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THE ROLE OF *CBF* ORTHOLOGS IN THE COLD ACCLIMATION OF BLOOMING SOUR CHERRY (*PRUNUS CERASUS*) AND STRAWBERRY (*FRAGARIA* X *ANANASSA*)

By

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ABSTRACT

THE ROLE OF *CBF* ORTHOLOGS IN THE COLD ACCLIMATION OF BLOOMING SOUR CHERRY (*PRUNUS CERASUS*) AND STRAWBERRY (*FRAGARIA* X *ANANASSA*)

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Many temperate fruit crops suffer yield reductions due to freeze damage. Several early blooming rosaceous crops, such as strawberry (*Fragaria* × *ananassa*), and many of the cultivated *Prunus* species, are particularly prone to floral freeze damage. The pistil and young fruitlet, is often the most susceptible to freezing injury. Many cultural techniques are employed to mitigate freeze damage to flowers, but these techniques are often costly and ineffective. Fruit cultivars with improved floral freezing tolerance would have a major effect on solving this problem.

Cold acclimation is the process by which a plant exposed to low temperature increases its freezing tolerance. Studies on cold acclimation of *Arabidopsis* have identified a family of cold-inducible transcription factors, CBF1, 2, and 3, that appear to be key regulators of cold acclimation and many cold regulated (*COR*) genes in a diverse number of plant families. Several *COR* genes appear to not be expressed in the pistils of *Arabidopsis* following exposure to low temperature despite expression in many vegetative and other reproductive tissues. Therefore, it was hypothesized that the *CBF-COR* gene system will be

conserved in the Rosaceae and that the expression of these genes will be compromised in pistils of rosaceous plants.

Following 16 days of 4°C treatment to whole plants, the freezing tolerance of strawberry receptacles was significantly enhanced, from -3.8 to -5.3 °C for 'Honeoye', with similar increases observed for many genotypes. In contrast, sour cherry pistils showed no significant increase in freezing tolerance for the 3 cultivars examined.

Orthologs of *CBF1* were cloned from strawberry and sour cherry with degenerate PCR primers. The putative orthologs *FaCBF* and *PcCBF* have 48% amino acid identity to *CBF1* and mRNA levels were up-regulated in leaves of both crops following exposure to 4° C from 15 minutes to 24 hours. mRNA expression of *FaCBF* and *PcCBF* in pistils of strawberry and sour cherry was not detected following 4° C exposure at any time point tested.

Agrobacterium-mediated transformation of a *CaMV35S-CBF1* construct was conducted on *Fragaria* × *ananassa* 'Honeoye' crown discs. Two transgenic lines were regenerated that expressed the transgene at low levels in both leaves and receptacles with pistils. Receptacles of the transgenic lines showed no significant change in freezing tolerance when compared to wild type plants. However, the temperature at which 50 % electrolyte leakage occurred in detached leaf-discs from the two transgenic lines was -8.2° C and -10.3° C, respectively. These freezing tolerance values were significantly greater than the value for the wild-type 'Honeoye' leaf discs of -6.4° C.

To Fergus and Ajax

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Introduction

Low temperature is one of the most important environmental factors limiting plant growth and development. Improved freezing tolerance is a major goal of plant breeders of many crops. The Rosaceae is a diverse plant family, consisting of many of the most economically important temperate fruit crops, such as apple (*Malus* × *domestica*), pear (*Pyrus communis*), peach (*Prunus persica*), sweet cherry (*Prunus avium*), sour cherry (*Prunus cerasus*), almond (*Prunus dulcis*), apricot (*Prunus armeniaca*), plum (*Prunus domestica*), strawberry (*Fragaria* × *ananassa*), and brambles (*Rubus spp.*). Almost all cultivated fruit species within this family are subject to freezing damage during bloom. Many rosaceous species bloom very early during the growing season, at a time in which fluctuating temperatures are often capable of dipping several degrees below freezing. In addition to the complete crop loss that can occur following severe spring frosts, the crop fluctuations that occur cause large swings in crop supply. Uncertain crop yields from year to year lead to insufficient crop in some years, and over-production and thus poor prices in others.

Within rosaceous flowers, the organ with the greatest susceptibility to freezing damage is often the pistil (Ki and Warmund, 1992; Darrow, 1966). For example, in strawberry, both receptacles and pistils have been shown to be killed by a temperatures as high as -2° C (Boyce and Marini, 1978). Field observations of sour cherry flowers following a freeze event in the orchard often show damaged pistils while the remaining floral tissues are undamaged (Dennis and

Howell, 1974). Since the pistil is often the most freezing susceptible tissue within the flower, breeding efforts directed at improving the physiological freezing tolerance of these flowers needs to first be directed at improving the freezing tolerance of the pistil.

Many cultural techniques are employed to prevent or reduce the damage that is caused by late frosts. These techniques include the use of field heaters, wind machines, overhead irrigation, and row-covers (Reiger, 1989). In addition, prevention of freezing damage during bloom is a major factor in orchard site selection. Fruit plantings are often established on hillsides where cool air can drain down the slope, away from the planting, and also near large bodies of water that help moderate temperatures, mitigating sudden dips in temperature. However, floral freezing damage within fruit plantings remains a major problem. Many of the cultural techniques employed to reduce freeze damage are either costly or not very effective (Perry, 1998). A more lasting solution to this problem would be the development of cultivars with enhanced floral freezing tolerance. A breeding approach to this problem could provide valuable new tools for the prevention of this serious problem.

Efficient breeding of fruit cultivars for improved floral freezing tolerance is dependent on the available information on the classical and molecular genetics of floral freezing tolerance in the Rosaceae. Freezing tolerance in rosaceous species is complexly inherited (Cain and Andersen, 1980; Watkins and Spangelo, 1970; Mowrey 1964; Powers, 1945; Dorsey and Bushnell, 1925). Little information is available on the inheritance of floral freezing tolerance in fruit

crops, although variation in floral freezing tolerance has been reported for several rosaceous crops: strawberry (Hummel and Moore, 1997; Boyce and Marini, 1978), apple (Janick and Moore, 1996), and peach (Scott and Cullinan, 1939). More detailed genetic analyses of floral freezing tolerance would be desirable to help provide information for the breeding of this trait in fruit cultivars.

Breeding for improved freezing tolerance:

Several approaches can be taken by plant breeders and plant scientists to attempt to improve the freezing tolerance of cultivated plants. Classical plant breeding has attempted to utilize naturally existing variation for freezing tolerance in the selection of parents and in the ascertainment of the quantitative genetics of freezing tolerance. However, for many important crops such as wheat and barley, whole plant freezing tolerance of current cultivars has not significantly changed in decades (Fowler and Gusta, 1979). Breeding for freezing tolerance in fruit crops has been more successful than the other major crops. However, most fruit crops are large in size, take up a large amount of space, and have long-cycling generations. As a result, even though genetic variability for floral freezing tolerance has not been exhausted, fruit crops have the disadvantage of taking an extremely long time to be bred through traditional methods, especially considering that many important traits, in addition to improved floral freezing tolerance need to be addressed by the fruit breeder.

Understanding the molecular basis of the variation in freezing tolerance in native germplasm could lead to the development of strategies for improving

freezing tolerance in elite germplasm. This approach has received little attention and is perhaps best suited for a crop that has sufficient naturally existing variation for freezing tolerance, such as several fruit species. While little work has been conducted on the genetics of freeze tolerance in fruit crops, several studies have shown that desirable alleles for quantitative traits do exist in wild accessions of several crop plants, even if those traits are not phenotypically evident (Xiao et al, 1998; Bernacchi et al., 1998; Tanksley and McCouch, 1997). For example, in tomato, QTL for fruit size and color have been identified and back-crossed from a wild accession of *Lycopersicon pimpinellifolium* to the cultivated *L. esculentum*, neither of which was observed in the wild selection (Bernacchi et al., 1998). Therefore, characterization and utilization of wild germplasm for crop improvement is likely to be another avenue for fruit breeders interested in improving floral freezing tolerance.

A third approach that is receiving attention is to attempt to understand how individual plants protect themselves from freezing temperatures. Many plants have the ability to cold acclimate, or develop increased freezing tolerance following exposure to low temperature. By understanding the physiological and molecular nature of cold acclimation it may be possible to develop novel strategies for improving plant cold hardiness. For some of our important agronomic crops in which freezing tolerance in new cultivars has plateaued, this approach is of significant interest. In *Arabidopsis*, manipulation of one regulatory gene, identified through this avenue of inquiry has lead to a whole plant

improvement in freezing tolerance through transgenic approaches (Jaglo-Ottosen et al., 1998).

Although variation for whole plant freezing tolerance exists within the available germplasm for many rosaceous species, variability for floral freezing tolerance appears to be much more limited. No major advances in floral freezing tolerance have been made in many fruit cultivars over the last several decades. Despite some variation, the floral freezing tolerance of strawberry cultivars introduced in the 1970s is little better than cultivars introduced in the 1920s (Boyce and Marini, 1978). Therefore, an approach attempting to determine if manipulation of the cold acclimation machinery is capable of improving the floral freezing tolerance of these plants is warranted.

Cold acclimation in plants:

Sensing of cold temperature:

There are still many gaps in our understanding of how plants sense low temperature, translate this information into alterations of gene expression, plant biochemistry and physiology, and eventually change plant phenotype (ie. cold acclimation). The specific nature of how plants first sense low temperatures is poorly understood. It has been postulated that changes in membrane fluidity during exposure to low temperature may lead to conformational changes in membrane bound proteins (Murata and Los, 1997; Monroy and Dhindsa, 1995; Minorsky, 1989). Changes in protein conformation could lead to changes in protein activity, binding, or perhaps even localization. One specific change could

be in the activity of membrane bound Ca^{2+} channels. Ca^{2+} has been implicated as an important signal transduction pathway component in response to low temperature. Transient increases in cytosolic Ca^{2+} caused by influxes of Ca^{2+} from extracellular locations have been demonstrated in response to low temperatures (Torrecilla et al., 2000; Monroy and Dhindsa, 1995; Knight et al., 1991). Experiments preventing this influx of Ca^{2+}, through the use of Ca^{2+} chelators or Ca^{2+} channel blockers, has been shown to prevent expression of low-temperature induced genes and to reduce the plant's ability to cold acclimate (Monroy and Dhindsa, 1995).

A full description of the events both up and downstream of these Ca^{2+} increases waits to be determined, but additional experiments have suggested a signal transduction cascade involving protein phosphorylation (Monroy et al., 1998), Ca-dependent protein kinases (Sheen, 1996), mitogen-activated protein kinases (Jonak et al., 1996) and a calcineurin B-like protein (Kudla et al., 1999). The likely end result of such a cascade is the activation of transcription factors necessary for the induction of genes conferring freezing tolerance to the plant.

Regulation of cold-induced gene expression:

Many genes have been identified that are regulated in response to low temperature. Our knowledge of how these genes are regulated and their exact role in freezing tolerance is still incomplete. Two main approaches have been taken to better understand the factors that regulate this large number of cold-induced genes. One approach, based on the differential expression of either

proteins or mRNA transcripts has either directly or indirectly lead to the identification of regulatory proteins involved in the regulation of cold-induced gene expression: *CBF1*, *2*, and *3* in *Arabidopsis thaliana* (Stockinger et al., 1997, Gilmour et al., 1998) and *SCOF1* in *Glycine soja* (Kim et al., 2001). A second approach has been to identify mutants with altered cold acclimation or freezing tolerance phenotypes. In *Arabidopsis*, this mutational approach has identified: sensitivity to freezing (*sfr*) mutants that do not cold acclimate (Warren et al., 1996), *eskimo1* that shows constitutive freezing tolerance even at warm temperatures (Xin and Browse, 1998), and *HOS1* which was identified by possessing heightened expression of several cold-regulated genes following cold treatment (Ishitani et al., 1998).

The combined data from both of these approaches is beginning to provide a framework for understanding plant gene expression in response to cold. Cold signaling pathways appear to be complex, and have many points in parallel as well as many interactions (Ishitani et al., 1997). However, there appear to be at least 3 partially distinct pathways leading to the induction of cold regulated gene expression: ABA-independent and ABA-dependent pathways that ultimately up-regulate many of the same genes, and a separate pathway defined by *eskimo1*.

<u>CBF-COR genes/sfr6/hos1/ABA-independent pathway:</u>

Perhaps the best characterized cold-induced genes are the *Arabidopsis cor* genes. In *Arabidopsis*, studies of the molecular basis of cold acclimation have identified a common *cis*-acting regulatory element, the C-

repeat/Dehydration Responsive Element (CRT/DRE) which is defined as a CCGAC, within the promoters of several cold induced (*COR*) genes (Stockinger et al., 1997). In turn, all of the currently characterized *Arabidopsis COR* genes are coordinately up-regulated by *CBF1*, *CBF2*, or *CBF3*, a family of cold and drought inducible transcriptional activators that bind to promoters containing a CRT/DRE (Thomashow et al., 2001; Kasuga et al., 1999; Liu et al., 1998; Jaglo-Ottosen et al., 1998). Even though freezing tolerance is a complexly inherited trait, manipulation of just one gene, *CBF1*, has been shown to improve the whole plant freezing tolerance of *Arabidopsis* and *Brassica napus* (Jaglo, 2001; Jaglo-Ottosen, et al., 1998). Additionally, it is known that these *COR* genes can be up-regulated by additional transcription factors DREB2A and DREB2B, which are important in the response to dehydration (Liu et al., 1998).

The CBF family of transcriptional activators are up-regulated in response to cold and drought stress, but not by exogenous ABA (Gilmour et al., 1998). CBF1, 2, and 3 are AP2/EREBP domain containing proteins. How *CBF* is regulated and how it is connected to upstream signaling components is not known.

SFR6 is a protein that may interact with CBF1 in the cold-induced regulation of the COR genes (Knight et al., 1999). Evidence for this comes from the *sfr6* mutant in *Arabidopsis* which is deficient in freezing tolerance following cold acclimation, and does not show expression of *COR6.6*, *COR15a*, or *COR78* in response to cold temperature, even though *CBF3* expression following cold exposure is normal (McCown et al., 1996; Warren et al., 1996; Knight et al.,

1999). It has been postulated that SFR6 is required for cold-induced activation of the CBF3 protein. The exact nature of the interaction between SFR6 and CBF3 is not known, nor has the SFR6 protein been characterized.

Another mutant, *hos1*, exhibits enhanced expression of several *COR* genes in response to cold temperature (Ishitanit et al., 1998). In addition, CBF2, CBF3, but not CBF1 were shown to have enhanced gene expression in the *hos1* mutants. HOS1 encodes a novel ring-finger protein that is constitutively expressed in *Arabidopsis*, and has been shown to be targeted to the nucleus following cold temperature exposure (Lee et al., 2001). The native HOS1 transcript is transiently expressed in response to cold, but quickly recovers and is expressed for up to 2 days before declining. Ring-fingers are small, zinc-binding domains found in many proteins with diverse function, and may be involved in ubiquitination and subsequent degradation of proteins. It has been speculated that HOS1 may be a regulator of the up-stream signaling components leading to cold-induced CBF expression (Lee et al., 2001).

SCOF1/ABA-dependent pathway

SCOF1 is a soybean regulatory protein that defines a related cold signal transduction pathway that appears to be ABA-dependent (Kim et al., 2001). SCOF1 is a cold and ABA-inducible zinc-finger protein, that has been shown to interact with a G-box protein in soybean to up-regulate genes that contain G-boxes in their promoter regions (Kim et al., 2001). G-boxes are a common

promoter element which share the same core sequence as ABA-responsive-elements (ABRE) – ACGT. Many characterized COR genes, such as *COR15a* and *COR78* possess both ABRE and CRT/DRE in their promoters (Stockinger et al., 1997; Baker et al., 1994). Expression of *SCOF1* in *Arabidopsis* leads to *COR* gene expression in the absence of a cold stimulus and enhanced freezing tolerance when compared to non-transgenic controls (Kim et al., 2001). However, SCOF1 does not appear to regulate COR gene expression through the CRT/DRE as is the case with CBF1, 2, and 3. SCOF1 appears to define a cold-sensing pathway that leads to the induction of many genes similar to those on the CBF-ABA independent pathway, but does so through a different set of intermediaries, possibly involving ABA.

eskimo1

At least one additional cold signaling pathway may be associated with *eskimo1*. *eskimo1* is an *Arabidopsis* mutant that shows constitutive freezing tolerance in the absence of a prior cold temperature stimulus (Xin and Browse, 1998). Non-acclimated *eskimo1* plants possess approximately 70% of the freezing tolerance of acclimated wild type plants, and acclimated *eskimo1* plants possess freezing tolerance greater than acclimated wild type plants. Significantly, 4 *COR* genes are not expressed in the non-acclimated *eskimo1* plants, suggesting that these mutants achieve increased freezing tolerance without up-regulating the genes in the CBF-COR gene pathway. *Eskimo1* mutants show enhanced levels of proline and soluble sugars, known compatible

osmolytes involved in providing increased freezing tolerance, increased expression of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), the key regulator of proline biosynthesis, and decreased expression of proline oxidase (AtPOX), a key enzyme of proline degradation (Xin and Browse, 1998).

Integrating gene expression to physiological responses to cold:

Many physiological and biochemical changes are known to occur in plants in response to low temperature. These changes include: alterations in lipid metabolism, changes in the properties of membranes (Murata and Los, 1997), shifts in carbohydrate production and localization, accumulation of proline, betaine, and other compatible osmolytes (Levitt, 1980) , and shifts in plant secondary metabolism (Guy, 1990). However, very few of the cold-induced genes characterized in plants have been directly tied to a known physiological function. Linking the large number of cold-induced physiological and biochemical changes to the molecular genetics of cold acclimation is a major future challenge.

Cold induced genes fall into many categories including: lipid desaturases, antifreeze proteins, and superoxide dismutases; the largest number having unknown functions. A large subset of the genes of unknown function share similarity with a class of proteins known as LEA proteins, which were first described in cereals and are transcribed late in embryogenesis during seed desiccation (Thomashow, 1999). Even though some of these cold-induced genes share homology with LEA proteins and some do not, many of them share several common characteristics. Common traits include: proteins that are highly

hydrophilic, possess simple amino acid compositions, remain soluble upon boiling in aqueous buffer, and have many simple repeated amino acid motifs (Thomashow, 1999). More detailed studies of one of these genes, *cor15a* from *Arabidopsis*, suggest that the COR15am protein has the ability to shift downward the temperature at which the lamellar to hexagonal II phase transition occurs in plant membranes (Steponkus et al., 1998). This phase transition is known to lead to extensive cellular damage upon return of the cells to non-freezing temperatures. This temperature shift allows the cells to withstand lower freezing temperatures without suffering damage. In the case of COR15am, the inner membrane of the chloroplast is thought to be the binding target, and it is hypothesized that this protein has the ability to alter the intrinsic curvature of the membrane leading to the temperature shift of the phase transition (Steponkus et al., 1998). Since many of the cold-induced genes share properties with COR15a it is possible that membrane stability is a common mode of freezing tolerance in plants. However, little is known about the physiological role of most cold-induced plant genes.

CBF as a key regulator of cold acclimation:

Considering that *CBF* genes are some of the best characterized cold acclimation related genes, appear to be key regulators of cold acclimation, and their over-expression is capable of improving the freezing tolerance of *Arabidopsis*, there is significant interest in determining if orthologs of the CBF

gene family exist and function in a similar fashion within cultivated plants, including the Rosaceae.

Indeed, CBF-like proteins have been identified in *Brassica napus, Triticum aestivum*, and *Secale cereale*, and the chilling-sensitive *Lycopersicon esculentum* (Jaglo et al., 2001). In these crops, these CBF-like proteins are cold-regulated in a fashion similar to CBF1, 2, and 3 of *Arabidopsis*, and possess high levels of amino acid identity. Additionally, overexpression of *CBF1*, *2*, or *3* in *Brassica napus* increases its freezing tolerance and up-regulates *Bn115* and *Bn28*, homologs of *COR15a* and *COR 6.6* of *Arabidopsis*. These findings suggest the CBF-COR system is highly conserved in diverse plant families, and may be a key regulator of cold temperature stress tolerance in many plants. If this set of genes is conserved in the Rosaceae, perhaps these genes can be manipulated to improve floral freezing tolerance.

Cold induced gene expression in the Rosaceae has not received much attention. However, a small number of cold induced genes have been identified through analyses of differential expression of proteins and mRNA in response to cold. A cold-induced dehydrin gene, *ppdhn1*, has been found to be expressed in the bark and xylem parenchyma cells of peach, shows increased expression during winter months and is associated with differences in cold hardiness in sibling genotypes of evergreen and deciduous peach (Artlip et al., 1997; Wisniewski et al., 1999). Three cold induced genes have also been identified from leaves of strawberry, *Fcor1*, *2*, and *3* (Ndong et al., 1997). *Fcor1* appears homologous to *LTI6b*, a low-temperature induced gene of Arabidopsis. *Fcor2*

appears to code for an hydroxyproline rich glycoprotein, and *Fcor3* which shows reduced transcript expression in response to low temperature has some similarity with subunit V from spinach photosystem I (Ndong et al., 1997).

Perhaps the most efficient approach to elucidating the molecular basis of cold acclimation in the Rosaceae, would be to take advantage of the wealth of information that is being generated from studies in model plant species, such as *Arabidopsis*. As mentioned above, the CBF family of transcriptional activators are important regulators of cold acclimation in *Arabidopsis*. When over-expressed these genes have been shown to enhance the freezing tolerance of both *Arabidopsis* and *Brassica napus*, and orthologs of the *CBF* gene family have been identified in a diverse array of plant species, such as wheat, rye, and tomato (Jaglo et al, 2001). Identifying and characterizing *CBF* orthologs in the Rosaceae would be a good starting point to transfer the information gained from model species to this important family of cultivated plants.

The interaction between cold temperature and photoperiod on cold acclimation of woody fruit crops has been extensively studied (Howell and Weiser, 1970; Weiser, 1970), and it is known that the over-wintering structures of perennial, temperate fruit crops possess the ability to cold acclimate. However, the ability of reproductive tissues of perennial plants to cold acclimate is largely unknown, although there is indirect evidence that rosaceous flowers may have that ability. Some cultivars of peach trees de-acclimated under a fluctuating 15/5° C temperature regimen have marginally better floral freezing tolerance than those brought out of dormancy at a constant 25° C (Lu and Rieger, 1993). There

is anecdotal evidence that peach flowers in the orchard survive frosts better following periods of cool temperature (Scott and Cullinan, 1939). Additionally, flowers from day-neutral strawberries collected at the end of a two-month period in autumn had slight improvements in floral freezing tolerance compared to the flowers collected at the onset of the study period (Boyce and Marini, 1978). While these studies provide circumstantial evidence that floral tissues can cold acclimate, there have been no direct comparisons of the freezing tolerance of non-acclimated rosaceous flowers to those exposed to low, non-lethal temperature.

The objectives of this research were to:

1) determine if the cold acclimation response observed in *Arabidopsis* also occurs in newly formed strawberry and sour cherry pistils and leaf tissue.

2) determine if expression of CBF1 increases freezing tolerance in the pistils, and vegetative tissues of strawberries, which is transformable.

3) determine if strawberry and sour cherry possess homologs of CBF and if so: (a) whether these genes are turned on in response to cold temperatures, (b) whether these cold regulated genes are differentially expressed between the pistils and the vegetative tissues, and (c) if differential gene expression will correlate with lower frost tolerance of the pistils compared to the vegetative tissues.

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Chapter 1 – Cold Acclimation in Blooming Strawberry (Fragaria × ananassa) and Sour Cherry (Prunus cerasus))

Many temperate fruit crops suffer yield reductions due to freeze damage. Early season freezes during or just prior to bloom are the main cause of low-temperature induced crop loss (Rodrigo, 2000). Several early blooming rosaceous crops, such as strawberry (*Fragaria* × *ananassa*), and many of the cultivated *Prunus* species, are particularly prone to floral freeze damage. The economically viable part of the plant, the pistil and young fruitlet, is often the most susceptible to freezing injury (Ki and Warmund, 1992; Darrow, 1966). Site selection, row covers, overhead irrigation, and other cultural techniques are often employed to mitigate freeze damage during bloom. However, these techniques are often labor intensive, costly, or not very effective (Perry 1998; Anderson and Whitworth, 1993; Rieger, 1989). Fruit cultivars with improved floral freezing tolerance would provide lasting and more cost-effective means of preventing damage.

Many plants have the ability to cold acclimate, or develop improved freezing tolerance following exposure to low, but non-lethal temperatures. Cold acclimation is associated with numerous biochemical changes in plants: increases in proline concentration, sugars, and other compatible solutes; changes in lipid metabolism (Thomashow, 1999; Hughes and Dunn, 1990); and, significantly, changes in gene expression. Genes thought to be associated with the plants adaptation to freezing temperatures have been identified in many plant

species, such as alfalfa (*Medicago sativa*), spinach (*Spinacia oleracea)*, barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and *Arabidopsis thaliana* (Monroy et al. 1993; Hong et al. 1992; Houde et al. 1992; Guy et al., 1985).

One approach to develop cultivars with enhanced freezing tolerance is to identify and utilize candidate genes playing an important role in this trait. Although freezing tolerance is a complexly inherited trait, whole plant freezing tolerance of *Arabidopsis* can be improved by the over-expression of a single cold-induced transcription factor (Jaglo-Ottosen et al., 1998). A large number of cold regulated genes have been identified from many crop plants, including a few from rosaceous fruit crops (Wisniewski et al., 1999; NDong et al., 1997). It is plausible that cold-induced genes exist within the Rosaceae that are orthologous to those in *Arabidopsis* capable of improving freezing tolerance. Whether these genes can be utilized, through either biotechnological manipulation or exploitation of natural allelic variation, to improve floral freezing tolerance in rosaceous crops is unknown. It is also unknown if the same relationship exists between cold-induced gene expression and onset of cold-acclimation in rosaceous flowers. Before an attempt to identify genes associated with cold-acclimation in rosaceous flowers, it is desirable to first determine if these flowers are capable of cold acclimation.

The interaction between cold temperature and photoperiod on cold acclimation of woody fruit crops has been extensively studied (Howell and Weiser, 1970; Weiser, 1970), and it is known that the over-wintering structures of perennial, temperate fruit crops possess the ability to cold acclimate. However,

the ability of reproductive tissues of perennial plants to cold acclimate is largely unknown. There is indirect evidence that rosaceous flowers may have an ability to cold acclimate. Some cultivars of peach trees de-acclimated under a fluctuating 15/5° C temperature regimen have marginally better floral freezing tolerance than those brought out of dormancy at a constant 25° C (Lu and Rieger, 1993). There is anecdotal evidence that peach flowers in the orchard survive frosts better following periods of cool temperature (Scott and Cullinan, 1939). Additionally, flowers from day-neutral strawberries collected at the end of a two-month period in autumn had slight improvements in floral freezing tolerance compared to the flowers collected at the onset of the study period (Boyce and Marini, 1978). While these studies provide circumstantial evidence that floral tissues can cold acclimate, there have been no direct comparisons of the freezing tolerance of non-acclimated rosaceous flowers to those exposed to low, non-lethal temperature.

The objectives of this study were to determine if the flowers of sour cherry (*Prunus cerasus*) and strawberry (*Fragaria x ananassa*) are able to cold acclimate. The specific objectives of this research were to: 1) determine if the leaves of fully blooming, non-acclimated strawberry and sour cherry grown under long-days have the ability to cold acclimate, 2) determine if the receptacles and pistils of blooming, non-acclimated strawberry and sour cherry, respectively, grown under long-days have the ability to cold acclimate, and 3) determine if there is any genotypic variability among cultivars in the ability of strawberry and sour cherry pistils to cold acclimate.

Materials and Methods

Plant Material, growth conditions:

Containerized, dormant, rooted runners of 'Honeoye' and 'Chandler' *Fragaria* × *ananassa*; RH-18, EBB1, NC 95-16-6, NC 95-18-5, NC 96-29-1, and NC 96-28-1 *F. virginiana*; FRA 0883, and FRA 368 *F. chiloensis*; as well as 3 year old, dormant plants grown from rooted cuttings of 'Montmorency', 'Rheinische Schattenmorelle', and 'Erdi Botermo' *Prunus cerasus* were grown in growth chambers maintained at 24 °C/18 °C with 14 h day-lengths at 800 $mmol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) and 8 h nights until the onset of flowering. Four degree cold treatments were administered by transferring plants to a cooler maintained at a constant 4 °C with 14 h day-lengths at 200 $mmol \cdot m^{-2} \cdot s^{-1}$ PAR and 8 h nights.

Table 1. Geographic origin of strawberry selections utilized in freezing tolerance experiments

Selection	Geographic Origin
Fragaria × anannasa	
Honeoye	New York
Chandler	California
Fragaria virginiana	
EBB1	Alaska
RH-18	Wyoming
NC 95-16-6	Mississippi
NC 95-18-5	Mississippi
NC 96-28-1	Alabama
NC 96-29-1	Alabama
Fragaria chiloensis	
FRA 0883	British Columbia
FRA 368	British Columbia

Determination of Freezing Tolerance:

Freezing tolerance was determined on detached leaf-discs and pistils (sour cherry) or receptacles with pistils (strawberry) from fully open flowers by two methods : visual scoring and electrolyte leakage (Sukumaran and Weiser 1972). Detached pistils were visually scored as either dead (brown) or alive (green). For the electrolyte leakage experiments leaf-discs, pistils, or receptacles with pistils were detached from the whole plant, and stored in de-ionized, distilled water until all had been placed in 16 mm diameter x 125 mm long glass culture tubes. Three leaf discs, 5 cherry pistils, or 3 strawberry receptacles with pistils

were placed in each culture tube. Data from 3 tubes were pooled for each temperature point of the controlled freezing run.

Freezing-runs were conducted by placing the tubes in freezing-water baths containing a mixture of ethylene glycol and water. The tubes were maintained at 0 °C for 1 h, after which a small amount of de-ionized ice was placed in contact with the plant material in each tube to nucleate ice formation. The tubes were kept for an additional hour at 0 °C, and the temperature was then decreased at the rate of 4 $°C \cdot h^{-1}$. Every 30 minutes a set of tubes was transferred to a separate freezing-bath kept at the same temperature as the bath it came from, and maintained at that temperature for an additional 30 minutes (for a total of 1 h at each temperature). After 1 hour of freezing, tubes were placed on ice until the completion of the freezing experiment. Freezing runs were carried out to a final temperature of –20 °C for leaf-discs, and –10 °C for pistils and pistils/receptacle.

Following completion of the freezing run, tubes were placed in a rack on top of ice, wrapped with plastic, and kept at 4 °C overnight. Following thawing, 3 mL of de-ionized, distilled water were added to each tube, and the tubes were then shaken for 3 hours. Electrical conductivity of the water was measured from each tube with a bench-top electrical conductivity meter (Corning 441, Acton, MA), the plant material in the tubes was placed at –80 °C for 1 hour, and the original water was then added back to each tube for an additional 3 hours, with agitation. A final electrical conductivity was then determined for the water in each tube.

Three sets of data points were generated for each temperature point of the freezing run, with 3 tubes representing each data point. These data were used to generate an electrolyte leakage curve over the range of temperatures used in the freezing run. Three to four curves were generated per treatment.

Determination of Relative Water Content:

The fresh weight and dry weight of strawberry receptacles were determined for 'Honeoye' and 'Chandler' following either exposure to 4° C for 16 days or 0 days to determine the relative water content of each.

Data Analysis:

The electrolyte leakage and visual damage data were fit to a logistic model ($y = a/(1 + be^{-cx})$) with CurveExpert 1.3 (Microsoft Corp.). The temperature at which 50 percentage electrolyte leakage (EL^{50}) occurred was used to compare treatments. The EL^{50} was determined by solving the logistic equation fit to each curve with y set to 50 % for each curve. (Linden et al., 2000)

Statistical significance of treatment comparisons was made with either a two-sample t-test or one-way analysis of variance with Minitab 13.0 (Minitab Inc. State College, PA).

Results

Freezing tolerance of leaves:

Both sour cherry and strawberry leaf discs from fully blooming plants showed a significant increase in freezing tolerance following 8 to 16 days of exposure to 4° C (Table 2). The highest freezing tolerance was observed after 16 days of exposure to cold temperature.

Table 2. Temperature at which 50% electrolyte leakage occurred for leaf discs removed from blooming, whole plants exposed to 4° C for 0, 2, 4, 8, or 16 days.

Days of exposure to 4° C	Sour Cherry[a]	Strawberry
0	-5.4 a	-6.4 a
2	-5.5 a	-6.9 a
4	-5.2 a	-7.0 a
8	-7.1 b	-8.5 b
16	-10.9 c	-13.2 c

[a] Values within columns followed by the same letter are not significantly different by LSD, α= 0.05. Cultivars used were 'Montmorency' sour cherry and 'Honeoye' strawberry.

Freezing tolerance of flowers:

Since 16 days of exposure to 4° C provided the greatest increase in freezing tolerance in strawberry and sour cherry leaves, this treatment was utilized to determine the effect of low-temperature exposure on pistil freezing tolerance. In strawberry, significant increases in receptacle freezing tolerance were observed in about half of the selections examined when measured by electrolyte leakage after 16 days of cold treatment (Table 3). When measured by visual scoring of receptacle and pistil viability, all of the genotypes except RH-18

showed a significant increase in freezing tolerance (Table 1.3). There was no significant correlation between the 50% electrolyte leakage values for leaf discs and the pistil damage values, regardless of the method utilized to determine the freezing tolerance (data not shown). No significant differences were observed between the freezing tolerance of any sour cherry pistils exposed to cold temperature and those that were not, regardless of genotype (Table 1.3).

Variation in floral freezing tolerance:

Significant variation in strawberry receptacle freezing tolerance was observed for all methods utilized to determine the freezing tolerance, and for both non-acclimated and non-acclimated plants. The freezing tolerance of non-acclimated receptacles with pistils ranged from -1.0° C to -4.2° C by visual scoring, and acclimated receptacles with pistils ranged from -4.0° C to -7.6° C (Table 1.3). The rankings of the genotypes for receptacle freezing tolerance was not correlated between the two methods utilized for measuring freezing tolerance. No discernible patterns were observed between floral freezing tolerance and geographic origin of the strawberry genotypes or species.

When measured at the α =0.10 significance level, there were significant genotypic differences among the three sour cherry cultivars for pistil freezing tolerance when measured by electrolyte leakage following 16 days of 4° C treatment (Table 3). No other significant genotypic differences in pistil freezing tolerance were observed in sour cherry.

Table 3. Temperature at which 50% electrolyte leakage or half of the pistils or receptacles plus pistils were dead occurred for strawberry receptacles, leaf discs, and sour cherry pistils removed from blooming, whole plants exposed to 4° C.

Genotype	Visual Scoring		Electrolyte Leakage		
	Receptacles		Receptacles		Leaf Discs
	0 days of 4° C	16 days of 4° C	0 days of 4° C	16 days of 4° C	16 days of 4° C
Honeoye	-3.8	-5.3 **	-3.6	-6.7 **	-13.2
Chandler	-2.6	-4.5 **	-3.0	-6.5 **	-10.6
EBB1	-1.0	-4.0 **	-3.8	-4.8 ns	-15.3
NC 96-28-1	-2.5	-5.1 **	-3.4	-4.7 ns	-14.4
NC 95-18-5	-2.5	-6.0 **	-6.3	-6.3 ns	-11.7
NC 95-16-6	-3.1	-5.3 **	-4.2	-5.3 ns	-11.4
NC 96-29-1	-3.5	-6.1 **	-5.3	-4.0 ns	-11.4
RH-18	-4.2	-5.0 ns	-4.5	-5.7 ns	-12.1
FRA 0883	-3.0	-7.6 **	-2.9	-6.0 **	-12.2
FRA 368	-3.2	-6.3 **	-3.8	-7.0 **	-11.5
F-test within column	**	**	**	**	**
	Pistils		Pistils		Leaf Discs
Montmorency	-1.5	-2.5 ns	-1.9	-2.3 ns	-10.9
Rheinische Schattenmorelle	-2.0	-2.8 ns	-2.7	-3.4 ns	-11.4
Erdi Botermo	-2.2	-2.7 ns	-2.0	-2.9 ns	-11.2
F-test within column	ns	ns	ns	*	ns

**, ns: within row represent significant at $\alpha = 0.05$ or non-significant t-test for comparisons of 0 days and 16 days of cold treatment for both electrolyte leakage and visual scoring. *, ns: within column represent significant at $\alpha = 0.10$ or non-significant F-test.

Relative Water Content (RWC) of acclimated strawberry receptacles:

No significant differences in the RWC were detected between non-acclimated and acclimated 'Honeoye' or 'Chandler' receptacles (Table 4).

Table 4. Relative water content of strawberry receptacles/pistils from non-acclimated and acclimated plants.

Cultivar	0 days of 4° C	16 days of 4° C
Honeoye	67.4%	67.8%^{ns}
Chandler	77.4%	78.6%^{ns}

ns: non-significant t-test within row at $\alpha = 0.05$.

Discussion

The results from this study demonstrate that fully expanded leaves of actively growing, flowering strawberry and sour cherry possess the ability to cold acclimate after 8 to 16 days of exposure to 4° C with a 14 hour photoperiod (Table 1.2), with maximal cold hardiness levels attained of –10.9° C and –13.2° C after 16 days of 4° C exposure for sour cherry and strawberry, respectively. By comparison, *Arabidopsis* leaves reach a maximal cold hardiness, measured as the 50 % electrolyte leakage point, of approximately –9.0° C following 12 days of exposure to 4° C, with no additional increase in freezing tolerance observed following 4° C up to 25 days (Gilmour et al., 1988).

Receptacles and their attached pistils of most of the strawberry genotypes examined had enhanced freezing tolerance following exposure to 4° C for 16

days (Table1.3). After this 16 day period, the strawberry pistils/receptacles often showed statistically significant improvement in freezing tolerance when measured by electrolyte leakage or by visual scoring.

In contrast, the three sour cherry cultivars examined in this study showed no statistically significant enhancement in pistil freezing tolerance under the same conditions reported for strawberry. Although not a direct comparison, these data are consistent with the reported literature on floral freezing tolerance in strawberry and *Prunus*. Flowers of day-neutral strawberry have been observed to have enhanced freezing tolerance in the autumn, compared to flowers borne earlier in the season (Boyce and Marini, 1978). Conversely, peach flowers have been reported to either not possess, or show only slight differences in, the ability to de-acclimate when brought out of dormancy in cool conditions as opposed to warm (Lu and Rieger, 1993). Data from field observations place the freezing tolerance of sour cherry pistils at full bloom to be approximately –2.2° C (Dennis and Howell, 1974), close to the values determined under controlled conditions in this study. Taken together these findings suggest sour cherry pistils are unlikely to be able to withstand freezing temperatures below approximately –2 to -3°C at full bloom. Perhaps sour cherry selections have developed adaptive changes in bloom date in order to avoid freezes instead of developing physiological freezing tolerance per se.

Some differences were observed in the freezing tolerance of strawberry and receptacles when measured by electrolyte leakage as opposed to visual scoring. For many genotypes these differences were minor, often less negative

values being measured by visual scoring than by electrolyte leakage. However, for two strawberry genotypes, visual scoring seemed to be a better indicator of freezing damage. NC 95-18-5 receptacles showed no significant change in freezing tolerance when measured by electrolyte leakage, with non-acclimated plants showing a surprisingly high freezing tolerance. The visual scoring of these same samples indicate that there was a clear difference in the freezing damage between the 0 and 16 days of exposure to 4° C. Also, NC 96-29-1 pistils, showed a high freezing tolerance at 0 days of exposure to 4° C and low freezing tolerance after 16 days of 4° C when measured by electrolyte leakage. The cause of these discrepancies is not known. Since visual scoring is an un-ambiguous method for determining the viability of these tissues, as well as being much more rapid than electrolyte leakage, it is recommended that visual scoring be utilized as a method for determining strawberry and sour cherry pistil damage following controlled freezes.

Significant variation for floral freezing tolerance was observed in the strawberry genotypes examined at either the non-acclimated or acclimated state, approximately 3° C between the extreme genotypes. Similarly, significant variation in the scale of freezing tolerance improvement following low temperature exposure was seen in strawberry. These results suggest there may be useful variation for floral freezing tolerance and floral cold acclimation in strawberry. Variation in freezing tolerance did not appear as great in sour cherry, but only a small number of genotypes were examined.

Although significant variation in floral freezing tolerance was observed among the strawberry genotypes tested, no discernible eco-geographical patterns could explain this variation. The strawberry selections tested were chosen to examine a wide range of latitudes and elevations of origin. However, these factors may not be the best predictors of temperature during bloom. Strawberries are often covered by snow in northern areas and at upper elevations. The potential for an insulating snow cover and also for the variance in spring-temperature fluctuations may be better indicators of a plants ability to withstand freezing temperatures during bloom. Additionally, the variability measured in these experiments may be suggestive of ecological variation during the autumn. Strawberry flowers of short-day genotypes are initiated in the autumn in response to decreasing day-lengths and temperature. The cold acclimation ability observed here probably exists as part of the plant's mechanism for withstanding autumn and winter temperatures. As mentioned earlier, the length of cold treatment required to observe an increase in pistil freezing tolerance in strawberry is longer than is likely to occur in the spring. Such a prolonged, but non-damaging cold period is more likely to occur in the autumn. Therefore, the variation in floral cold acclimation ability may be better explained by exploring climatic and ecological variation of these genotypes at the ends of the growing season as the plants are beginning to harden off.

The freezing tolerance of leaves was not significantly correlated with the freezing tolerance of pistils. These results suggest freezing tolerance of leaves is not a good indicator of floral freezing tolerance or of the ability for flowers to cold

acclimated. Probably the best way to screen for floral freezing tolerance in breeding parents is to visually assess damage in non-acclimated plants, considering the length of time required to observed a significant increase in floral freezing tolerance. The plants have the ability to genetic capability to cold acclimate so an important question is why are pistils either unable or less proficient in the ability to acclimate?

Several studies have shown a correlation between tissue water content and freezing tolerance (Graham and Mullin, 1976; Johnston 1923). Our results showed no significant difference in the tissue water content of non-acclimated and acclimated 'Honeoye' and 'Chandler' receptacles and pistils. Although, only two cultivars were examined, this evidence suggests that genetic and physiological factors other than water content play a role in freezing tolerance following cold acclimation.

The reason why strawberry and sour cherry flowers' differ in their ability to cold acclimate is not known. One possibility is that the addition of receptacle tissue enhances the pistil freezing tolerance in strawberries. No observations were made of non-viable styles attached to viable receptacle tissue following a freezing event. However, other reports of controlled freezes of strawberry floral parts have shown no significant difference in the freezing tolerance of pistils and receptacles at 5 stages of floral development (Ki and Warmund, 1992). In fact, there was a striking correspondence in the critical temperature for freezing damage between the 2 tissues for 4 of the 5 developmental stages examined. It

therefore seems unlikely that the ability of strawberry pistils to acclimate is an artifact of the inclusion of receptacle tissue in the freezing damage assessment.

Some authors have suggested that leaves are critical for the sensing of, and attainment of cold acclimation in fruit trees (Weiser, 1970). Defoliation of apple trees has been shown to limit the ability of these plants to cold acclimate as they enter dormancy (Howell and Stackhouse, 1973; Weiser, 1970). One speculation is that sour cherry pistils have a reduced ability to acclimate, compared to strawberry, because of the reduced canopy development of these plants compared to strawberry, which maintain a fuller canopy during flowering. This hypothesis could be tested by examining the effects of reduced leaf area on strawberry floral cold acclimation.

Manipulation of one key regulator of cold acclimation in *Arabidopsis* has led to an enhancement of freezing tolerance of this plant (Jaglo-Ottosen et al., 1998). Knowledge that strawberry pistils also exhibit the ability to cold acclimate raises the possibility that these plants may have a similar molecular machinery underlying the cold acclimation response, and that it may be active in these tissues. Regardless of the validity of this hypothesis, there is likely to be a molecular component underlying the cold acclimation response in strawberry. By identifying these components, it may be possible to improve floral freezing tolerance of these plants and these tissues. In contrast, sour cherry pistils do not appear to acclimate, suggesting that there may be a deficiency in the signal transduction pathway between the sensing of cold temperature and the onset of acclimation, a deficiency which apparently does not exist in leaves. Identifying

which of these components, if any, is non-functional may ultimately lead to the ability to manipulate the underlying genes and improve floral freezing tolerance.

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Chapter 2 – Identification of CBF orthologs in strawberry (Fragaria x ananassa) and sour cherry (Prunus cerasus)

Low temperature is one of the most important environmental factors limiting crop plant growth, distribution, and productivity (Sakai and Larcher, 1987). Plant species differ greatly in their ability to develop freezing tolerance through a process known as cold acclimation (Thomashow, 1999). Traditional breeding of many major crops has met with only limited success in improving freezing tolerance. For example, the freezing tolerance of wheat cultivars has not been substantially improved in the past 50 years (Fowler and Gusta, 1979). As a result, considerable effort has been spent to understand the nature of cold acclimation, in the hopes that novel strategies for improving crop freezing tolerance may be developed.

Many physiological and biochemical changes occur in plants in response to cold temperature, including changes in gene expression (Alberdi and Corcuera, 1990; Guy, 1990). Many cold-induced genes have been identified in several crop species, including alfalfa (*Medicago sativa*), spinach (*Spinacia oleracea*), barley (*Hordeum vulgare*), and peach (*Prunus persica*) (Wisniewski et al., 1999; Monroy et al., 1993; Hong et al., 1992; Guy et al., 1985). In *Arabidopsis*, studies of the molecular basis of cold acclimation have identified a common *cis*-acting regulatory element, the C-repeat/Dehydration Responsive Element (CRT/DRE) which has the conserved core sequence CCGAC, and found within the promoters of several cold induced (*COR*) genes (Stockinger et

al., 1997). This element is present in one to multiple copies in the promoters of many cold-induced plant genes, including COR6.6, COR15a, COR47, and COR78 of *Arabidopsis* and BN115 of *Brassica napus* (Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki 1994; Baker et al., 1994; Jiang et al., 1996). In turn, all of the currently characterized *Arabidopsis COR* genes are coordinately up-regulated by CBF1, CBF2, or CBF3, a family of cold and drought inducible transcriptional activators that bind to promoters containing a CRT/DRE (Thomashow et al., 2001; Kasuga et al., 1999; Liu et al., 1998; Jaglo-Ottosen et al., 1997). Even though freezing tolerance is a complexly inherited trait, manipulation of just one gene, *CBF1*, has been shown to improve the whole plant freezing tolerance of *Arabidopsis* (Jaglo-Ottosen, et al., 1997). Because *CBF* genes are key regulators of cold acclimation and over-expression of CBF1, 2, or 3 is capable of improving the freezing tolerance of *Arabidopsis*, there is significant interest in determining if orthologs of the CBF gene family exist in cultivated species and function in a similar fashion to improve freeze tolerance.

Many temperate fruit crops are in the Rosaceae and their distribution is restricted by low temperature stress. Floral damage is often the main form of freeze damage, particularly in several of the early blooming members of the Rosaceae (e.g. strawberry, cherry, peach, almond, apricot) (Rodrigo, 2000). Many cultural techniques are employed to mitigate the damage caused by freezes during bloom (Rieger, 1989). However, improved cultivars with enhanced floral freezing tolerance would be of considerable interest to help

prevent crop loss caused by freeze damage and to help stabilize yearly yield fluctuations.

One approach to develop cultivars with enhanced freezing tolerance is to identify and utilize candidate genes playing an important role in this trait. It is plausible that cold-induced genes exist within the Rosaceae that are orthologous to those in *Arabidopsis* capable of improving freezing tolerance. Whether these genes can be utilized, through either biotechnological manipulation or exploitation of natural variation, to improve floral freezing tolerance in rosaceous crops is unknown. It is also unknown if the same relationship exists between cold-induced gene expression and onset of cold-acclimation in rosaceous flowers.

There is evidence from *Arabidopsis* suggesting that some of the genes involved in cold acclimation may not be expressed in pistils. In transgenic *Arabidopsis* containing reporter genes driven by the *cor15a* and *cor78* promoters, no expression of the reporter was observed in ovaries following whole-plant exposure to 4 °C despite reporter expression in many vegetative and reproductive tissues (Baker et al., 1994; Horvath et al., 1993). It is unknown what effect the lack of expression of these genes has on the ability to cold acclimate or develop freezing tolerance in *Arabidopsis* flowers. Considering the extreme freezing susceptibility of rosaceous and other species' pistils, it is tempting to speculate that reduced expression of cold-acclimation genes is partly responsible for the high sensitivity of these organs to freezing.

The interaction between cold temperature and photoperiod on cold acclimation of woody fruit crops has been extensively studied (Howell and Weiser, 1970; Weiser, 1970), and it is known that the over-wintering structures of perennial, temperate fruit crops possess the ability to cold acclimate. When tested under controlled conditions, detached pistils from sour cherry (*Prunus cerasus*) do not appear to have the ability to cold acclimate (Owens, Chapter 1). In contrast, receptacles with attached pistils of strawberry show an improvement in freezing tolerance of approximately 3° C following whole plant exposure to 4° C for 16 days (Owens, Chapter 1). If orthologs of the CBF gene family can be identified in strawberry and sour cherry, the relationship between expression of these CBF orthologs and cold acclimation in pistils of both these crops can be determined.

The objectives of this study were to identify orthologs of the CBF gene family in strawberry (*Fragaria* × *ananassa*) and sour cherry (*Prunus cerasus*), and to determine if any putative orthologs were expressed in response to low temperature, and if so, were there any differences in expression levels in leaves and pistils of these two crops.

Materials and Methods

Plant Material and Growing Conditions:

Containerized, dormant, rooted runners of 'Honeoye' *Fragaria* × *ananassa* and 3 year old, dormant plants from rooted cuttings of *Prunus cerasus* 'Montmorency' were grown in growth chambers maintained at 24 °C/18 °C with

14 h day-lengths at 800 $mmol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) and 10 h nights until the onset of flowering. Four degree cold treatments were administered by transferring plants to a cooler maintained at a constant 4 °C with 14 h day-lengths at 200 $mmol \cdot m^{-2} \cdot s^{-1}$ PAR and 10 h nights.

RNA extractions, northerns:

RNA extractions followed the protocol reported by Tao et al. (1999). Total RNA was extracted from both young, developing sour cherry and strawberry leaflets, pistils and receptacles from fully open flowers by grinding fresh tissue under liquid nitrogen, homogenized in phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), mercaptoehtanol, and 0.1 M Tris (pH 9.0), 0.1 M LiCl, 1% SDS, and 10 mM EDTA. The aqueous phase was extracted with phenol: chloroform: isoamyl alcohol 3 times, and then precipitated with LiCl at a final concentration of 4 M overnight at -20° C. The RNA was recovered, re-suspended, and precipitated with sodium acetate and isopropanol, re-suspended and precipitated a second time with sodium acetate and ethanol.

Northern analyses were carried out according to standard molecular biology methodology texts (Sambrook et al. 2001) Twenty micrograms total RNA was separated on agarose under denaturing conditions, transferred to a nylon filter, and probed with randomly-primed ^{32}P-labeled probes. The final stringency of washes was 60° C without formamide, and 0.2% SSC.

Degenerate primer PCR, iPCR, sequencing:

Degenerate primers for CBF were designed by aligning CBF or putative CBF orthologs from *Arabidopsis thaliana* (Stockinger et al., 1997), *Brassica napus* (Zhou et al., unpublished submission to GenBank), *and Nicotiana tabacum* (Durrant et al., unpublished submission to GenBank) (Figure 1). Three regions of similarity were found between these sequences and used to design nested primers for PCR. The degenerate primers used to amplify partial genomic fragments of putative *CBF* orthologs in strawberry and sour cherry are listed below (Table 1).

Figure 1. Amino acid alignment of CBF1 and conceptual translations of CBF-like cDNAs reported in GenBank (*Brassica napus* DREBP AF084185, *Nicotiana tabacum* AP2 containing protein AAG43548). Identical residues are indicated by shaded boxes. Positions of similarity used to design degenerate primers are indicated by the boxed areas in the consensus sequence, which is the CBF1 sequence minus gaps.

Table 5. PCR primers utilized in this study.

	Forward[a]	Reverse
CBF Degenerate	CCNAARAARCCNGCNGGNAG (43)	GGNARNARCATNCCYTCNGCC (194)
CBF Degenerate nested	CCNAARAARCCNGCNGGNAG (43)	TCNGCRAARTTYAARCA (112)
iPCR cherry	TCGCGCTGAGAGGTCGGTCT	AGTCTCCTTGAAATTCTTCC
iPCR strawberry	TTGCCTTGAGGGGCCGGTCT	AGTCTCCTTGAAATTCTTCC
iPCR nested	GCKTGCTTRAACTTCGCMGAA	CCTACCAGCAGGYTTCTTAGG

[a] Degenerate nucleotides were N = (AGTC), R = (AG), Y = (CT), K = (GT), M = (AC). Numbers in parentheses indicate amino acid start position in Figure 1.

The first round of amplification was run with the first set of primers reported in Table 1. Reactions were carried out in 25 µL volumes at final concentrations of: buffer (10 mM Tris, 50 mM KCl, 0.001% gelatin, pH8.3), 0.05 mM dNTPs, 0.4 µM each primer, 3.0 mM $MgCl_2$, 0.04 $U \cdot \mu^{L-1}$ Taq polymerase, and 100 ng genomic DNA as template. The reactions were run at 94°C for 5 min, followed by 35 cycles of 94° for 1 min, 50° C for 1 min, and 72° C for 1 min, with a final elongation of 72° C for 5 min. One microliter of these reaction products were then utilized as template for a nested PCR reaction possessing the same conditions as listed above, but utilizing the nested primers.

PCR products of interest were excised from 1.0% agarose, and purified with Qiagen's Gel Extraction Kit (Qiagen Corp., Valencia, CA). Purified PCR products were then cloned into the vector pGEM-T Easy (Promega Corp., Madison, WI) and sequenced by the Michigan State University DNA sequencing facility.

The resultant sequence was then used to design inverse PCR (iPCR) primers for obtaining flanking genomic sequence of the fragment already

obtained. The iPCR protocol followed a modified version of the protocol reported by Willis et al. (1997). One microgram of total genomic DNA was restriction digested with 5 U HindIII in 10 µL overnight at 37° C, phenol:chlorofrom (1:1) extracted, precipitated in sodium acetate and ethanol, and re-suspended in water. Three hundred micrograms of digested DNA were self-ligated at a concentration of 0.5 $\mu g \cdot mL^{-1}$ and 0.02 $U \cdot \mu L^{-1}$ overnight at 15° C. PCR was then conducted with the primers listed in Table 1. A nested PCR reaction was then run with 1 µL of product from the first reaction as template for the second, using the nested iPCR primers reported in Table 1. Nested PCR products were cloned and sequenced as described above.

Results

Nested, degenerate primer-PCR yielded small, 225 bp fragments from strawberry 'Honeoye' and sour cherry 'Montmorency' genomic DNA that were the same size as a fragment from *Arabidopsis* genomic DNA. This fragment was predicted to contain the entire AP2 domain plus a small 5' portion of the gene based on the position of the nested PCR primers. Sequencing of the cloned fragment revealed that the degenerate PCR products had predicted approximately 95% amino acid identity to the AP2 domain of CBF1 with a small portion 5' to the AP2 domain (data not shown). Nested inverse PCR (iPCR) primers were designed from this sequence and iPCR reactions yielded fragments ranging from 1 to 1.5 kb in length. Sequencing of these fragments yielded ORFs that contained the full predicted amino acid coding region of these genes (Figure

2). Primers from this full length sequence were designed and used to amplify and clone full-length genomic clones of the putative CBF orthologs from strawberry and sour cherry. These putative orthologs were named *Prunus cerasus CBF* (*PcCBF*) and *Fragaria* × *ananassa CBF* (*FaCBF*).

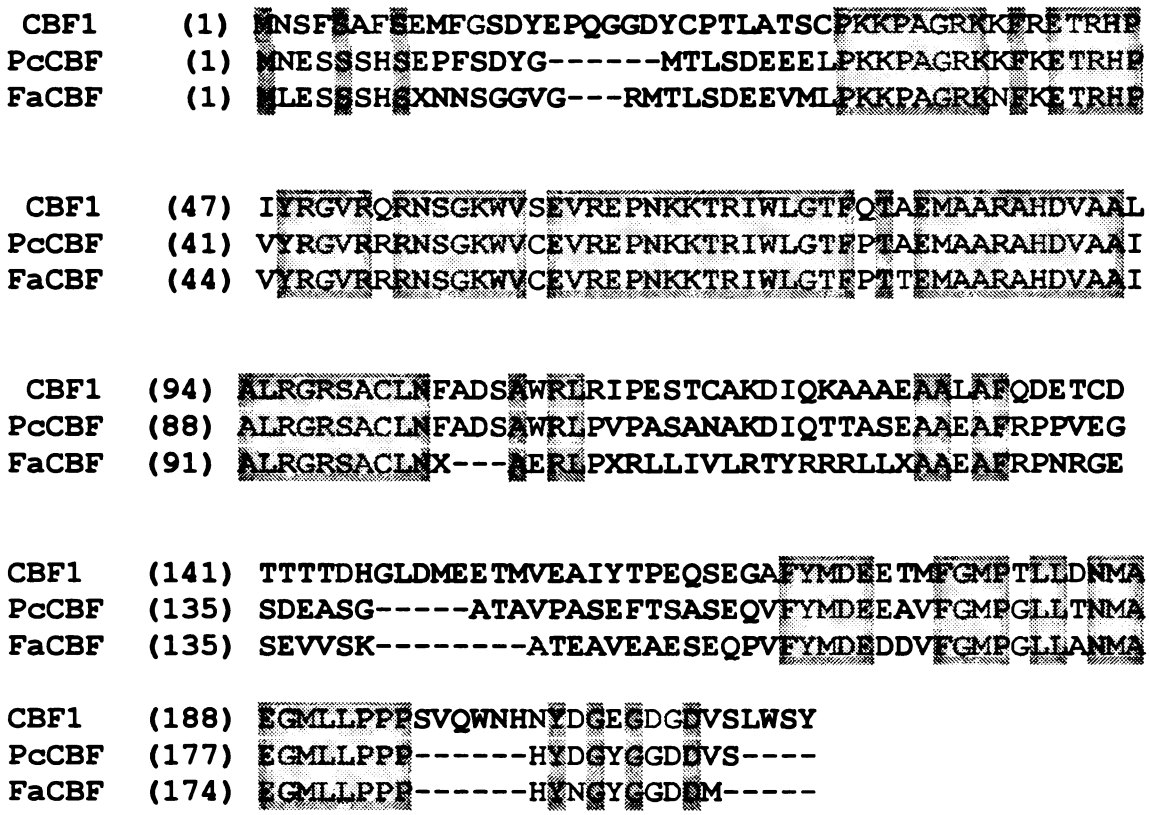

Figure 2. Amino acid sequence alignment of CBF1, PcCBF, and FaCBF. Identical residues are shaded.

Northern analyses were conducted to determine the effects of 4° C treatment on the mRNA expression of *PcCBF* and *FaCBF*. No expression of these two genes was evident in non-acclimated leaf tissue of either species (Figure 3). Following whole plant exposure to 4° C for 1 hour, faint expression of

PcCBF was observed in sour cherry leaves, and strong expression of *FaCBF* in strawberry leaves. Of the time points measured in this experiment, 4 hours of 4° C treatment yielded the highest level of expression for both *PcCBF* and *FaCBF* in sour cherry and strawberry leaves, respectively. Expression appeared to begin to taper off after 4 hours of cold treatment, as the mRNA expression was reduced after 24 hours of exposure to 4° C in both strawberry and sour cherry leaves.

No *PcCBF* or *FaCBF* expression was detected by northern analysis in either sour cherry pistils or strawberry receptacle tissue in any of the treatments of 4°C (Figure 4).

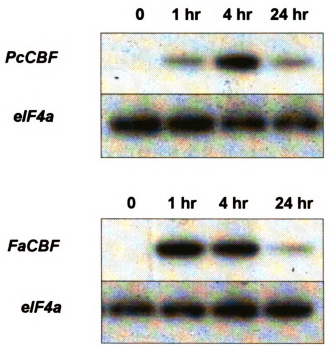

Figure 3. *PcCBF* and *FaCBF* transcript levels determined by northern analysis in leaves of sour cherry 'Montmorency' and strawberry 'Honeoye' following 0 hour, 1 hour, 4 hours, and 24 hours of exposure to 4°C. Eukaryotic elongation factor 4a (eIF4a) was used as a RNA loading control.

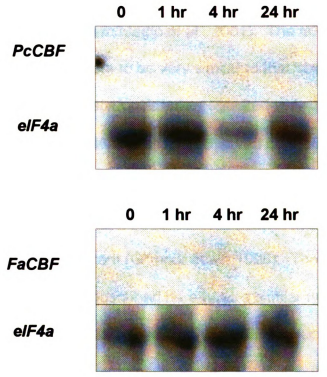

Figure 4. *PcCBF* and *FaCBF* transcript levels determined by northern analysisin pistils of sour cherry 'Montmorency' and receptacles with pistils of strawberry 'Honeoye' following 0 hour, 1 hour, 4 hours, and 24 hours of exposure to 4°C. eIF4a was used as a RNA loading control.

Discussion

The putative CBF orthologs identified in this study have 48% deduced amino acid sequence identity with the characterized CBF 1, 2, and 3 genes from *Arabidopsis thaliana*. These proteins have very high similarity through the entire AP2 domain. There are several additional conserved stretches: a PKKPAGRK 5' to the AP2 domain, a AWRL 3' to the AP2 domain, and a NMAEGMLLPP near

the carboxy-terminus. Similar conserved regions have been reported for CBF-like proteins from *Secale cereale*, *Brassica napus*, *Triticum aestivum*, and *Lycopersicon esculentum* (Jaglo et al., 2001). The mRNA expression profile in response to cold appears to be very similar to that observed in *Arabidopsis*. The *CBF* gene family of *Arabidopsis* is up-regulated very quickly in response to cold temperature, with mRNA expression observed after 15 min of exposure to 4° C (Gilmour et al, 1998). mRNA expression hits a peak approximately 4 hours after constant 4° C and then gradually tails off, often being low, but present after 24 h of constant 4° C treatment (Gilmour et al., 1998). The strawberry and sour cherry *CBF* orthologs described here have a similar mRNA expression profile, at least in vegetative tissues. *FaCBF* and *PcCBF* are up-regulated within 1 hour of exposure to 4° C, appear to have peaked at 4 h, and then decrease substantially, but are still present after 24 h. Gene expression at further time points, beyond 24 hours of exposure to 4° C was not measured in these experiments.

Arabidopsis, an ancient polyploid, has 3 characterized cold-inducible *CBF* genes (Gilmour et al., 1999). Only one putative *CBF* ortholog was identified from both strawberry and sour cherry. Considering that *CBF* exists as a multi-gene family in *Arabidopsis*, strawberry, an octoploid, and sour cherry, a tetraploid, may have additional, functional, cold-inducible *CBF*s within their genomes. Southern analysis of the sour cherry and strawberry genome with the *CBF* orthologs characterized in this study reveal complex banding patterns of varying intensities (data not shown). Considering the polyploid nature of these crops it may be difficult to determine the total number of functional CBF or CBF-like proteins

operating in these species. Whether or not the 3 cold-inducible *CBF* gene family members present in *Arabidopsis* possess unique or redundant functions is not known. Determining whether or not there are functional redundancies between theoretical *CBF* gene family members within rosaceous species will be challenging. One of the theoretical advantages of polyploidy is the relaxation of selection pressure on duplicated genes (Soltis and Soltis, 1993). The possibility exists that sour cherry and strawberry may possess several *CBF*-like pseudo-genes or paralogs with diverged function.

Despite the lack of *FaCBF* expression in strawberry receptacles with pistils, these organs are capable of cold acclimation (Owens, Chapter 1). One fundamental question is: are the *CBF* gene family members required for cold acclimation in higher plants? *Arabidopsis* has 3 cold-induced *CBF* genes that are linked in tandem, have high sequence identity with each other, and thus far appear to have redundant functions (Medina et al., 1999; Gilmour et al, 1998). It is currently unknown what effect the silencing or the mutation of these genes will have on the capability of *Arabidopsis* to acclimate. Strawberry receptacles may be a system in which *CBF* expression is not evident, but cold acclimation does occur. However, it is unknown what effect CBF expression would have on the ability of strawberry receptacles to cold acclimate.

The molecular mechanisms underlying the cold acclimation response in strawberry receptacles are not known. There are many characterized cold-induced genes that do not contain CRT/DRE and are not directly regulated by CBF. Several cold-induced genes possess ABA-responsive elements (ABRE)

within their promoters, and are up-regulated in an ABA dependent fashion. Sixteen days of 4° C treatment are required to observe a significant increase in freezing tolerance in strawberry receptacles when compared to non-acclimated plants. Open strawberry flowers are not normally subjected to this extensive of a cold treatment under field conditions. Perhaps a general stress response, including an increase in ABA production, leads to the induction of many ABRE containing genes or other genes responsible for providing increased freezing tolerance in a CBF-independent fashion. Information on the expression of both CRT/DRE and non-CRT/DRE containing, cold-inducible genes within the Rosaceae should provide additional information for testing some of these alternative hypotheses.

Several general approaches could be taken to utilize the information obtained in this study to ultimately improve the floral freezing tolerance of rosaceous fruit cultivars. One fundamental question is, why do strawberry and sour cherry pistils acclimate less so, or not at all, while the leaves acclimate to a greater extent? Differential expression of CBF orthologs may help explain the difference between these two tissues. Putative cold-inducible *CBF* orthologs exist within the Rosaceae, but do not appear to be expressed in pistils of either strawberry or sour cherry. However, strawberry receptacles and pistils appear to have the ability to cold acclimate in the absence of *CBF* expression. If the ability of these organs to acclimate is not due to *CBF* expression, an elucidation of the molecular mechanisms leading to improved floral freezing tolerance of strawberry following exposure to cold temperature could lead to novel methods for improving

floral freezing tolerance in this crop. In contrast, in sour cherry, in which pistil cold acclimation is not evident, and interesting question is what differs between strawberry and sour cherry that prevents sour cherry pistils from acclimating in response to cold? An additional approach would be to observe the effects of *CBF* over-expression on pistil freezing tolerance. In short, studies on the basis of the cold acclimation ability in strawberry receptacles, or the basis of the differences observed between strawberry and sour cherry pistils may provide information useful for the development of cultivars with enhanced floral freezing tolerance.

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Chapter 3 -- Enhancement of freezing tolerance of strawberry by heterologous expression of CBF1

Many temperate fruit crops suffer yield reductions due to freeze damage. Early season freezes during or just prior to bloom are the main cause of low-temperature induced crop loss (Rodrigo, 2000). Several early blooming rosaceous crops, such as strawberry (*Fragaria* × *ananassa*), and many of the cultivated *Prunus* species, are particularly prone to floral freeze damage. Plant species differ greatly in their ability to develop freezing tolerance through a process known as cold acclimation (Thomashow, 1999). Many physiological and biochemical changes occur in plants in response to cold temperature, including changes in gene expression (Alberdi and Corcuera, 1990; Guy, 1990). In *Arabidopsis*, studies of the molecular basis of cold acclimation have identified a common *cis*-acting regulatory element, the C-repeat/Dehydration Responsive Element (CRT/DRE) which is defined as a CCGAC, and found within the promoters of several cold induced (*COR*) genes (Stockinger et al., 1997). In turn, all of the currently characterized *Arabidopsis COR* genes are coordinately up-regulated by CBF1, CBF2, or CBF3, a family of cold and drought inducible transcriptional activators that bind to promoters containing a CRT/DRE (Thomashow et al., 2001; Kasuga et al., 1999; Liu et al., 1998; Jaglo-Ottosen et al., 1998).

CBF1 orthologs have been identified in two cultivated members of the Rosaceae: sour cherry (*Prunus cerasus*) and strawberry (*Fragaria* × *ananassa*), suggesting the *CBF-COR* gene system may be conserved in this diverse family (Owens, Chapter 2). The putative orthologs, *Prunus cerasus CBF* (*PcCBF*) and *Fragaria* × *ananassa CBF* (*FaCBF*), share 48% amino acid identity with *Arabidopsis CBF*1 and are up-regulated in leaves in response to cold temperature. The goal of this study was to provide additional support for the hypothesis that the *CBF-COR* gene system is conserved in the Rosaceae by the heterologous expression of *CBF*1. The expected downstream events would be a) an increase in freezing tolerance, and b) the up-regulation of CRT/DRE containing, cold-inducible genes. Even though freezing tolerance is a complexly inherited trait, manipulation of just one gene, *CBF1*, has been shown to improve the whole plant freezing tolerance of *Arabidopsis* (Jaglo-Ottosen, et al., 1997). Because *CBF* genes are key regulators of cold acclimation and over-expression of CBF1, 2, or 3 is capable of improving the freezing tolerance of *Arabidopsis*, there is significant interest in determining if orthologs of the CBF gene family exist in cultivated species and function in a similar fashion to improve freeze tolerance.

Knowledge of the effects of *CBF1* manipulation on the floral or whole plant freezing tolerance of rosaceous plants is of significant interest. Many temperate fruit crops are in the Rosaceae and their distribution is restricted by low temperature stress. Commercially, however, floral damage is often the main form of freeze damage, particularly in several of the early blooming members of

the Rosaceae (e.g. strawberry, cherry, peach, almond, apricot) (Rodrigo, 2000). Many cultural techniques are employed to mitigate the damage caused by freezes during bloom (Rieger, 1989). However, improved cultivars with enhanced floral freezing tolerance would be of considerable interest to help prevent crop loss caused by freeze damage and to help stabilize yearly yield fluctuations.

The objectives of this study were to determine the effects of over-expression of *CBF1* on the freezing tolerance of cultivated strawberry vegetative and reproductive tissues.

Materials and Methods

Plant Material/Conditions:

Transformation was conducted on crowns from tissue culture stocks of 'Honeoye' *Fragaria* × *ananassa* plants that had been maintained on a Murashige and Skoog (MS) salts media with vitamins, but no hormones for approximately 20 weeks (4.3 $g \cdot L^{-1}$ MS salts (GIBCO), 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine, 0.1 $mg \cdot L^{-1}$ thiamine, 2 $mg \cdot L^{-1}$ glycine, 100 $mg \cdot L^{-1}$ myo-inositol, 20 $g \cdot L^{-1}$ sucrose, 8 $g \cdot L^{-1}$ agar, pH 5.6) (Graham et al., 1995). Following transformation and regeneration, rooted explants were transferred to 10 x 10 x 20 cm plastic pots containing sterile, soil-less potting media enclosed in a plastic bag, and gradually hardened off by opening the bags slightly each day. Tissue culture plants, regenerants, and rooted plants were maintained at 24°C, 16 h photoperiods.

Plants were induced to flower by placing them under an 8 h photoperiod at constant 18° C for 2 weeks. After the 8 h photoperiod treatment, the plants were transferred back to a 16 h photoperiod.

Transformation:

The transformation protocol was that of Graham et al.(1995). Briefly, 1 to 2 mm transverse crown cross-sections were taken from tissue cultured 'Honeoye' plants, inoculated for 24 hours with *Agrobacterium tumefaciens* isolate LBA4404 containing the binary vector with *CaMV35S-CBF1* which contains the NPTII marker, and placed on regeneration media (4.3 $g \cdot L^{-1}$ MS salts, 0.2 $mg \cdot L^{-1}$ benzylamino purine, 0.2 $mg \cdot L^{-1}$ 2,4-D, 20 $g \cdot L^{-1}$ sucrose, 8 $g \cdot L^{-1}$ agar, 250 $mg \cdot L^{-1}$ ticarcillin, and 250 $mg \cdot L^{-1}$ kanamycin sulfate, pH 5.6). The crown discs were transferred to fresh regeneration media every 7 days with selection for 12 weeks. Surviving healthy shoot tips were transferred to MS media with vitamins.

Control, wild-type plants used in these experiments, were non-transformed 'Honeoye' plants that had been regenerated from crown-discs in a similar fashion as the transgenics.

DNA extraction, RNA extraction, Southerns, northerns:

Genomic DNA was extracted from young strawberry leaflets by grinding fresh tissue under liquid nitrogen, homogenizing in buffer containing 2% CTAB, 100mM Tris, 1.4 M NaCl, 20 mM EDTA, 0.1 % DTT, 1% PVPP, extracting with

chloroform/IAA, and precipitating once with isopropanol, and a second time with 70% ethanol (Graham et al., 1994).

Total RNA was extracted from both young, developing strawberry leaflets, and receptacles with pistils from fully open flowers by grinding fresh tissue under liquid nitrogen, and homogenizing in phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), mercaptoehtanol, and 0.1 M Tris (pH 9.0), 0.1 M LiCl, 1% SDS, and 10 mM EDTA. The aqueous phase was extracted with phenol: chloroform: isoamyl alcohol 3 times, and then precipitated with LiCl at a final concentration of 4 M overnight at -20° C. The RNA was recovered, resuspended, and precipitated with sodium acetate and isopropanol, resuspended and precipitated a second time with sodium acetate and ethanol (Tao et al., 1999)

Northern transfers were made by fractionating 20 µg of total RNA in 1% agarose formaldehyde gels (Sambrook et al. 2001) and transferring it to nylon membranes by capillary action. The filters were hybridized with randomly-primed ^{32}P-labeled probes. Probes were made from full-length cDNA of CBF1. eIF4a was used to probe the northern membranes to determine even loading of RNA. The final stringency of washes was 60° C without formamide, and 0.2% SSC.

Southern transfers were made by fractionating 6 µg of genomic DNA digested overnight at 37°C with HindIII in 1% agarose gels (Sambrook et al. 2001) and transferring it to nylon membranes. The filters were hybridized with randomly-primed ^{32}P-labeled probes. Probes were made from full-length CBF1 cDNA.

Determination of freezing tolerance/Data analysis:

Freezing tolerance was determined on detached leaf-discs and receptacle tissue. The leaf discs were detached from the whole plant, and stored in de-ionized, distilled water until all had been placed in 16 mm diameter x 125 mm long glass culture tubes. Three leaf discs or receptacles plus pistils were placed in each culture tube. Data from 3 tubes were pooled for each temperature point of the controlled freezing run (Sukumaran and Weiser, 1972).

Freezing-runs were conducted by placing the tubes in freezing-water baths containing a mixture of ethylene glycol and water. The tubes were maintained at 0 °C for 1 h, after which a small amount of de-ionized ice was placed in contact with the plant material in each tube to nucleate ice formation. The tubes were kept for an additional hour at 0 °C, and the temperature was then decreased at the rate of 4 $°C \cdot h^{-1}$. Every 30 minutes a set of tubes was transferred to a separate freezing-bath kept at the same temperature as the bath it came from, and maintained at that temperature for an additional 30 minutes (for a total of 1 h at each temperature). After 1 hour of freezing, tubes were placed on ice until the completion of the freezing experiment. Freezing runs were carried out to a final temperature of –20° C.

Following completion of the freezing run, tubes were placed in a rack on top of ice, wrapped with plastic, and kept at 4 °C overnight. Following thawing, 3 mL of de-ionized, distilled water were added to each tube, and the tubes were then shaken for 3 hours. Electrical conductivity of the water was measured from each tube, the plant material in the tubes was placed at –80 °C for 1 hour, and

the original water was then added back to each tube for an additional 3 hours, with agitation. A final electrical conductivity was then determined for the water in each tube.

Three sets of data points were generated for each temperature point of the freezing run, with 3 tubes representing each data point. These data were used to generate an electrolyte leakage curve over the range of temperatures used in the freezing run. Three curves were generated per treatment.

Statistical significance of treatments was determined with a two-sample t-test within each tissue type using Minitab 13.0 (Minitab Inc., State College, PA)

Results

Two transgenic strawberry lines transformed with a *CaMV 35S-CBF1* construct were recovered from the selection media, designated 35-1-1 and 35-1-2. PCR results confirmed the presence of the transgene in these two lines (data not shown). Southern analysis with a full-length *CBF1* cDNA as probe revealed a single insertion of the transgene in each of the two lines (Figure 1).

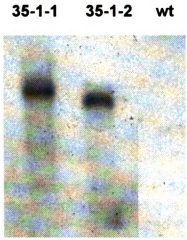

Figure 5. Southern analysis of wild-type and 35-1-1 and 35-1-2 'Honeoye' strawberry plants transformed with *CBF1*. Restriction digestion conducted with HindIII

Northern analysis of the two transgenic lines probed with a full-length *CBF1* cDNA showed expression of the transgene in both actively growing leaf tissue as well as receptacle tissue of fully open flowers (Figure 2).

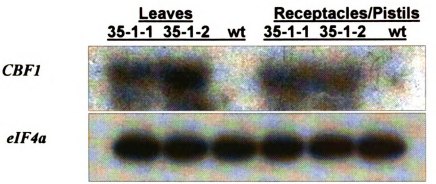

Figure 6. *CBF1* transcript levels in wild-type plants and 35-1-1 and 35-1-2 transgenic plants which over-express *CBF1*. Eukaryotic elongation factor 4a (eIF4a) was used as a RNA loading control.

The freezing tolerance of leaf-discs and receptacle tissues was determined for the two transgenic lines. Both lines showed a significant increase in freezing tolerance of leaf-discs compared to the wild type plants when measured by electrolyte leakage (Table 1). The temperature at which 50% electrolyte leakage occurred was –8.3°C and –10.4° C for non-acclimated lines 35-1-1 and 35-1-2, respectively. These values were approximately 4-5° C below the freezing tolerance of wild type leaf discs. Leaf-discs taken from transgenic whole plants exposed to 4° C for 16 days showed a less significant increase in freezing tolerance when compared to wild type plants. All plants showed a significant increase in freezing tolerance following 16 days of exposure to 4° C, when compared to non-acclimated plants.

Freezing tolerance values of the receptacle and pistils of the transgenic lines were not significantly different from those of wild type plants acclimated for 16 days or for 0 days (Table 1), although the freezing tolerance of receptacles did increase following cold acclimation.

Table 6. Temperature at which 50% electrolyte leakage occurred for wild-type 'Honeoye' plants and 35-1-1 and 35-1-2 plants which over-express *CBF1*.

	Non-Acclimated		Acclimated[a]	
	Leaf Discs	Receptacles + Pistils	Leaf Discs	Receptacles + Pistils
wild type	-4.8 a	-2.0	-12.3 a	-5.1
35-1-1	-8.3 b	-2.6	-13.4 b	-5.6
35-1-2	-10.4 c	-2.1	-13.8 b	-5.4
F-test within column	**	ns	*	ns

**, *, ns within column represent significant at α = 0.05, 0.10 or non-significant F-test. [a] acclimated whole plants exposed to 4° C for 16 days. Values within column followed by the same letter are not significantly different at α = 0.05 with an LSD.

Discussion

The level of freezing tolerance enhancement observed in this study between strawberry plants expressing *CBF1* and the wild-type, non-transgenic regenerated 'Honeoye' plants is of a similar order of magnitude as that observed between transgenic and wild type *Arabidopsis* (Jaglo-Ottosen et al., 1997; Gilmour et al., 2000). A range of freezing tolerance values has been observed among different transgenic *Arabidopsis* lines often showing approximately a 3 to 4 °C increase in the 50% electrolyte leakage point of detached leaves between non-acclimated transgenic and wild type plants. Although data from a large number of transgenic lines has not been reported, there appears to often be a positive correlation between transgene expression and degree of freezing tolerance enhancement (Jaglo-Ottosen et al., 1997; Gilmour et al., 2000). In this

study, only 2 transgenic lines with similar transgene expression levels were identified and they had similar freezing tolerances.

In *Arabidopsis*, high *CBF3* transgene expression appears to be negatively correlated with plant vigor (Gilmour et al., 2000). Several lines expressing *CBF3* at high levels have shown a marked increase in freezing tolerance and a significant reduction in plant size compared to the wild type plants. Among our transgenic strawberry lines, no apparent phenotypic differences were observed, although the generation of additional strawberry transformants expressing higher levels of *CBF*1, 2 or 3 might lead to a wider range of phenotypes.

No enhancement in freezing tolerance was observed in the receptacle tissue, despite expression of the transgene in the receptacles at similar levels as was observed in developing leaves. The reason for the lack of freezing tolerance improvement in receptacle tissue, despite a significant improvement in leaves is not known. However, there is evidence from *Arabidopsis* suggesting that some of the genes involved in cold acclimation are not expressed in pistils. In transgenic *Arabidopsis* containing reporter genes driven by the *cor15a* and *cor78* promoters, no expression of the reporter was observed in ovaries following whole-plant exposure to 4 °C despite reporter expression in many vegetative and reproductive tissues (Baker et al., 1994; Horvath et al., 1993). Freezing tolerance of *Arabidopsis* flowers has not been directly measured, but it is possible that the signal transduction steps or components between *CBF* induction and *COR* gene activation are not functional. Evidence for this comes from the *sfr6* mutant in *Arabidopsis* which is deficient in freezing tolerance

following cold acclimation, does not show expression of *COR6.6*, *COR15a*, or *COR78* in response to cold temperature, even though *CBF3* expression following cold exposure is normal (McCown et al., 1996; Warren et al., 1996; Knight et al., 1999). It has been postulated that SFR6 is required for cold-induced activation of the CBF3 protein. It is possible SFR6 or some as yet unidentified component of the cold acclimation molecular response is functioning differently in pistils and leaves. However, strawberry receptacles appear to be able to acclimate in the absence of expression of a strawberry *CBF* ortholog (Owens, Chapter 1 and 2). This raises the possibility that there is either some defect in the signal transduction pathway leading to the induction of *CBF* in strawberry pistils and receptacles, but that cold acclimation is still possible through a non-*CBF* mediated pathway.

The data reported in this study support the hypothesis that the *CBF-COR* gene system is conserved in the Rosaceae. CBF1 is a cold-inducible transcriptional activator that has been shown to increase the freezing tolerance of *Arabidopsis* when over-expressed by up-regulating a battery of genes containing CRT/DRE in their promoters (Jaglo-Ottosen, et al., 1997). Although, a rosaceous CRT/DRE containing gene was not identified, the enhancement in freezing tolerance suggests there may be an intermediary step in which several CRT/DRE containing proteins are up-regulated leading to the observed enhancement in freezing tolerance. Alternatively, the freezing tolerance improvement in the transgenic plants could be do to a positional effect of the transgene. Considering

that two transgenic lines were generated that both showed an improvement in freezing tolerance, this seems unlikely.

The probability that the *CBF-COR* gene system is conserved in the Rosaceae, is further supported by the identification of putative orthologs of *CBF* in both strawberry and sour cherry that have similar mRNA expression profiles as the *CBF* gene family in *Arabidopsis* in response to cold temperature, in vegetative tissues. Additional support for this hypothesis could be gained by the identification of COR gene orthologs that possess CRT/DRE in their promoters, a demonstration of up-regulation of the COR gene orthologs by over-expression of either Arabidopsis CBF1, 2, or 3, or over-expression of a native *CBF* from a rosaceous plant. Also, a demonstration of the direct binding of the rosaceous *CBF* orthologs to the promoters of COR gene orthologs would provide support for conservation of a similar molecular mechanism of these genes.

The demonstration of an enhancement in freezing tolerance of an important perennial fruit crop is of major practical significance. In the case of strawberry, freezing damage can occur both to flowers, due to its early bloom, and also to the crown tissue of the plant due to mid-winter temperatures below its critical threshold for survival. Strawberries have a wide cultivated range, and in colder climates plantings are often mulched to protect them from damaging winter temperatures where snow cover is unpredictable. Additional physiological protection from mid-winter temperatures would allow a wider range of cultivars to be grown in cool climates.

Although flowers of the transgenic plants did not show an improvement in freezing tolerance, it remains important to know that the *CBF* genes are conserved in the Rosaceae. Further study is required to determine if manipulation of the *CBF* system in strawberry, or other fruit crops, is capable of improvement the freezing tolerance of pistils. Additional experiments, attempting to develop lines with higher transgene expression levels, or targeted expression directly to the pistils may provide some additional information to answer this question. Significantly, if the *CBF-COR* gene system is closely conserved between *Arabidopsis* and rosaceous species, than *Arabidopsis* can be used as a model for elucidating the nature of the cold acclimation molecular signal transduction pathway in these important cultivated plants. Progress will be more rapid in *Arabidopsis* and utilization of the information generated in this model species will be of major utility to horticultural scientists and breeders of horticultural plants.

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