved to a second antifreeze bath at -1 °C, kept there

for 40 min, and removed to ice. Meanwhile, the temperature of the master bath was lowered to -2 °C. After 20 min, three tubes of each line were moved from the master bath to another antifreeze bath set at -2 °C for 40 min and then removed to ice (a total of 60 min at the test temperature). This process continued for all temperatures tested (generally -1 °C to -9 °C; except -1 °C to -14 °C for acclimated pMPS13-7 lines). Tubes were then placed in racks on top of ice and kept at 2.5 °C to thaw slowly overnight.

The following day, 6 ml of deionized water were added to each tube followed by shaking for three hours at room temperature to allow released electrolytes to dissolve. The water was then transferred to a new culture tube and electrical conductivity (L_1) was measured using a CON110 conductivity meter (Oakton Instruments, Vernon Hills IL, USA). Plant discs remained in the original tube and were frozen to -80°C overnight to release all electrolytes. The next day, the water from the previously measured tubes was poured back into the corresponding plant disc tube, followed by shaking for three hours at room temperature. Conductivity of the water was measured again to obtain the reading for total electrolytes leakage (L_2). Percent of total electrolyte leakage at each test temperature was calculated by $(L_1 \div L_2) * 100$. Data analysis was carried out using Sigmaplot (SPSS Inc., USA) and SAS (SAS Institute Inc., USA) software. A sigmoidal curve was fitted to the leakage data for each species according to the equation:

$y = a1 + (a2 \div (1 + \exp(a3 - a4 * T)))$, where y is the average percent electrolyte leakage of the three tubes at each temperature T , using the curve fitting function of Sigmaplot. The initial parameters were specified as $a1=0.1$, $a2=99.9$, $a3=0.1$ and $a4=0.1$ with constraints imposed such that $a1>0$ and $0<a2<100$. The temperature at which 50% of total electrolytes were released (EL_{50}) was calculated by solving the equation $EL_{50} =$

$((a_3 - \log((a_2 \div (50 - a_1)) - 1)) \div a_4)$ where the parameters a_1 , a_2 , a_3 and a_4 are given by the equation of the fitted curve. A total of at least two EL_{50} values were calculated for each transgenic line from two separate replications of the experiment. ANOVA analysis and mean separation using Fisher's LSD ($\alpha=0.05$) were conducted on the EL_{50} values using PROC GLM in SAS.

Characterization of endogenous CBF pathway

Expression of putative endogenous petCBF transcription factors

RT-PCR was used to examine expression of putative endogenous CBF genes in *Petunia hybrida* 'Mitchell.' Four putative petCBF transcription factors have been identified in *P. hybrida* 'V26' by Goldman *et al.* (2007) whose expression is induced in response to cold (*petCBF1-4*) as well as drought and salinity (*petCBF4* only). Sequences from these genes were used to design primers to examine expression patterns in *P. hybrida* 'Mitchell'. Seeds of *P. hybrida* 'Mitchell' were surface planted onto 288-cell plug trays filled with 70% peat moss, 21% perlite, 9% vermiculite (Suremix, Michigan Grower Products Inc., Galesburg MI, USA) and placed under intermittent mist irrigation until at least two true leaves had developed. Plants were then transferred to a greenhouse at 20 °C with a 16 h photoperiod until plugs were pullable (root ball holds together upon removal from plug tray). Seedlings were transplanted to 5.7 x 5.7 x 7.6 cm pots and grown at 20 °C, 16 h photoperiod for 4 weeks. Plants were then transplanted to 15.2 cm pots and moved to a controlled environment growth chamber at 22 °C, 16 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) for 1 week prior to the beginning of cold treatments. RNA was extracted and reverse transcribed as described above from plants grown at 22 °C and from plants subjected to chilling at 3 °C for various periods of time (15 min, 2 h, or 24 h).

Two micrograms of the cDNA product was used as template in subsequent PCR analysis. Primer sequences and reaction conditions are listed in Table 3.4.

This experiment was repeated using 7 week-old plants. Seeds from the WT3-2 line (a wild type *P. hybrida* 'Mitchell' control line taken through tissue culture), pSPUD74-24B-5, and pSPUD74-24B-13 were grown as described above for detached leaf freezing tolerance assays. Seven weeks post-planting, plants were subjected to the same chilling procedure and RT-PCR was performed as described for the first replication.

Downstream components of the CBF pathway

RT-PCR was performed to assess the impact of CBF overexpression on putative downstream components of the CBF pathway in *Petunia*. A BLAST search was conducted against the TIGR Petunia Gene Index (<http://compbio.dfci.harvard.edu/tgi/>) using sequences for known CBF-responsive genes from other Solanaceous plants. Using the lowest expect values (likelihood of finding such an alignment by chance alone) as criteria, four tentative consensus sequences were selected for further investigation (Table 3.5). These sequences were used to develop gene specific primers (Table 3.6) for semi-quantitative RT-PCR reactions aimed at determining the cold-responsiveness and CBF-responsiveness of these sequences. Initial reactions were carried out on cDNA template derived from warm grown (22 °C) and cold treated (3 °C for 15 min, 2 h, and 24 h) *P. hybrida* 'Mitchell'. If it appeared that a particular sequence was upregulated in response to cold treatment, then additional reactions were carried out using cDNA template from nonacclimated constitutive CBF-expressing transgenic lines. All reactions were normalized by running control reactions with actin primers designed to the tomato *Tom51* gene (Jones *et al.* 2005).

Identification of putative endogenous CBF transcription factors in *Petunia* spp.

Southern hybridization was performed to determine whether endogenous CBF transcription factors may be present in selected *Petunia* species. DNA from *P. hybrida* 'Mitchell', *P. axillaris* (USDA 28546 and USDA 28548), *P. exserta*, and *P. integrifolia* was extracted using a CTAB extraction method as previously described. Total DNA was digested with either PstI or EcoRI (New England Biolabs, Beverly MA, USA) restriction enzymes at 37 °C according to manufacturer's instructions. Digested DNA (15 µg) was separated and affixed to a nylon membrane as previously described.

Nucleotide sequences for known CBF genes from Solanaceous species including *LeCBF1* from *L. esculentum* (Genbank accession no. AY497899), the CBF3-CBF1-CBF2 clusters from *S. tuberosum* (Genbank EU365384) and *S. commersonii* (Genbank accession no. EU365383) and *petCBF1-4* from *P. hybrida* 'V26' (Goldman *et al.* 2007) were aligned using the AlignX function of Vector NTI Advance 10 (Invitrogen Corp., Carlsbad CA, USA). A region of high conservation was selected including 65 nucleotides encoding 37% of the AP2 domain, 15 nucleotides encoding the DSAWR signature sequence plus an additional 83 nucleotides of the sequence flanking the 3' end. Primers were designed to be specific to the *petCBF1* sequence for this region. Primer sequences are: Forward-5' CGGCTAGAGCTCATGACGTG 3'; Reverse-5' GACTCCAAAGGCCTAAAAGC 3'. An initial PCR reaction using *P. hybrida* 'Mitchell' genomic DNA as template was carried out with these primers and conditions consisting of 40 cycles of 94 °C, 30 s; 57 °C, 1 min; 72 °C, 2.5 min; plus a final extension of 72 °C, 10min. This PCR product was separated on a 1% agarose gel and extracted using a Gel Extraction kit (Qiagen Inc., Valencia CA, USA) Using 30 ng of gel purified

PCR product as template, a DIG-labeled probe was created using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Germany) according to the manufacturer's instructions and using the same reaction conditions as above. Southern hybridization was performed as previously described.

RESULTS

Development of *LeCBF1* constitutive expression lines

Three unique single-insertion events of the *35S::LeCBF1* construct were recovered from tissue culture and taken to the T₁ generation. Progeny testing of T₂ seed for segregation on 100 µg/ml kanamycin plates found homozygous T₁ individuals (pXIN1-17B-20 and BpXIN1-120-2) representing two of the events. No homozygous T₁ plants were found for the BpXIN1-110 line, so seed from a hemizygous T₁ individual (BpXIN1-110-5) was used for subsequent tests. In order to get more lines for testing, seed from a double-insertion T₁ plant (pXIN1-25B-12) was also included in subsequent tests.

Development of *AtCBF3* constitutively expressing lines

One single-insertion event of the *35S::AtCBF3* construct (pMPS13-7) was recovered from tissue culture and taken to the T₁ generation. Progeny testing of T₁ individuals for segregation on 100 µl/ml kanamycin plates failed to find any homozygous plants. Therefore, a homogenous population was created by crossing a hemizygous T₁ individual (pMPS13-7-12) to a wild type parent. In subsequent tests, growth of the seeds from this cross on 100 µg/ml kanamycin plates resulted in a population consisting of only hemizygous individuals. In order to have more lines for testing, T₂ seed from a triple-

insertion T₁ individual (pMPS13-10-8) and T₁ seed from a double-insertion T₀ plant (BpMPS13-101) was also included. Growth of all seeds on kanamycin assured that the transgenic construct was present in all individuals used for testing.

Development of cold inducible *AtCBF1* lines

pSPUD74

No single insertions and only one unique double-insertion of the *AtCor15a::AtCBF1* construct were recovered from tissue culture. Southern analysis in the T₁ generation revealed one individual (pSPUD74-24B-13) in which a single insertion had segregated away. Progeny testing of T₂ seed showed that this plant was hemizygous for the insertion. T₂ seed from pSPUD74-24B-13 as well as T₂ seed from a double insertion T₁ (pSPUD74-24B-5) was used in subsequent testing.

pSPUD73

Only two transgenic plants containing the *AtCor78::AtCBF1* construct were recovered from tissue culture and each line had three insertions. Neither the T₁ nor T₂ seed from either of these lines showed any kanamycin resistance even at concentrations as low as 25mg/L, making them very difficult to work with. Therefore, no plants from this construct were used in later experiments.

CBF expression in transgenic lines used in experiments

Transgene expression level varied among the recovered constitutive expression lines (Fig. 3.2). In the hemizygous pMPS13-7-12 line, expression is higher compared to all of the other *AtCBF3* over-expressing lines. Expression in the pMPS13-10-8 and BpMPS13-101 lines is much lower although there is clear evidence of expression in these lines. Of the *LeCBF1* over-expressing lines, pXIN1-17B-20 has the highest expression

level, but the other lines also appear to be transcribing the transgene at moderate levels. Expression of *AtCBF1* was not detected in either of the transgenic lines containing *AtCBF1* under control of the cold-inducible *Cor15a* promoter with or without cold exposure (Fig. 3.3).

Basal freezing tolerance of one line is increased by constitutive expression of *AtCBF3*

Basal freezing tolerance was increased in one *AtCBF3* over-expressing line, pMPS13-7, to a level similar to acclimated wild type plants (Fig. 3.4 and 3.5). The nonacclimated EL₅₀ temperature for the pMPS13-7 line was -4.3°C, while wild type plants had a basal EL₅₀ = -1.7°C (Fig. 3.5), a significant increase compared to nonacclimated wild type plants (Table 3.7A and 3.8), and not significantly different from the EL₅₀ temperature for acclimated wild type plants of -5.8°C. The basal freezing tolerance of pMPS13-10 and BpMPS13-101 did not differ from wild type. The acclimated EL₅₀ temperature for controls and all pMPS13 transgenic lines was also not statistically different (Table 3.7B).

Constitutive expression of *LeCBF1* does not affect *Petunia* freezing tolerance

Comparison of all *LeCBF1* expressing lines with wild type and empty vector controls indicates that expression of *LeCBF1* did not confer a significant increase in basal freezing tolerance as tested by electrolyte leakage assay (Fig. 3.6 and Fig. 3.7, Table 3.9A). As with the *AtCBF3* expressing lines, the EL₅₀ value of the acclimated plants did not differ from the controls (Table 3.9B).

Petunia appears to encode endogenous CBF transcription factors

Examination of amino acid sequences for four putative *Petunia* CBF transcription factors (*petCBF1-4*) from Goldman *et al.* (2007) reveals that the

PKK/RPAGRxKFxETRHP and DSAWR CBF signature sequences (Jaglo *et al.* 2001) are largely conserved (Fig. 3.8). Expression of these *petCBF* genes was induced in wild type plants in response to cold treatment at 3 °C (Fig. 3.9). Transcripts for *petCBF3* and *petCBF4* began to accumulate after just 15 min, and after 2 h all four *petCBF* genes were expressed at higher than basal levels. The cold induction of *petCBF2* expression appears to be less than that of the other three genes. After 24 h at cold temperatures, the expression of these genes began to taper off but levels of *petCBF1*, 3, and 4 remained elevated compared to basal levels. After 24 h of chilling, the expression of *petCBF2* was similar to basal levels. A similar expression pattern was seen in the pSPUD74 transgenic lines (Fig. 3.9).

Southern hybridization using a probe created from a highly conserved region of the already identified *petCBF1* in *P. hybrida* 'Mitchell' revealed at least three, and potentially more, cross-hybridizing DNA regions within each of the other wild *Petunia* species (Fig. 3.10). This suggests the presence of multiple endogenous CBF transcription factors in each of these species.

Downstream components of the CBF cold-response pathway are conserved in *Petunia*

Among the CBF-responsive genes in *S. tuberosum* and *S. commersonii* is the dehydrin-like gene, DHN10, which is upregulated in response to both cold exposure and CBF over-expression (Pino *et al.* 2008). A homolog, TC116174, is also upregulated in *S. lycopersicum* in response to CBF over-expression (Zhang *et al.* 2004). A putative homolog to DHN10 identified in *Petunia*, TC2800 (hereafter *PhDHN10*), is responsive to cold exposure at 3 °C (Fig. 3.11A) as well as *LeCBF1* and *AtCBF3* over-expression (Fig. 3.11B). In fact, there appears to be a correlation between the level of heterologous CBF

expression and the expression level of *PhDHN10*. The highest *AtCBF3* expressing line, pMPS13-7, shows the highest level of expression of *PhDHN10* in nonacclimated plants (Fig. 3.11B). Likewise, in the *LeCBF1* expressing lines, pXIN1-17B-20 has the highest level of expression of the transgene and has the highest expression of *PhDHN10* in the absence of cold treatment (Fig. 3.11B). Another putative dehydrin-like tentative sequence from petunia, TC2907 (hereafter *PhDHN2*), which is similar to *DHN2* from *S. commersonii* (GenBank accession no. AF386075), shows similar expression patterns as *PhDHN10* (Fig. 3.11). TC1671 was identified as a putative petunia homolog to a CBF-responsive ω 9 stearoyl ACP desaturase from *S. commersonii* (GenBank accession no. X92847). Analysis of this tentative consensus sequence in petunia shows that it is slightly induced in response to cold temperatures (Fig. 3.11A). However, RT-PCR analysis failed to show that this sequence was upregulated in any of the transgenic lines (Fig. 3.11B). A putative homolog for a CBF-responsive proteinase inhibitor, TC3472, was identified in the TIGR petunia database by performing a BLAST search with sequences for known CBF-responsive proteinase inhibitors found in tomato (Zhang *et al.* 2004), potato and *S. commersonii* (Pino *et al.* 2008). Analysis of the expression of this TC sequence in petunia failed to show that it was upregulated in response to chilling temperatures of 3 °C at any of the time points tested (data not shown).

DISCUSSION AND CONCLUSIONS

The CBF cold-response pathway is conserved in a number of species, including members of the Solanaceae family such as *S. lycopersicum* (Zhang *et al.* 2004), *S. tuberosum*, and *S. commersonii* (Pino *et al.* 2007 and 2008). Our experiments show that

a CBF regulon is likely conserved in *Petunia hybrida* as well. At least four, and potentially more, CBF transcription factors appear to be present and are upregulated in response to a cold treatment in *P. hybrida* 'Mitchell' (Fig. 3.9). Even after 15 min at 3 °C, transcription of these endogenous CBF genes begins to increase and remains elevated for at least 2 h (*petCBF2*) or 24 h (*petCBF1*, 3 and 4). This is similar to the expression pattern of CBF in *Arabidopsis* where exposure to temperatures of 2.5 °C induces expression within 15 min, peak expression at 2 h, and elevated expression for at least 24 h (Gilmour *et al.* 1998).

Additional support for the presence of a functional petunia CBF pathway comes from evidence that at least two downstream genes are activated by cold exposure as well as CBF over-expression (Fig. 3.11). The dehydrin genes, DHN2 and DHN10, have been shown to be both cold and CBF-responsive in other Solanaceous species (Pino *et al.* 2008 and Zhang *et al.* 2004), and we have shown that putative petunia dehydrins, *PhDHN2* and *PhDHN10*, are upregulated in response to chilling. These genes are noticeably upregulated after 24 hours of exposure to 3 °C and levels remain elevated even after the three week rampdown acclimation regime (Fig. 3.11A). These genes are also upregulated in non-chilled transgenic lines constitutively expressing *AtCBF3* or *LeCBF1* (Fig. 3.11B). The expression level of these putative downstream genes correlates to the expression level of the heterologous CBF genes in the transgenic lines (Fig. 3.2 and Fig. 3.11B), as transgenic lines with the highest transgene expression also show the highest expression of *PhDHN2* (TC2907) and *PhDHN10* (TC2800).

The pMPS13-7 transgenic line provides further evidence for a functional CBF pathway in petunia. This line constitutively expresses *AtCBF3* at very high levels and

also shows increased basal freezing tolerance. Wild type basal freezing tolerance is near $EL_{50} = -2.0\text{ }^{\circ}\text{C}$, while the pMPS13-7 transgenic line shows basal freezing tolerance of $EL_{50} = -4.3\text{ }^{\circ}\text{C}$. The basal freezing tolerance of pMPS13-7 is not statistically different from the freezing tolerance of fully acclimated wild type plants. Furthermore, the pMPS13-7 plants do not significantly increase in freezing tolerance when exposed to acclimating conditions. Therefore, endogenous acclimation mechanisms are not capable of increasing freezing tolerance beyond the point to which CBF over-expression does. This does not necessarily indicate that the CBF regulon controls all of the acclimation processes in *Petunia*. There may be other pathways present which function in the acclimation process, but it appears that effects of the CBF pathway and any other potential pathways are not additive.

There appears to be a dosage-dependent effect of *AtCBF3* expression level on freezing tolerance in transgenic *P. hybrida*. Two other *AtCBF3* expressing lines (pMPS13-10 and BpMPS13-101) express the transgene, but at levels much lower than pMPS13-7, and these lines show no increase in freezing tolerance. It seems that there is a level of CBF expression that must be reached before increased freezing tolerance is conferred. Unfortunately, there also seems to be a fitness cost to constitutively expressing this transgene at such high levels. The pMPS13-7 line is severely stunted and no homozygous plants reached maturity and set seed. This line is only able to be maintained in the hemizygous state. This severe stunting associated with high levels of CBF expression may hinder recovery of highly-expressing lines from tissue culture. Cells expressing CBF at high levels are probably less likely to produce healthy plantlets and therefore recovery of high expressing lines is difficult.

Negative phenotypic effects of CBF expression, such as stunting and delayed flowering, have previously been reported in *Arabidopsis* (Kasuga *et al.* 1999; Gilmour *et al.* 2000) and *S. tuberosum* (Pino *et al.* 2007). In both of these species, the use of a cold inducible promoter minimized these negative effects (Kasuga *et al.* 1999; Pino *et al.* 2007). We attempted to employ the *Cor15a* cold-inducible promoter to drive expression of *AtCBF1* but were unable to verify expression of the transgene following exposure to 3 °C for up to 24 h (Fig. 3.3). In *Arabidopsis*, transcripts for *Cor15a* begin to accumulate after 4 h of exposure to 2.5 °C (Gilmour *et al.* 1998). *Cor15a* is itself a CBF-responsive gene (Jaglo-Ottosen *et al.* 1998) and the successful use of a *Cor15a* promoter would require the presence of native CBF transcription factors in petunia to activate it in response to cold temperatures. We have shown that putative petCBF transcription factors are expressed in the pSPUD74 lines (Fig. 3.9), yet the *AtCor15a::AtCBF1* transgene is not induced during cold treatment. The petCBF transcription factors may have diverged enough from those in *Arabidopsis* that they no longer bind the CRT/DRE elements within the *AtCor15a* promoter. Alternatively, transcription of *AtCBF1* may be inhibited by mutations within our transgenic construct. There is also the possibility that additional transcription factors not present in petunia are required for activation of the *Cor15a* promoter. Creation of a double transgenic line by crossing the constitutive expression pMPS13-7 line to our pSPUD74-24B lines would reveal whether the lack of *AtCBF1* transcription is due to mutations within the construct or the lack of promoter activation by endogenous CBF transcription factors.

It is interesting that heterologous expression of *LeCBF1* from tomato did not impact freezing tolerance while high expression levels of *AtCBF3* did. Tomato belongs

to the same botanical family as petunia and would be expected to have genes that are more closely related. This is indeed the case at the nucleic acid level, where the petCBF genes identified by Goldman *et al.* (2007) cluster closer to the *LeCBF1* gene sequence than those of the AtCBF genes (Fig. 3.12). Over-expression of *LeCBF1* in *Arabidopsis* confers an increase in freezing tolerance, yet expression of *LeCBF1* or *AtCBF1* in tomato has no impact freezing tolerance (Zhang *et al.* 2004). It was concluded that CBF over-expression does not noticeably impact tomato freezing tolerance because the downstream portion of the CBF regulon in tomato is much smaller than in *Arabidopsis* with only four genes being upregulated in response to CBF over-expression (Zhang *et al.* 2004). However, this reasoning cannot explain why *LeCBF1* does not change petunia freezing tolerance while *AtCBF3* does. If *AtCBF3* expression increases freezing tolerance, then enough of the downstream components must be present in petunia for cold acclimation. So why does *LeCBF1* not have the same effect? Again, there are several possibilities. If there is indeed a threshold of expression that must be reached before we see increased cold tolerance, then perhaps none of our *LeCBF1*-expressing lines have reached this level of expression. It is also possible that the *LeCBF1* protein is less stable, resulting in the presence of fewer functional proteins despite high transcription levels. Alternatively, there may be differences between *LeCBF1* and *AtCBF3* in critical regions of the gene. *LeCBF1* is more similar to petCBF as a whole, but there may be specific regions where *LeCBF1* is divergent enough to make it non-functional in activating downstream petunia genes.

In conclusion, we have shown that manipulation of the CBF cold-response pathway in *P. hybrida* has the potential to increase freezing tolerance. However, the

negative phenotypic changes that accompany high constitutive expression of *AtCBF3* calls into question the feasibility of this method for improving the freezing tolerance of commercial cultivars. Further work is needed to determine whether the use of the *Cor15a* or another cold-inducible promoter might over-come this limitation.

Table 3.1. Primer sequences and reaction conditions used for screening putative transgenic *P. hybrida* 'Mitchell' plants for transgene presence in the T₀ generation.

Primer	Sequence	Reaction Conditions
AtCBF1 F	ACGAGTTGTCCGAAGAAACC	40 cycles of 94°C, 30s; 57°C, 30s; 72°C, 1m; plus final extension of 72°C, 4 min
AtCBF1 R	TCAGCGAAGTTGAGACATGC	
AtCBF3 F	AATGTTTGGCTCCGATTACG	
AtCBF3 R	CCTCACAAACCCACTTACCG	
LeCBF1 F	TTCGAATAACCCGAAAAAGC	40 cycles of 94°C, 30s; 56°C, 1m; 72°C, 2.5m; plus final extension of 72°C, 10 min
LeCBF1 R	AAAAGATCGCCTCCTCATCC	
NPT II F	GAGGCTATTCGGCTATGACTG	40 cycles of 94°C, 30s; 60°C, 1.5m; 72°C, 3.5m; plus final extension of 72°C, 10 min
NPT II R	ATCGGGAGCGGCGATACCGTA	

Table 3.2. Primer sequences and reaction conditions used in RT-PCR analysis of CBF expression in transgenic *P. hybrida* 'Mitchell'.

Primer	Sequence	Reaction Conditions
AtCBF1 F	ATTCCCAACTGCTGAAATGG	26 cycles of 94°C, 2m; 57°C, 2m; 72°C, 2m; plus final extension of 72°C, 10min
AtCBF1 R	AATCCAGGCATGCAGAAAAG	
AtCBF3 F	TGCTTGCTTGAACCTTGCTG	30 cycles of 94°C, 2m; 60°C, 2m; 72°C, 2m; plus final extension of 72°C, 10 min
AtCBF3 R	GGCATGTCAACATCAGCATC	
LeCBF1 F	GCATCAGCTGCTACTTTTGG	30 cycles of 94°C, 30s; 56°C, 1m; 72°C, 2.5m; plus final extension of 72°C, 10 min
LeCBF1 R	ACAAACCCACTTGCCTGAAC	
Actin F	GTGTTGGACTCTGGTGATGG	26 cycles of 94°C, 2m; 60°C, 2m; 72°C, 2m; plus final extension of 72°C, 10 min
Actin R	TCAGCAGTGGTGGTGAACAT	

Table 3.3. Plant generation and transgene copy number for the transgenic *P. hybrida* ‘Mitchell’ lines tested in freezing tolerance experiments.

Line ID	Generation and Zygosity of Tested Individuals	Transgene Copy Number
<u>35S::AtCBF3</u>		
Mitchell 6 (WT) × pMPS13-7-12	all hemizygous seed from cross	1
BpMPS13-101	T ₁ seed from hemizygous T ₀	2
pMPS13-10-8	T ₂ seed from a 3 insertion T ₁	3
<u>35S::LeCBF1</u>		
BpXIN1-110-5	T ₂ seed from hemizygous T ₁	1
BpXIN1-120-2	T ₂ seed from homozygous T ₁	1
pXIN1-17B-20	T ₂ seed from homozygous T ₁	1
pXIN1-25B-12	T ₂ seed from 2 insertion T ₁	2
<u>AtCor15a::AtCBF1</u>		
pSPUD74-24B-5	T ₂ seed from 2 insertion T ₁	2
pSPUD74-24B-13	T ₂ seed from a hemizygous T ₁	1

Table 3.4. Primer sequences and reaction conditions for RT-PCR of putative endogenous petunia CBF genes. Reaction conditions consisted of 26 cycles of 94 °C, 30 s; 56 °C, 1 min; 72 °C, 2.5 min; plus final extension of 72 °C, 10 min.

Primer	Sequence
petCBF1 F	ATTCCCAACTGCTGAAATGG
petCBF1 R	AATCCAGGCATGCAGAAAAG
petCBF2 F	TGCTTGCTTGAAC TTTGCTG
petCBF2 R	GGCATGTCAACATCAGCATC
petCBF3 F	GCATCAGCTGCTACTTTTGG
petCBF3 R	ACAAACCCACTTGCCTGAAC
petCBF4 F	TGCAGAAATGGCAGCTAGAG
petCBF4 R	AGGAAGCCGGGATAGGTAAC
Actin F	GTGTTGGA CTCTGGTGATGG
Actin R	TCAGCAGTGGTGGTGAACAT

Table 3.5. Putative orthologs of CBF-regulated genes and their expression in response to cold and CBF overexpression in *Petunia hybrida*

Known CBF Responsive Genes				Potential Petunia Orthologs		
TC number/ Genbank ID ^a	Source Species	Reference	Description	Petunia TC number ^a	Cold-responsive ^b	CBF-responsive ^c
TC116174	<i>L. esculentum</i>	Zhang <i>et al</i> 2004	Dehydrin-like	TC2800	yes	yes
TC103027	<i>S. tuberosum</i>	Pino <i>et al</i> 2007	protein (DHN10)			
TC115955	<i>L. esculentum</i>	Zhang <i>et al</i> 2004	Putative proteinase	TC3472	no	x
CK854013	<i>S. tuberosum</i>	Pino <i>et al</i> 2007	inhibitor			
X92847	<i>S. commersonii</i>	Pino <i>et al</i> 2008	ω 9 stearyl ACP desaturase	TC1671	yes	no
AF386075	<i>S. commersonii</i>	Pino <i>et al</i> 2008	Dehydrin (DHN2)	TC2907	yes	yes

^a TC number from <http://compbio.dfci.harvard.edu/tgi/>; Genbank ID from <http://www.ncbi.nlm.nih.gov>

^b Cold-responsiveness determined by RT-PCR on RNA extracted from petunia chilled at 3 °C for 0 min, 15 min, 2 hr, and 24 hr

^c CBF-responsiveness determined by RT-PCR on RNA extracted from *AtCBF3* and *LeCBF1* overexpressing lines grown at 22 °C; x indicates CBF-responsiveness was not determined.

Table 3.6. Primer sequences and reaction conditions used in RT-PCR analysis of putative downstream genes in the endogenous CBF-pathway in *Petunia hybrida* 'Mitchell'.

Primer	Sequence	Reaction Conditions
TC2800 F	GGGATGCACTTCACATTGG	17 cycles of 94°C, 2m; 55°C, 2m; 72°C, 2m; plus final extension of 72°C, 10 min
TC2800 R	TCCTTCTTATCCTTCTTGTCCTTC	
TC3472 F	ACCAGGCCATGCTAACACC	
TC3472 R	GGCAGAAACCAAAAATACACC	
TC1671 F	AAGGCTGATGTCCTCCAAAG	16 cycles of 94°C, 2m; 55°C, 2m; 72°C, 2m; plus final extension of 72°C, 10 min
TC1671 R	GCAATGACCCAAACTCCTG	
TC2907 F	CGAGTGATGAGGAGGAGGAAG	
TC2907 F	GTGGTGGTGGTGGTGGTTG	
Actin F	GTGTTGGACTCTGGTGATGG	20 cycles of 94°C, 2m; 60°C, 2m; 72°C, 2m; plus final extension of 72°C, 10 min
Actin R	TCAGCAGTGGTGGTGAACAT	

Table 3.7. ANOVA for effect of transgenic line on non-acclimated (A) and acclimated (B) EL₅₀ temperature of *AtCBF3*-expressing *P. hybrida* 'Mitchell' lines.

A. Dependent variable:			Sum of	Mean	F	
EL ₅₀ temperature	Source	DF	Squares	Square	Value	Pr > F
	Model	4	13.34	3.34	8.67	0.00
	Error	11	4.23	0.38		
	Total	15	17.58			
B. Dependent variable:			Sum of	Mean	F	
EL ₅₀ temperature	Source	DF	Squares	Square	Value	Pr > F
	Model	4	4.55	1.14	1.72	0.22
	Error	11	7.27	0.66		
	Total	15	11.83			

Table 3.8. Pairwise comparisons with Fisher's LSD between non-acclimated EL₅₀ values of different *AtCBF3* expressing lines and controls. Shown are p-values for each comparison.

	Wild Type	Empty Vector	pMPS13-7	pMPS13-10
Empty Vector	0.555			
pMPS13-7	0.000	0.001		
pMPS13-10	0.637	0.924	0.001	
BpMPS13-101	0.185	0.138	0.009	0.161

Table 3.9. ANOVA for effect of transgenic line on non-acclimated (A) and acclimated (B) EL₅₀ temperature of *LeCBF1*-expressing *P. hybrida* 'Mitchell' lines.

A. Dependent variable:						
EL ₅₀ temperature	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	5	3.49	0.7	1.73	0.2
	Error	12	4.84	0.4		
	Total	17	8.33			
B. Dependent variable:						
EL ₅₀ temperature	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	5	5.37	1.07	1.77	0.19
	Error	12	7.28	0.61		
	Total	17	12.65			

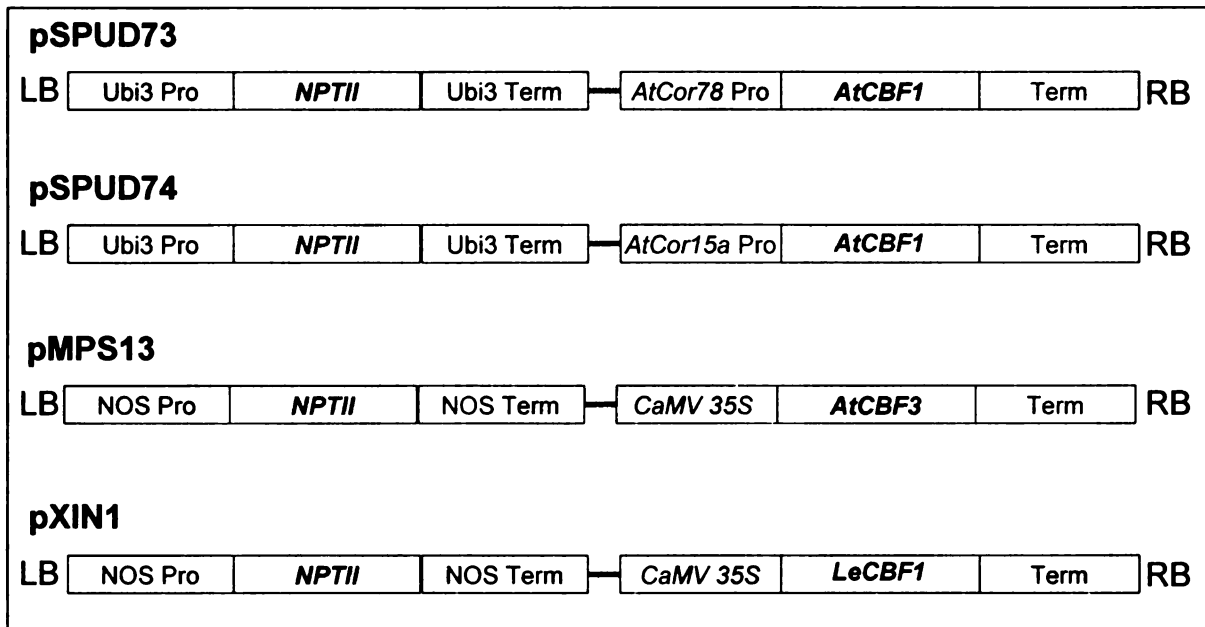


Figure 3.1. Diagram of constructs used for *Agrobacterium*-mediated transformation of *P. hybrida* ‘Mitchell.’ All constructs contain *NPTII* as a selectable marker for kanamycin resistance. pSPUD73 contains *Arabidopsis thaliana* *CBF1* behind the cold-inducible *AtCor78* promoter. pSPUD74 contains *Arabidopsis thaliana* *CBF1* behind the cold-inducible *AtCor15a* promoter. pMPS13 and pXIN1 contain *Arabidopsis thaliana* *CBF3* and *Lycopersicon esculentum* *CBF1*, respectively, behind the strong constitutive *CaMV* 35S promoter.

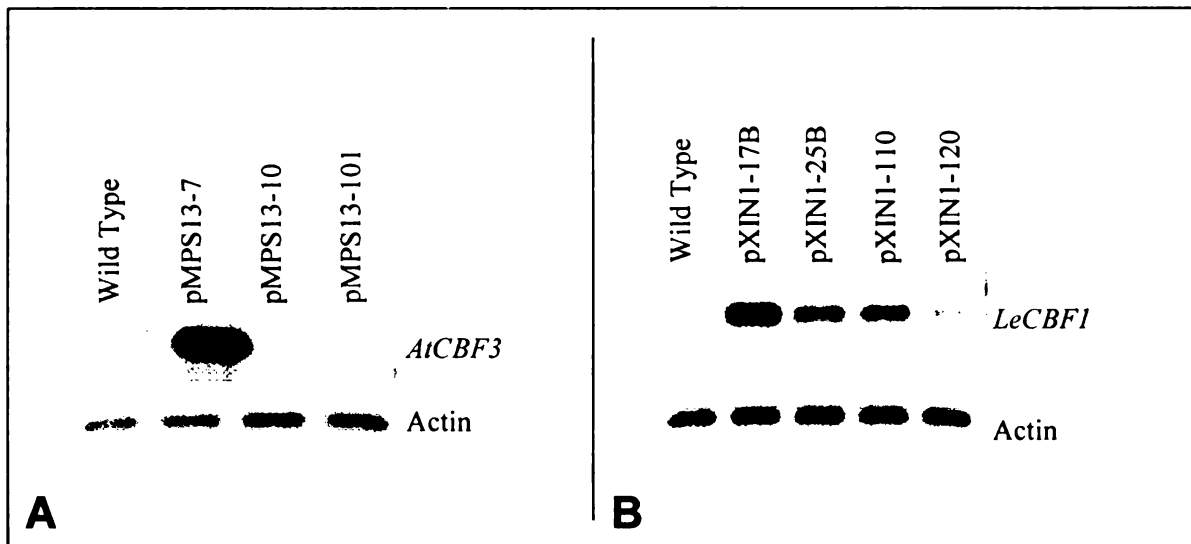


Figure 3.2. Gene expression analysis by semi-quantitative RT-PCR of *P. hybrida* ‘Mitchell’ transgenic lines containing 35S::*AtCBF3* (A) or 35S::*LeCBF1* (B). RNA was isolated from plants grown at 22 °C.

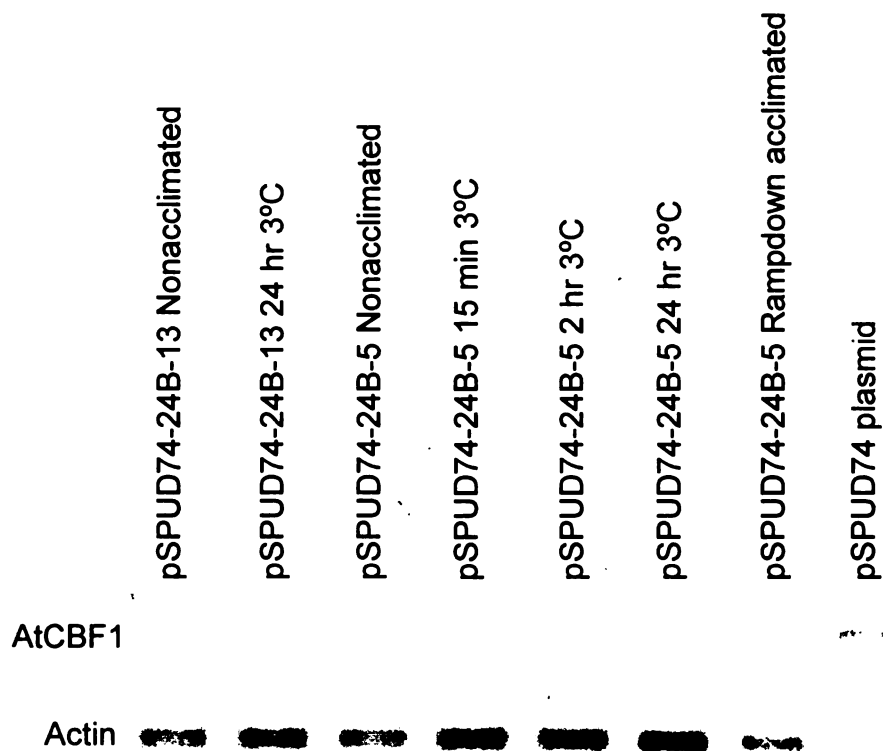


Figure 3.3. RT-PCR analysis of *AtCBF1* expression in pSPUD74 transgenic lines following various exposures to cold temperatures. Nonacclimated plants were grown at 22 °C and rampdown acclimated plants were grown 7 d at 15 °C SD, 7 d at 10 °C SD, and 7 d at 3 °C SD.

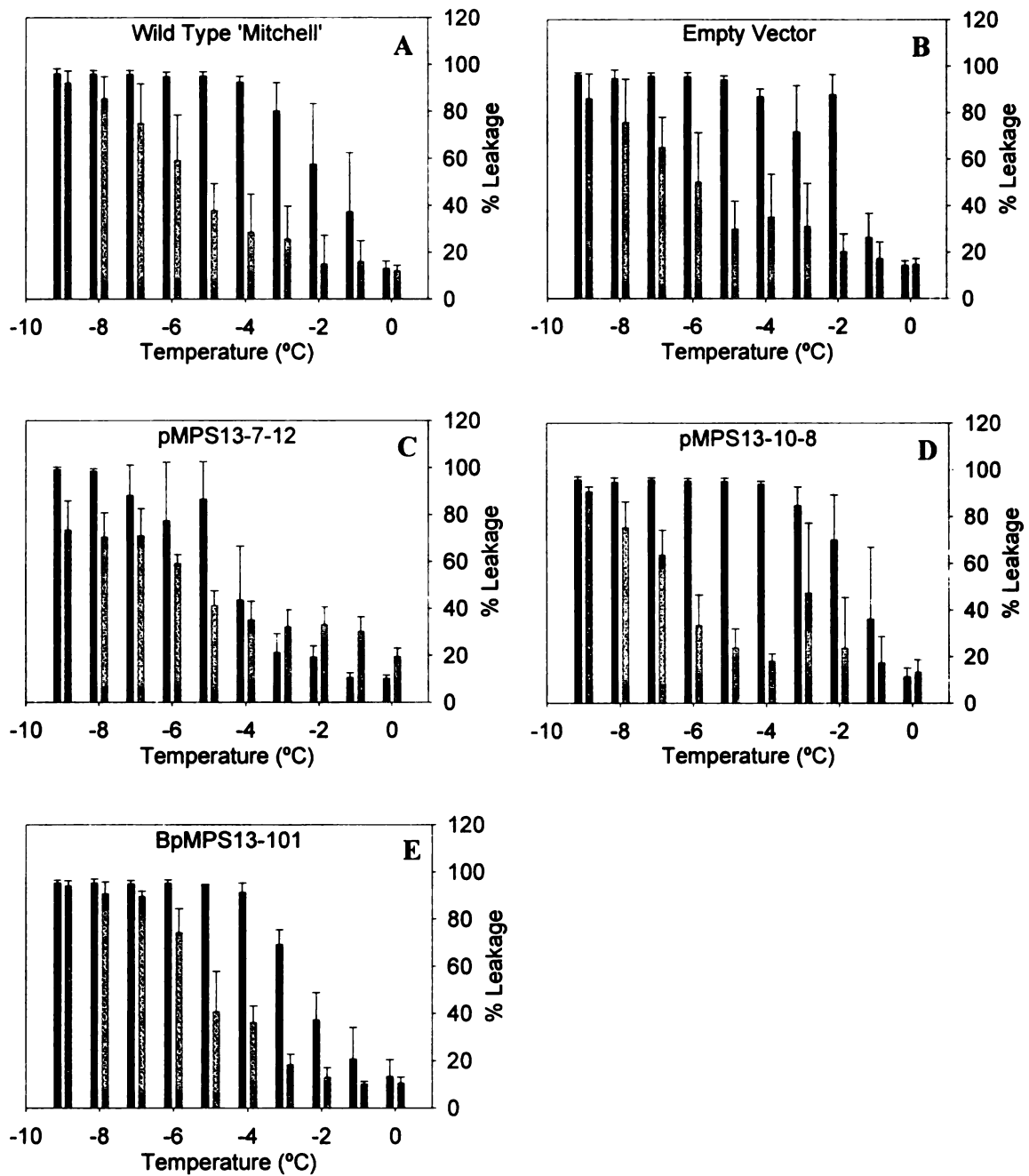


Figure 3.4. Percent electrolyte leakage at each temperature tested for wild type (A), empty vector control (B), and transgenic lines (C-E) containing the *35S::AtCBF3* (pMPS13) construct. Nonacclimated plants (black bars) were grown at 22 °C LD and acclimation was accomplished by exposing plants to 15 °C SD for 1 week, 10 °C SD for 1 week, then 3 °C SD for 1 week (grey bars). Leakage data at each temperature averaged over 6 measurements for empty vector and transgenic lines; 24 measurements for wild type. Standard deviation shown by error bars.

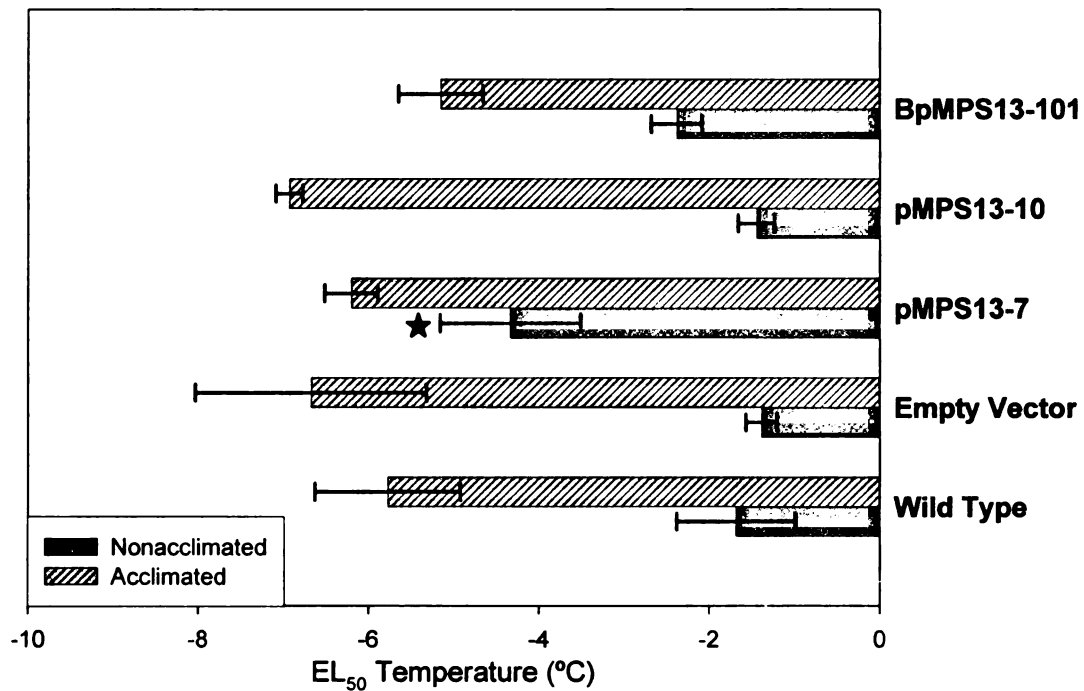


Figure 3.5. EL_{50} temperatures of nonacclimated and acclimated *AtCBF3* constitutively over-expressing lines. pMPS13-7 is significantly more freezing tolerant than the control lines prior to acclimation (starred bar). Following our acclimation regime (7 d at 15 °C SD, 7 d at 10 °C SD, and 7 d at 3 °C SD), there is no significant difference between any of the transgenic lines and the control lines. Standard deviation shown by error bars.

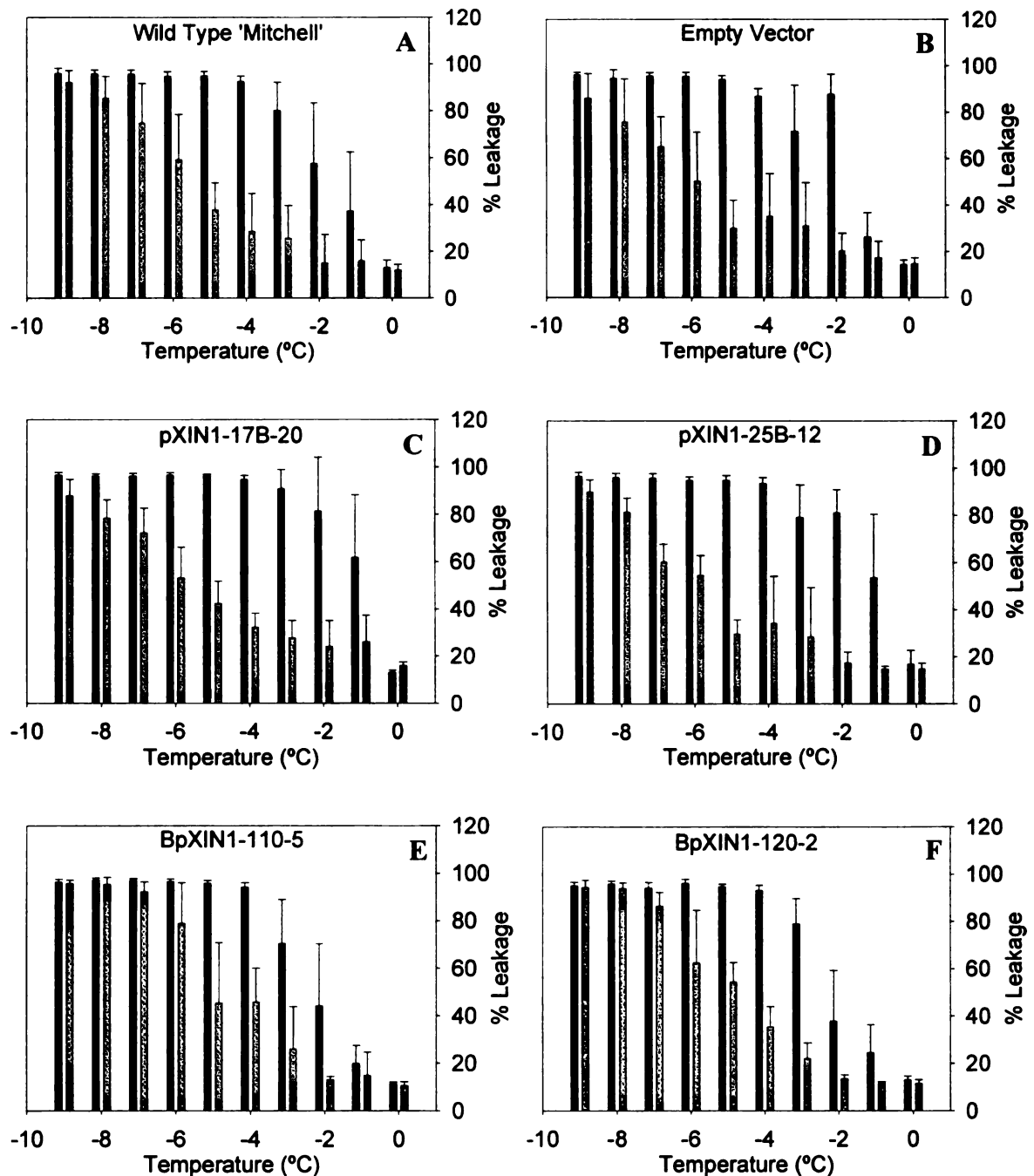


Figure 3.6. Percent electrolyte leakage at each temperature tested for wild type (A), empty vector control (B), and transgenic lines (C-F) containing the *35S::LeCBF1* (pXIN1) construct. Nonacclimated plants (black bars) were grown at 22 °C LD and acclimation was accomplished by exposing plants to 15 °C SD for 1 week, 10 °C SD for 1 week, then 3 °C SD for 1 week (grey bars). Leakage data at each temperature averaged over 6 measurements for empty vector and transgenic lines; 24 measurements for wild type. Standard deviation shown by error bars.

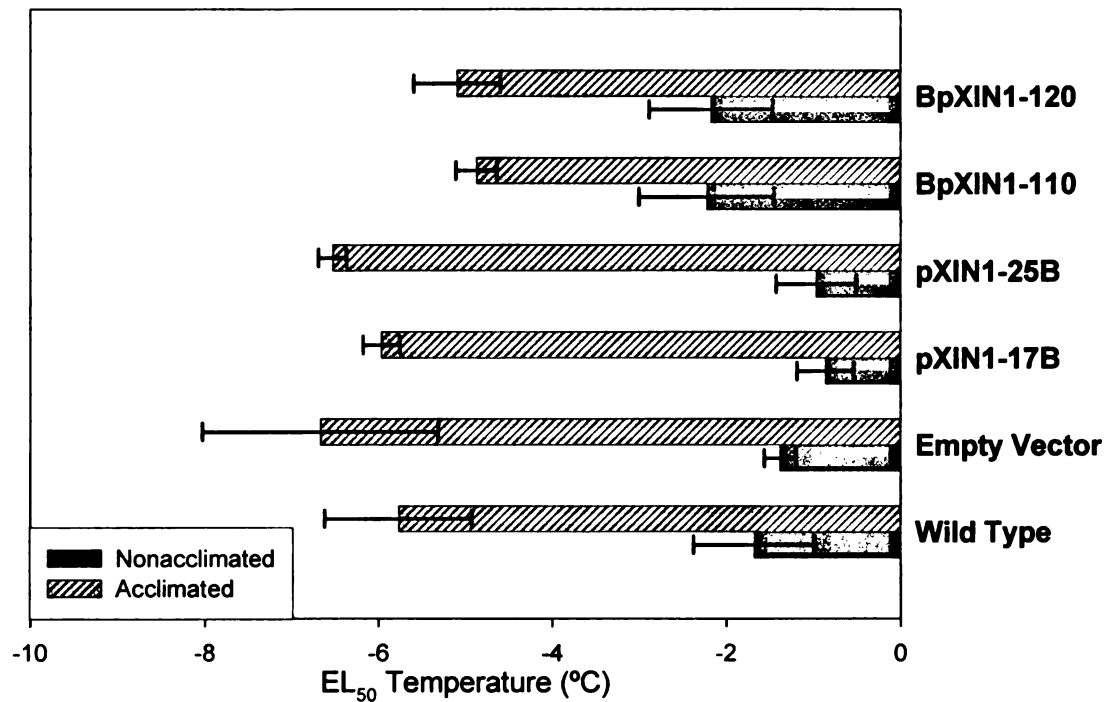


Figure 3.7. EL_{50} temperatures of nonacclimated and acclimated *LeCBF1* constitutively over-expressing lines. Nonacclimated plants were grown at 22 °C LD and acclimation was accomplished by exposing plants to 15 °C SD for 1 week, 10 °C SD for 1 week, then 3 °C SD for 1 week. There are no significant differences between any of the transgenic lines and the control lines.

	1		50
petCBF1	MDIFGSYYSDTLPAASAPTFWPLDVPEYSSPISDNSSCSNNRANHSDEEV		
petCBF2	MDIFGSYYSDIL-----PIELPEYSSPMSDNSSCSNYRANHSDDDEV		
petCBF3	MDIFARYYSDQLPIASAATFWPLEVAEYSSPMSD---ISNNRANLSDEEV		
petCBF4	MDIFGRYYSDQLPIASAATFWPLEVAEYSDNSSS---SSNNRANVSDEEV		
AtCBF1	-----MNSFSAFSEMFGSDYEPQGDDYCP		
	51		100
petCBF1	MLASNNPKKRRAGRKKKRETRHPVYRGVRKRNSGKWVCEVREPNNKQSRIWL		
petCBF2	MLASNNPKKCAGRKKKRETRHPVYRGVRKRNGKWVCEVREPNNKKSRIWL		
petCBF3	MLASNNPKKRRAGRKKKRETRHPVYRGVRKRSSGKWVCEVREPNNKKSRIWL		
petCBF4	MLASNNPKKRRAGRKKKRETRHPVYRGVRKRNSGKWVCEVREPNNKKSRIWL		
AtCBF1	TLATSCPKKRAGRKKKRETRHPVYRGVRQRNSGKWVSEVREPNNKKTRIWL		
	101		150
petCBF1	GTFTAEMAARAHDVAAIAFRGRSACLNFA DSAW KLPTPASSDPKDIQKA		
petCBF2	GSFPTAEMAARAHDVAAIALRGRSACLNFA DSAW KLPIPASSNPDKDIQKA		
petCBF3	GTYITAEMAARAHDVAAIALRGRSACLNFA DSAW KLHIPASSKAKDIQKA		
petCBF4	GTYSTAEMAARAHDVAAIALRGRAACLNFA DSAW KLPIPASSKAKDIQKA		
AtCBF1	GTFTAEMAARAHDVAAIALRGRSACLNFA DSAW KLRIPESTCAKDIQKA		
	151		200
petCBF1	AAEAAEAFRPLESEGVHSAGEESKEESTTPETAES-----MYFMDEEALF		
petCBF2	AAEAAKAFR-----ESGEESKEESSTRETPEK-----MFFMDEEALF		
petCBF3	ATEAASAFQ-----ESKEEGTTPETPEK-----MLFMDEEALF		
petCBF4	ATEAAATAF-----LEPGE---PETRKKN-----MLFMDEEALF		
AtCBF1	AAEAALAFQDETCDTTTTNHGLDMEETMVEAIYTPEQSEGAFFYMDEETMF		
	201		242
petCBF1	CMPGLLANMAEGLMLPPP-QCSEVGDHFMEADADMPLWSYSV		
petCBF2	CMPELLANMAEGLMLPPPSQCSDVGEHFMDADVDMPLWSYSI		
petCBF3	YMPGLLANMAEGLMLPLPPQCSEVGDHFMEAAAADMPLWSYSF		
petCBF4	CMPGLLANMAEGLMLTPP---QCYGEHFMEADAEVPLWSY--		
AtCBF1	GMPTLLDNMAEGMLLPPP-SVQWNHNYDGEGLDGVSLWSY--		

Figure 3.8. Alignment of petCBF amino acid sequences obtained from Goldman *et al.* (2007) and *AtCBF1* sequence (Pubmed Gene ID: 828653). Shown in boxes is the matching of the petCBF sequences with the “CBF signature sequences”, PKK/RPAGR_xKFxETRHP and DSAWR (Jaglo *et al.* 2001). Grey shading denotes where the petCBF amino acids differ from the signature sequence.

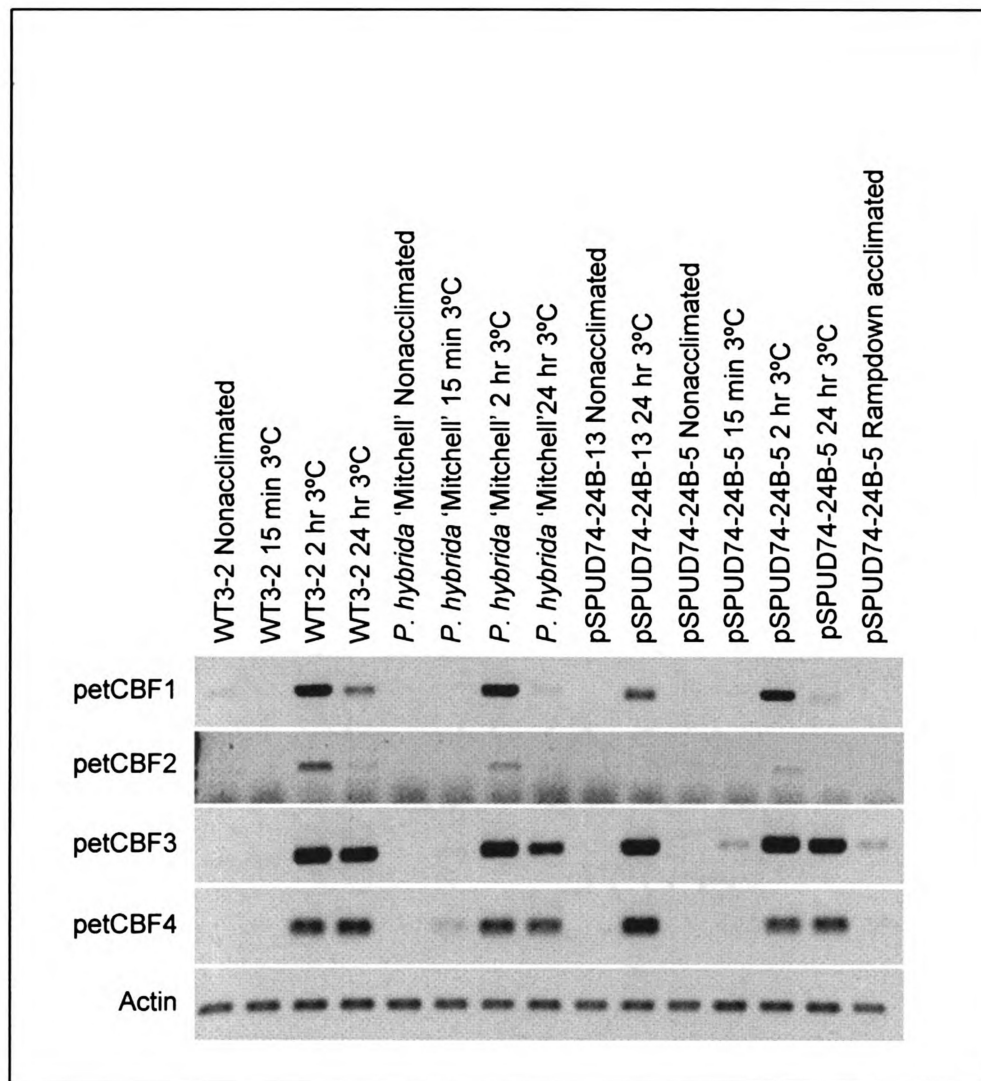


Figure 3.9. RT-PCR analysis for expression of *petCBF1-4* in wild type *P. hybrida* 'Mitchell' and pSPUD74 transgenic lines following chilling at 3 °C for various time periods. Nonacclimated plants were grown at 22 °C and rampdown acclimated plants were grown at 15 °C SD for 1 week, 10 °C SD for 1 week, then 3 °C SD for 1 week. WT3-2 is a wild type line recovered from tissue culture and *P. hybrida* 'Mitchell' is a wild type that has not undergone tissue culture.

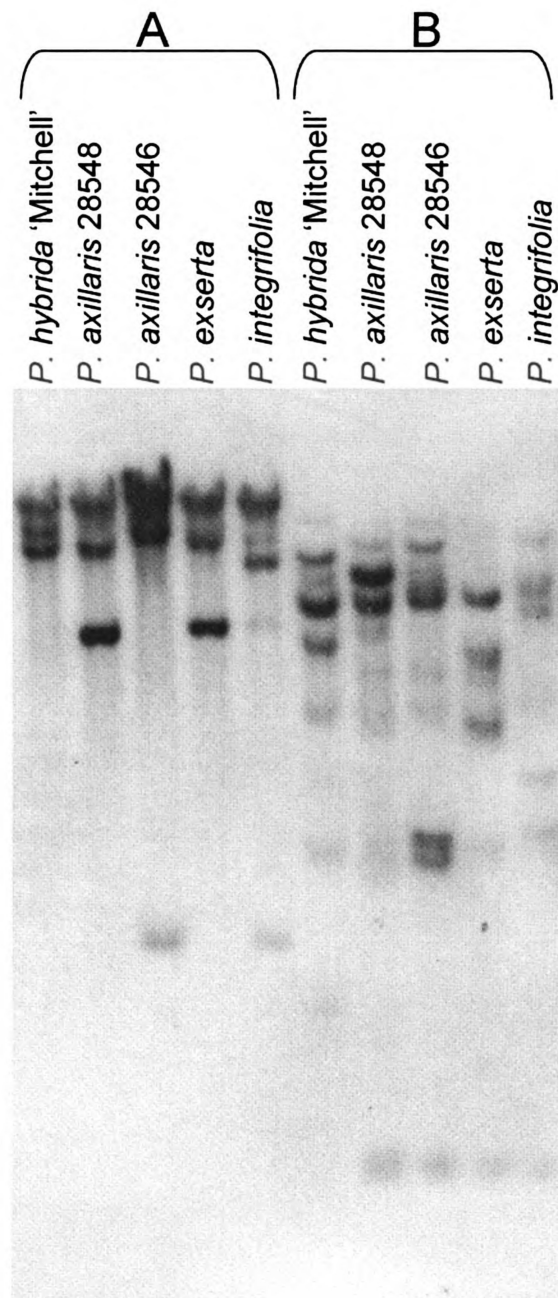


Figure 3.10. Southern hybridization with genomic DNA from four *Petunia* species digested with PST I (A) or ECO RI (B) restriction enzymes. Probe is a 163 nucleotide fragment from a highly conserved region of *petCBF1* from *P. hybrida* 'Mitchell'.

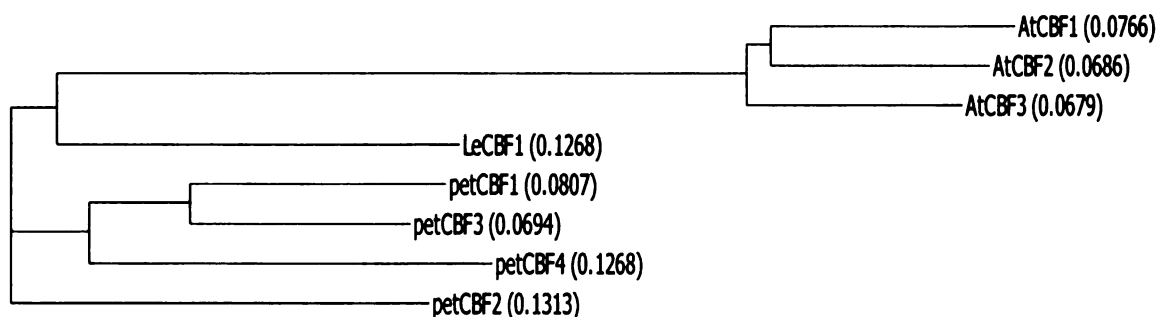
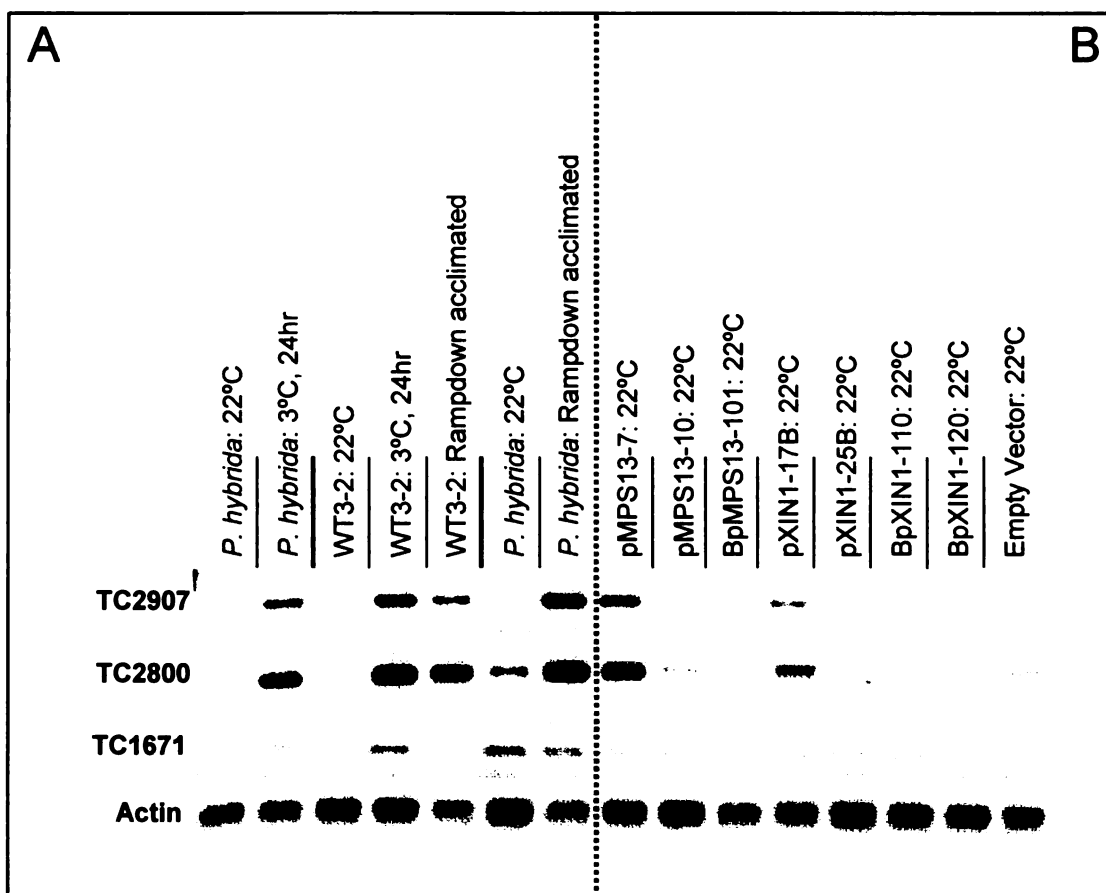


Figure 3.12. Phylogenetic tree showing relationship between nucleic acid sequences for CBF transcription factors from various species.

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CHAPTER 4

ASSESSING EFFECTS OF CBF OVER-EXPRESSION ON HORTICULTURAL TRAITS IN *PETUNIA HYBRIDA* 'MITCHELL'

ABSTRACT

Heterologous expression of CBF transcription factors has previously been shown to effectively increase freezing tolerance of many plant species, including petunia. However, strong constitutive CBF expression often results in negative phenotypic changes, such as stunting and delayed flowering. Here we show that while high constitutive expression of *AtCBF3* increases basal freezing tolerance of one transgenic petunia line, deleterious phenotypic changes occur such as increasing days to flower by over 30%, reduced flower size, and reduced biomass at flowering. These negative phenotypes were either less severe or not present in other transgenic lines, but transgene expression was lower in these lines and they showed no increase in freezing tolerance. It is concluded that constitutive expression of *AtCBF3* at levels high enough to increase freezing tolerance brings about negative effects on horticulturally important traits.

INTRODUCTION:

Frequent night temperatures below freezing severely limit the selection of ornamental bedding plant species that can be grown in early spring and late fall. However, in some species, exposures to low non-freezing temperatures induce an increase in freezing tolerance, referred to as cold acclimation. Following acclimation, plants survive temperatures below levels that were previously lethal (Thomashow 1999).

Increased freezing tolerance is the result of many genetic changes (Guy *et al.* 1985), including those regulated by the CBF cold response pathway. This well-characterized pathway in *Arabidopsis thaliana* L. consists of three functionally redundant transcription factors, *AtCBF1-3* (Gilmour *et al.* 2004). Downstream components of the pathway contain a *cis*-acting element called the C-repeat/Dehydration responsive element (CRT/DRE) to which CBF binds (Stockinger *et al.* 1997, Gilmour *et al.* 1998). Expression of *AtCBF1-3* is induced in response to low temperatures, bringing about an increase in expression of downstream genes, such as the COR (Cold-Responsive) genes, and ultimately leading to an increase in freezing tolerance (Jaglo-Ottosen *et al.* 1998).

Heterologous expression of *Arabidopsis* CBF genes has proven to be a useful method for increasing freezing tolerance of several plant species, including *Brassica napus* L. (Jaglo *et al.* 2001), *Fragaria ×ananassa* Duchesne (Owens *et al.* 2002) and *Solanum tuberosum* L. (Pino *et al.* 2007; Pino *et al.* 2008). However, expression of *AtCBF* transcription factors behind the strong constitutive cauliflower mosaic virus (*CaMV*) 35S promoter often results in deleterious phenotypic effects. For instance, when *AtCBF3* is over-expressed in *Arabidopsis*, stunting, delayed flowering, and prostrate growth habits have been observed (Liu *et al.* 1998; Kasuga *et al.* 1999; Gilmour *et al.* 2000). Constitutive expression of *AtCBF1-3* in *S. tuberosum* reduces or inhibits tuber formation, delays flowering, and causes stunting (Pino *et al.* 2007). *Solanum lycopersicum* L. plants expressing either *AtCBF3* or *LeCBF1* (a CBF homolog from *S. lycopersicum*) behind the *CaMV* 35S promoter also display stunted growth and delayed flowering (Zhang *et al.* 2004).

Petunia hybrida Vilm. (petunia) is a major horticultural crop with the ability to cold acclimate (Yelenosky and Guy 1989; Pennycooke *et al.* 2003). However, further improvements to the cold tolerance of petunia would benefit both consumers and commercial growers by allowing this species to be grown earlier in the spring and later into the fall, when cool temperatures limit the growth of other bedding plants. Therefore, one of two CBF transcription factors, either *AtCBF3* or *LeCBF1*, have been transformed into *P. hybrida* ‘Mitchell’ behind the *CaMV 35S* promoter in an effort to further increase the freezing tolerance of this species. One transgenic line, pMPS13-7, expressing *AtCBF3* at high levels has previously been shown to have increased basal freezing tolerance compared to wild type (chapter 3). The objective of this study is to determine whether constitutive expression of these genes has deleterious effects on traits of horticultural importance in petunia.

MATERIALS AND METHODS

Analysis of horticultural traits in transgenic lines

Transgenic *P. hybrida* ‘Mitchell’ lines expressing *AtCBF3* or *LeCBF1* behind the strong constitutive *CaMV 35S* promoter were created as described in chapter 3. These lines were tested to determine the effect of constitutive CBF expression on horticultural traits. Seeds from transgenic lines, empty vector control, and wild type were surface sterilized and planted onto petri plates as described in chapter 3. Seeds were plated at a density of ~100 seeds per plate. Plates were placed in a 22 °C, 16 h photoperiod (100-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) chamber for three weeks. After three weeks, 10 plants of each genotype were transplanted to 10 cm round pots filled with 70% peat moss, 21% perlite,

9% vermiculite (Suremix, Michigan Grower Products Inc., Galesburg MI, USA). Plants were left in a 22 °C, 16 h photoperiod chamber with increasing light intensity for 1 week to harden plants in preparation for transfer to a greenhouse. Plants were then transferred to a greenhouse at 20 °C with 16 h daylight extension lighting to grow until the first flower opened. On the day the first flower opened, days to flower, number of fully open flowers, flower bud number, length of flower-bearing stem, leaf number on the flower-bearing stem, length and width of longest leaf, open flower corolla tube length and corolla rim diameter were determined. Aboveground biomass was harvested and dried in an oven at 70 °C for five days for dry mass determination. In the second replication, additional measurements were made including length of the primary stem and leaf number on this stem. Genotype effects were determined by ANOVA using PROC GLM of SAS (SAS Institute Inc., USA) and mean separations were carried out using two-tailed Dunnett comparisons with wild type as the control. Two replications of this experiment were conducted.

RESULTS

CBF over-expression impacts horticultural traits

Constitutive CBF expression impacted several traits of horticultural importance compared to wild type (Tables 4.1 and 4.2). There was a significant effect of replication, so data from the two replications were analyzed independently. Time to flowering of the pMPS13-7 transgenic line was increased compared to wild type. In the first replication, wild type plants flowered an average of 70 d after planting, compared to 94 d for pMPS13-7 (Table 4.1). Days to flowering of all other transgenic lines in the first

replication did not differ from wild type. In the second replication, wild type plants took 77 d to flower while pMPS13-7 plants flowered 127 d after planting (Table 4.2).

The pMPS13-7 line flowered exclusively on lateral branches in both replications, while the first open flower for wild type and other transgenic lines occurred on the primary stem more often than on the lateral branches. In the first replication, length of the first flower-bearing stem was shorter than wild type for pMPS13-7 and pXIN1-25B, but the number of leaves on the flower-bearing stems did not differ (Table 4.1), indicating reduced internode length. In the second replication, pMPS13-10, BpMPS13-101, pXIN1-25B, and BpXIN1-110 all had shorter flower-bearing stems than wild type, but leaf number did not differ (Table 4.2). Leaf numbers on the primary stems at time of flowering were determined in the second replication (Table 4.2) and indicate that pMPS13-7 plants flowered at approximately the same physiological time as wild type, and the delayed flowering is the result of a reduced development rate.

Open flower number on the first day of flowering was not different from wild type in any lines (Tables 4.1 and 4.2). Flower bud number was decreased in pMPS13-7 during the first replication compared to wild type, with wild type plants having an average of 28 unopened visible flower buds on the day of flowering, compared to only 17 on pMPS13-7 (Table 4.1). In the second replication, pMPS13-7 showed a slight increase in visible buds on the day of flowering, having 31 per plant compared to 24 for wild type (Table 4.2).

Length of the largest leaf was decreased in the BpXIN1-120, pMPS13-7, and pXIN1-25B transgenic lines compared to wild type during the first replication (Table 4.1). The longest leaf on wild type plants measured 17.7 cm from node to leaf tip, while

BpXIN1-120, pMPS13-7, and pXIN1-25B had leaf lengths of 15.9, 10.9, and 15.9 cm, respectively. Leaf width was also altered in several lines (Table 4.1). pXIN1-17B leaves were wider than wild type, measuring nearly 6.5 cm across, compared to 5.5 cm for wild type. The largest leaves of pMPS13-7 and pXIN1-25B were significantly narrower than wild type, measuring 2.4 and 5.0 cm, respectively. In the second replication, pMPS13-7 again had shorter and narrower leaves compared to wild type. pMPS13-10 had slightly wider leaves and pXIN1-25B had slightly narrower leaves than wild type in this replication (Table 4.2).

Flower size was reduced in the pMPS13-7 line in both replications (Tables 4.1 and 4.2). In the first replication, length of the first fully open wild type flower was 5.4 cm from corolla rim to base of the receptacle, while pMPS13-7 flowers were 4.9 cm. Corolla rim diameter was also reduced from 5.4 cm in wild type to 4.3 cm in pMPS13-7. In the second replication, the first fully open pMPS13-7 flowers measured 4.5 cm long with a corolla diameter of 4.1 cm. This is significantly smaller than wild type flowers in this replication which were 5.5 cm long with a corolla diameter of 5.2 cm.

Aboveground biomass varied by genotype and replication (Tables 4.1 and 4.2). In the first replication, biomass of pMPS13-10, BpXIN1-110, pMPS13-7, and pXIN1-25B was 9.8, 10.4, 6.4, and 7.5 g, respectively, compared to 12.9 g for wild type. However, the empty vector control plants also had reduced biomass compared to wild type with an average mass of 9.5 g. In the second replication, only pMPS13-7 differed from wild type, with a biomass of 11.6 g compared with 8.7 g for wild type.

DISCUSSION AND CONCLUSIONS

Heterologous expression of CBF transcription factors results in increased freezing tolerance of many species (Jaglo *et al.* 2001, Kasuga *et al.* 1999, Owens *et al.* 2002, Pino *et al.* 2007, Pino *et al.* 2008). However, the use of the strong constitutive *CaMV 35S* promoter to drive expression of CBF often causes undesirable effects such as stunting and delayed flowering (Liu *et al.* 1998; Kasuga *et al.* 1999; Gilmour *et al.* 2000; Zhang *et al.* 2004, Pino *et al.* 2007). In tomato, constitutive expression of *AtCBF1* reduces fruit number, seed production, and plant biomass (Hsieh *et al.* 2002). In potato, constitutive expression of *AtCBF1-3* reduces biomass and tuber yield (Pino *et al.* 2007). Here we have shown that improved freezing tolerance of petunia through constitutive expression of *AtCBF3* comes at a cost to horticultural traits, especially flowering time.

Long periods between planting and flowering are undesirable in greenhouse crops due to the increased time and money that a grower must invest. High expression of *AtCBF3* in the pMPS13-7 transgenic line delayed flowering by several weeks, increasing time to flowering by more than 30% in the first and 65% in the second replications compared to wild type plants. Expression of *AtCBF3* or *LeCBF1* in the other transgenic lines did not delay flowering, but none of these lines showed increased freezing tolerance (chapter 3).

The length of the flower-bearing stem was reduced by more than half in the pMPS13-7 line in the first replication, with an average length of 15.6 cm compared to 32.2 cm for wild type. While the flower-bearing stem was shortened, the number of leaves present on the stem was unchanged, indicating that reduced stem length was the result of shortened internodes. Although the pMPS13-7 plants flowered several weeks

after wild type, the leaf/node numbers of both the flower-bearing and primary stems reveal that flowering still occurred at the same physiological point, but reduced development rate resulted in delayed flowering. Several other transgenic lines had reduced stem length, but none as extreme as pMPS13-7. The number of leaves below the first flower on these stems was also unchanged relative to wild type.

The number of visible buds was dramatically reduced in pMPS13-7 in the first replication, an undesirable result in petunia, a plant grown primarily for its abundant flowers. In the second replication, pMPS13-7 had slightly more visible flower buds at time of flowering, but this is likely a result of the additional 50 days of growing time that this line had relative to wild type. Flower length and width of the pMPS13-7 line was also reduced in both replications.

In addition to changes in flowering characteristics, the pMPS13-7 line was smaller in overall size and displayed a more prostrate growth habit with lateral branches that grow parallel to the ground rather than curving upward (Figure 4.1). The primary stem of this line was also very short, with an average length of just 3.3 cm at time of flowering. Several transgenic lines showed altered leaf length and width. Most notably, the largest leaves of pMPS13-7 were significantly shorter and narrower than wild type in both replications.

Aboveground dry biomass measurements were highly variable between different transgenic lines and also between replications of the same line. In the first replication, pMPS13-7 had the lowest biomass at the time of flowering, only half as much as wild type, while changes in biomass of other lines were not as dramatic. In the second replication, pMPS13-7 had increased biomass compared with wild type.

Heterologous expression of *AtCBF3* in *P. hybrida* can increase freezing tolerance if expressed at high levels (chapter 3). Unfortunately, plants expressing the transgene at these levels are also impacted by negative phenotypic changes. The pMPS13-7 line is more tolerant to freezing temperatures than wild type, but this line would not be marketable as a horticultural crop. Further work should be done to examine the feasibility of using a stress-inducible promoter to drive expression of *AtCBF3*. This technique has successfully been used to minimize the negative effects of constitutive CBF expression, while maintaining the benefits of increased freezing tolerance (Kasuga *et al.* 1999, Pino *et al.* 2007).

Table 4.1. Comparison of horticultural traits of transgenic lines with wild type *Petunia hybrida* 'Mitchell' in first replication. All measurements were taken the day the first flower was fully open.

Plant ID	Days to flower	Open flower number	Flower bud number	Leaf length (cm)	Leaf width (cm)	First open flower length (cm)	First open corolla diameter (cm)	Dry bio-mass (g)	Flower stem length (cm)	Flower stem number
Wild type	70.4	2.0	28.3	17.7	5.6	5.4	5.4	12.7	32.3	21.9
Empty vector	68.8	1.4	30.5	16.9	5.6	5.3	5.4	9.5**	26.5	21.9
pMPS13-7	94.0**	1.6	17.1**	10.9**	2.4**	4.9*	4.3**	6.4**	15.6**	19.6
pMPS13-10	70.0	1.6	28.5	17.5	5.5	5.5	5.5	9.8**	33.3	20.8
BpMPS13-101	68.8	1.4	27.1	17.0	5.7	5.2	5.4	11.2	34.9	22.1
pXINI-17B	74.1	1.0	22.4	17.6	6.6**	5.6	5.6	13.6	36.6	23.4
pXINI-25B	67.0	1.4	27.9	15.9**	5.0*	5.3	5.2	7.5**	25.0*	20.4
BpXINI-110	70.0	1.9	25.4	17.3	5.7	5.3	5.2	10.4*	28.0	21.9
BpXINI-120	72.1	1.7	27.3	15.9**	5.5	5.3	5.1	10.5	27.0	23.1

* and ** indicate significantly different from wild type at $\alpha=0.05$ and 0.01 , respectively, according to two-tailed Dunnett analysis.

Table 4.2. Comparison of horticultural traits of transgenic and wild type *Petunia hybrida* 'Mitchell' in second replication. All measurements were taken the day the first flower was fully open.

Plant ID	Days to flower	Open flower number	Flower bud number	Leaf length (cm)	Leaf width (cm)	First open flower length (cm)	First open corolla diameter (cm)	Dry bio-mass (g)	Flower stem length (cm)	Flower stem number	Primary stem length (cm)	Primary stem number
Wild type	76.8	1.5	24.4	13.8	4.5	5.5	5.2	8.7	26.6	22.3	25.4	26.5
Empty vector	75.7	1.3	25.5	13.6	4.7	5.5	5.5	7.6	22.2	21.6	19.5**	24.8
pMPS13-7	127.2**	1.4	31.3*	8.6**	2.3**	4.5**	4.1**	11.6**	24.4	25.7	3.3**	25.0
pMPS13-10	67.4**	1.4	25.9	14.5	5.2**	5.5	5.1	7.2	18.8**	18.5	18.8**	20.6**
BpMPS13-101	71.5	1.1	23.9	14.1	4.9	5.6	5.6	7.0	21.7*	21.4	21.8	22.0**
pXINI-17B	81.7	1.4	21.1	13.4	5.0	5.6	5.2	10.5	30.1	24.7	30.6**	28.4
pXINI-25B	80.8	1.4	30.0	12.8	4.0*	5.3	5.0	7.1	20.8**	23.4	20.5**	24.2
BpXINI-110	74.8	1.8	27.2	13.3	4.8	5.3	5.2	8.4	22.0*	21.4	21.2*	25.8
BpXINI-120	76.5	1.3	27.5	12.7	4.8	5.4	5.4	8.1	23.2	22.5	20.7**	28.7

* and ** indicate significantly different from wild type at $\alpha=0.05$ and 0.01 , respectively, according to two-tailed Dunnett analysis.

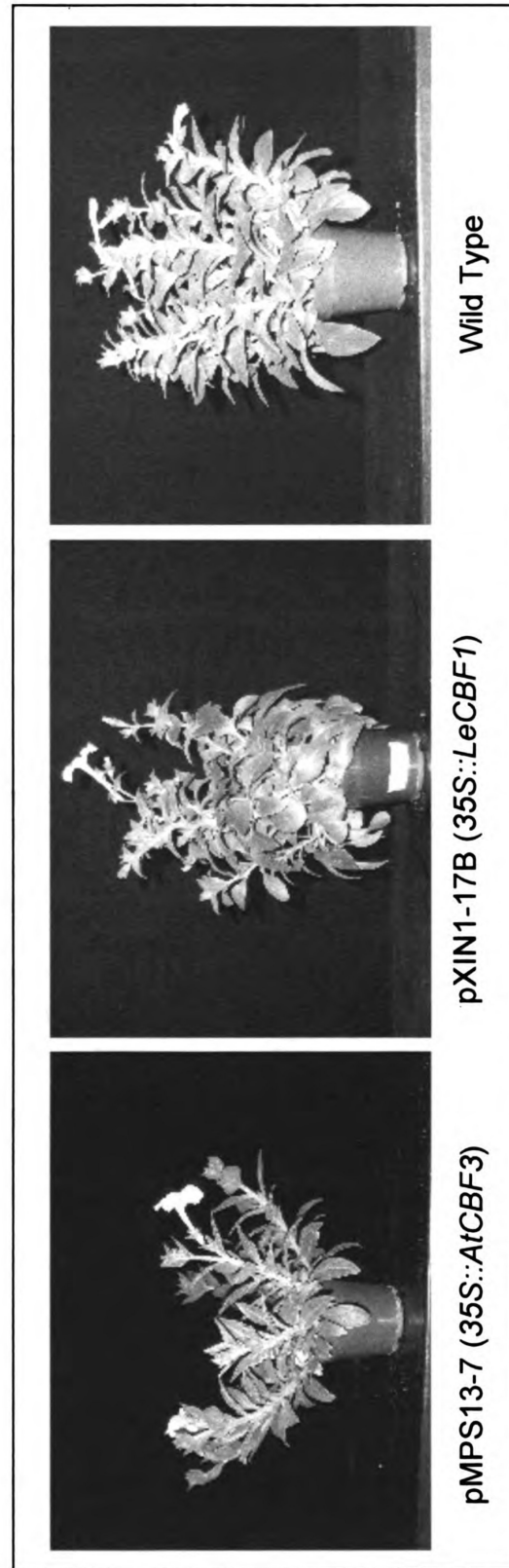


Figure 4.1. Photographs comparing phenotype of transgenic lines and wild type on the day the first flower opened. Shown are representative individuals from the transgenic lines with the highest level of heterologous CBF expression for each construct and wild type. Images are sized to the same approximate scale.

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CHAPTER 5

ASSESSING DROUGHT TOLERANCE OF CBF OVER-EXPRESSING *PETUNIA HYBRIDA* 'MITCHELL'

ABSTRACT

Ectopic expression of CBF transcription factors has been reported to improve drought tolerance in several species, including *Solanum lycopersicum* (tomato). The objective of this work was to determine whether constitutive heterologous expression of *AtCBF3* from *Arabidopsis thaliana* or *LeCBF1* from *S. lycopersicum* in *Petunia hybrida* results in increased tolerance to drought stress. Following 9 d of water withholding, survival of the transgenic lines was not different than wild type. After 12 d of water withholding, survival of the transgenic line with the highest expression of *AtCBF3*, pMPS13-7, was lower than wild type, while the survival of all other lines was not different. No differences in relative biomass gain were seen between wild type and transgenic lines following either 9 or 12 d of water withholding.

INTRODUCTION

Water deficits are a major stress facing plants in the garden. Recent water shortages, especially in the southeastern and western parts of the country, highlight the need for ornamental plants that are able to thrive under low water conditions. An increasing population (US Census Bureau 2004), coupled with decreasing water resources, will likely lead to increased water use restrictions in this country. It is

therefore wise to develop ornamental plants that can tolerate or thrive in low water conditions.

Drought tolerance is a complex trait involving many genetic factors (Shinozaki *et al.* 2003). Among these is the CBF family of transcription factors isolated from *Arabidopsis thaliana* L. consisting of three cold-induced members, *AtCBF1-3* (Gilmour *et al.* 1998), and a drought-induced member, *AtCBF4* (Haake *et al.* 2002).

Overexpression of *AtCBF3* (Liu *et al.* 1998; Kasuga *et al.* 1999) or *AtCBF4* (Haake *et al.* 2002) in *Arabidopsis* increases both drought and cold tolerance of transgenic plants.

Many species encode homologs of the *Arabidopsis* CBF genes, including *Solanum lycopersicum* L. (Zhang *et al.* 2004), *Brassica napus* L. (Jaglo *et al.* 2001; Gao *et al.* 2002), *Prunus avium* L. (Kitashiba *et al.* 2004), and *Zea mays* L. (Qin *et al.* 2004). Heterologous expression of *AtCBF1* in *S. lycopersicum* L. increases drought tolerance (Hsieh *et al.* 2002) and expression of *AtCBF1*, 2, or 3 enhances drought tolerance of *Oryza sativa* L. (Ito *et al.* 2006).

The objective of this study was to determine whether constitutive heterologous expression of *AtCBF3* or *LeCBF1* (a CBF homolog from *S. lycopersicum*) enhances the drought tolerance of transgenic *Petunia hybrida*.

MATERIALS AND METHODS

Analysis of drought tolerance of transgenic lines

Transgenic *P. hybrida* 'Mitchell' lines over-expressing *AtCBF3* or *LeCBF1* behind the constitutive cauliflower mosaic virus (*CaMV*) 35S promoter were created as previously described in chapter 3. These lines were tested to determine the impact of

heterologous CBF expression on drought tolerance. Seeds were surface sterilized by soaking for 10 to 15 min in a 50% bleach, 0.1% Triton-X-100 solution with gentle agitation. Seeds were rinsed with sterile deionized water 3 to 4 times to remove all traces of bleach and then suspended in a 0.1% sterile agar solution to facilitate pipetting. Seeds from transgenic lines were pipetted onto 100 x 15 mm disposable Petri plates containing Gamborg's B5 medium (minus sucrose, kinetin and 2,4-D; plus 0.7% agar) supplemented with 100 µg/ml kanamycin. Seeds from wild-type control plants were pipetted onto identical plates without the addition of kanamycin. Seeds were plated at a density of ~100 seeds per plate. Plates were placed in a 22 °C, 16 h photoperiod (100-130 µmol m⁻² s⁻¹ light) chamber for three weeks. After three weeks, three plants of each genotype including wild type and an empty vector control were transplanted in each of twenty 27 x 54 cm open flats filled with 4 cm of 70% peat moss, 21% perlite, 9% vermiculite (Suremix, Michigan Grower Products Inc., Galesburg MI, USA). Plants were placed randomly in each flat with a spacing of 5 cm between each plant. After one additional week in a 22 °C, 16 h photoperiod growth chamber with increasing light intensity to harden the plants off, the trays were transferred to a greenhouse at 20 °C with a 16 h photoperiod for 10 d in order to allow for the establishment of the plants. At the onset of the experiment, the plants from four trays were harvested for aboveground biomass measurement to determine relative initial plant size. The soil in the remaining trays was saturated to ensure uniform initial soil moisture conditions. Water was then withheld from four trays for each drought period, 9 d or 12 d, after which the soil was again saturated and plants were allowed to recover for one week. At the conclusion of the recovery period, survival and final biomass were determined. During the drought period,

four trays were watered normally to be harvested as unstressed controls along with the drought stressed plants for each time period. This experiment was conducted twice. Fisher's Exact Test was conducted to determine whether survival of transgenic lines differed from wild type. ANOVA analysis of the relative biomass gain for each line was conducted using PROC GLM with SAS (SAS Institute Inc., USA) software.

RESULTS

Heterologous expression of CBF does not confer drought tolerance

Survival of *P. hybrida* under drought conditions was not enhanced by high heterologous expression of CBF transcription factors. In fact, under these experimental conditions, the lowest survival rates were seen in lines expressing the transgenes at the highest levels (Table 5.1). Though not statistically different than wild type, the only plants that did not survive the 9 day drought in the first replication were pMPS13-7, while all plants survived the second replication. After 12 days of drought, the only plants that did not recover in the first replication were from the pMPS13-7 and pXIN1-17B lines, although only the survival of pMPS13-7 was statistically less than wild type. Likewise, in the second replication pMPS13-7 and pXIN1-17B had the lowest survival, but only pMPS13-7 was significantly lower than wild type. It should be noted that pMPS13-7 appears to have a much less extensive root system than wild type plants which may have contributed to the reduced survival under low water conditions.

In addition to survival data, relative biomass gain was used to determine how well the transgenic lines grew under water stress. Direct comparisons of biomass before and after drought are difficult because of the severe stunting seen in the pMPS13-7 line. The

pMPS13-7 line was smaller than wild type at the onset of the drought period and even under ideal conditions grew slower. Therefore, comparisons were made based on “relative gain” which was calculated as the percent of unstressed biomass gain that was achieved by stressed plants during the drought and recovery periods (Table 5.2). There was no significant variation between the biomass gained by the transgenic lines and the controls following either of the drought periods (Tables 5.3 and 5.4).

DISCUSSION AND CONCLUSIONS

Heterologous expression of CBF did not improve the survival of *P. hybrida* in our water withholding experiments. Survival of pMPS13-7 was significantly less than wild type following 12 days of water withholding. This may be due to the less extensive root system that was present on this high expressing transgenic line. The pXIN1-17B line also seems to have a less aggressive root system, but the survival of this line was not statistically different from wild type. This was a competitive survival study where transgenic lines were grown alongside wild type plants. The pMPS13-7 line is a severely stunted line that grows very slowly. This may have also reduced its survival because it was shaded by the taller wild type plants. However, this is not true for pXIN1-17B which has normal wild type growth. Under drought conditions typically encountered by a plant in the garden, it seems that the negative impact of high CBF expression on root growth may outweigh any potential benefit on drought tolerance at the cellular level due to CBF expression.

There was significant biomass variation between the two replications of this experiment, hindering the detection of differences between transgenic lines and controls.

There was no significant difference between the relative gain values for any of the lines (Tables 5.3 and 5.4). In the first replication, it appears that the pMPS13-7 line was less tolerant of water stress than wild type during 9 days of drought (73 % relative gain for pMPS13-7 vs. 93 % for wild type), but in the second replication it appeared to be more tolerant than wild type (336 % relative gain for pMPS13-7 vs. 109 % for wild type) (Table 5.2). The same was true for the 12 day drought period where wild type had 21 and 45 % relative gain in the first and second replications, respectively, while pMPS13-7 had 15 and 108 % (Table 5.2).

Table 5.1. Survival rates of transgenic *P. hybrida* 'Mitchell' plants expressing *AtCBF3* (pMPS13 and BpMPS13) or *LeCBF1* (pXIN1 and BpXIN1) following 9 or 12 days of water withholding and 7 days of recovery. Numbers shown represent percent survival of 12 individuals.

Genotype	9 day drought		12 day drought	
	Rep 1	Rep 2	Rep 1	Rep 2
Wild type	100	100	100	92
Empty vector	100	100	100	100
pMPS13-7-12	83	100	25*	17*
pMPS13-10-8	100	100	100	92
BpMPS13-101	100	100	100	100
pXIN1-17B-20	100	100	75	58
pXIN1-25B-12	100	100	100	83
BpXIN1-110-5	100	100	100	83
BpXIN1-120-2	100	100	100	92

* indicates significant variation from wild type survival as tested by Fisher's Exact Test (alpha = 0.05)

Table 5.2. Relative gain of above-ground dry biomass averaged for 12 plants per genotype in each drought treatment. Relative gain of drought plants was defined as the percent of non-stressed weight gain achieved by stressed plants; calculated as: $\text{Relative gain} = (\text{drought final} - \text{initial}) / (\text{nonstressed final} - \text{initial}) \times 100$

	Relative gain (%)			
	9-day drought		12-day drought	
	rep 1	rep 2	rep 1	rep 2
Wild type	93	109	21	45
Empty vector	59	75	38	44
pMPS13-7-12	73	336	15	108
pMPS13-10-8	79	148	28	45
BpMPS13-101	87	66	39	58
pXIN1-17B-20	158	72	15	36
pXIN1-25B-12	106	121	58	69
BpXIN1-110-5	83	81	32	54
BpXIN1-120-2	90	58	33	51

Table 5.3. ANOVA for effect of transgenic line on relative biomass gain for 9-day drought period.

Dependent variable: Relative Gain (%)	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	8	27813.11	3476.64	0.75	0.65
	Error	9	41766.00	4640.67		
	Total	17	69579.11			

Table 5.4. ANOVA for effect of transgenic line on relative biomass gain for 12-day drought period.

Dependent variable: Relative Gain (%)	Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
	Model	8	2480.00	310.00	0.49	0.83
	Error	9	5640.50	626.72		
	Total	17	8120.50			

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