Cytokinin Response Factor 4: A Role in Development During Cold Stress in *Arabidopsis thaliana*

by

Margaret Alpha Compton

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of Master of Science

Auburn, Alabama
August 4, 2012

Keywords: *Arabidopsis thaliana,* CRF4, Cold Stress, Freeze Treatment

Approved by

Aaron Rashotte, Chair, Assistant Professor of Biological Sciences
Joanna Diller, Associate Professor of Biological Sciences
Fenny Dane, Professor of Horticulture

Abstract

Cold stress is a major cause of crop loss annually in the United States such that a detailed study of the mechanisms and genes behind these stresses are essential for improvement of crop plants. This thesis investigated the role of Cytokinin Response Factor 4 (CRF4) in cold stress response. Transcript levels of CRFs at different developmental stages and times after cold treatment revealed that CRF4 is activated by the cold stress response pathway in the accumulation phase and possibly during freezing stress response. Examinations of plants under both cold and high sucrose revealed that CRF4 appears to be important in promoting germination, negatively regulates shoot development, and high CRF4 levels can repress root growth. It also appears thatCRF4 has different mechanisms of response to cold stress in shoots and roots, which can be seen in timing of expression response that shifts as development progresses.

Acknowledgments

The author would like to thank her advisor, Aaron Rashotte, for the financial and mental support, guidance, and encouragement. A thank you is extended to the other committee members, Dr. Joanna Diller and Dr. Fenny Dane, for direction, help, and encouragement on the projects undertaken. The lab members, Paul Zwack, Xiuling Shi, and Sarika Gupta, who were very patient and helpful during this process, are also greatly appreciated. Finally, the author could not have made it through this process without the love and patience of her friends and family.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

abi……..…..ABA-Insensitive protein

AREB/ABF……...……. ABA-Responsive Element Binding/ABA Binding Factor

CRF……... Cytokinin Response Factor

COR…….. Cold Responsive Gene

DRE/CRT……..... Dehydration Responsive Element/C-Repeat Sequence

ICE1……..……..Inducer of CBF Expression1

IP_3…….Inositol (1,4,5) Triphosphate

MAPk……...Mitogen-Activated Protein kinase

MYC/MYB……Myelocytomatosis oncogene/Myoblastosis oncogene

qPCR……..quantitative Polymerase Chain Reaction

ROS……...Reactive Oxygen Species

TCS……... Two Component Signaling System

Introduction

Plants encounter a variety of abiotic stresses, including cold, heat, drought, flooding, and salt and nutrient changes in soil throughout their life cycle. Since plants are sessile organisms, they have evolved unique mechanisms to cope with these stresses. Each of these different stresses induces responses with distinct characteristics, since the way in which the plant responds determines whether or not the plant will survive. It is important to note that responses are not always exclusive to one stress and there are also general plant stress responses.

These abiotic stresses are a major cause of crop losses around the world. In the United States an examination of crop losses amounts revealed that about 40% of crop loss is due to drought, 30% to excessive moisture, rain or floods, 10% to hail, and the remaining 10% to frost, freeze or cold weather (USDA; http://www.ers.usda.gov/Briefing/RiskManagement/questions.htm, 2009). Though frost, freezing, or cold events only account for a small portion of the total crop loss in the U.S., this 10% results in average annual losses reaching $690,000 to $736 billion (taken from 1975-1995) (Kunkel *et al.,* 1999). Specifically, the 2011 losses from this abiotic stress reached $400 million (Rain and Hail, 2012). Because of these stresses are highly detrimental to crops and because of the changing environmental conditions, great efforts have been made understand the mechanisms that plants have evolved to cope. This thesis will focus on gaining a better understanding of how cold stress functions in plants.

Cold stress can be defined into two distinct categories (Beck *et al.,* 2007). The first is characterized by the exposure of plants to low, positive temperatures slightly above freezing (0°C). This category is generally referred to as the acclimation phase, since these conditions often prepare the plant for further cold stress, specifically freezing. The acclimation phase involves the induction of protective mechanisms such as sucrose accumulation or transcriptional changes, which will be described further, later in this thesis. This category of cold stress involving low, positive temperatures will be the primary focus of this thesis represented by treatments of plants at 4°C. The second category of cold stress is freezing stress. This stress is characterized by the exposure of plants to less than or equal to 0°C temperatures: freezing. This stress is the most detrimental to plant survival since it can cause major osmotic stress and membrane damage to the plant as well as death (Theocharis *et al.,* 2012). In this thesis, this second category is referred to as freeze stress and the temperature representing this stress will range from -8°C to -13°C in the treatment of plants.

In general, cold stress affects plants in a variety of ways. For example, this stress slows photosynthesis and respiration that ultimately slows growth and can result in death. Cold stress also reduces transpiration, which may result in excess water storage that upon freezing could damage or kill the cells in which it is stored. One effect often seen in response to cold stress in the accumulation of sucrose that is used both in membrane stabilization by increasing the osmotic potential or by direct interaction with proteins or membrane lipids and signaling pathways for changing gene expression (Hincha and Hagemann, 2004; Rekarte-Cowie *et al.,* 2008). Another major effect of cold stress is an altered gene expression and protein activity, which contribute to the slowed growth of plants exposed to cold environments through repressed expression of proteins involved in growth and metabolic activity.

Cold stress has also been shown to trigger some protective and growth strategies. One instance is the cold induction of dormancy during which growth and development are slowed and metabolic needs are reduced (Evans; http://www.ces.ncsu.edu/depts/hort/consumer/weather/tempeffect-plants.html, retrieved 2012). A prime example is that many trees become dormant during the winter in order to preserve nutrients and energy. Cold is also involved in two different mechanisms by which the plant regulates major developmental shifts: germination (through stratification) or flowering (through vernalization) (Evans; http://www.ces.ncsu.edu/depts/hort/consumer/weather/tempeffect-plants.html, retrieved 2012). Stratification is the treatment of seeds with cold temperatures that in turn results induces seed germination. This cold exposure can shift seeds from a dormant to active state. Many plants require vernalization or an extended cold exposure of vegetative tissues in order to transition to flowering. Both of these strategies, stratification and vernalization can be and are regularly manipulated by researchers in the study and general growth of plants (Evans; http://www.ces.ncsu.edu/depts/hort/consumer/weather/tempeffect-plants.html, retrieved 2012).

Cold stress is linked to other abiotic stress processes and other stress pathways can be induced when plants are exposed to cold. One example of this is that osmotic stress responses that can be induced during cold stress (Beck *et al.,* 2007). This can occur in the following way. When encountering low, positive temperatures, the plant experiences a cold acclimation phase, during which many biochemical and metabolic changes occur. During this phase, the plant prepares for possible freezing temperatures, by accumulating amino acids, solutes, polyamines, and glycine betaine (Draper, 1972; Kaplan and Guy, 2004; Capell *et al.,* 2004; Kaplan *et al.,* 2007; Alcázar *et al.,* 2010; Chen and Murata, 2011; Alet *et al.,* 2011; Krasensky and Jonak, 2012). These accumulating molecules serve a protective mechanism against the cold, freezing

stress being expected. For example, sucrose can stabilize the cell membrane or act as a signaling molecule regulating gene expression (Hincha and Hagemann, 2004; Rekarte-Cowie *et al.,* 2008). However, the accumulation of solutes and other molecules in response to cold stress, accounts for the possible osmotic imbalance in cells that results in further temperature decreases to subzero temperatures. Thus, the accumulation of solutes is a preemptive response to cope with further stress that may follow.

Some plants are more tolerant of specific stresses than others. For example lettuce is more tolerant of cold than tomato, and cacti are more tolerant of heat and drought conditions than grasses. Plants that are able to cope better with specific stresses often have an initial defense against the stress sometimes in the form of basic anatomy, physiology, and metabolism. Plants also have different strategies to deal with stress. Cacti, for example have stress avoidance strategy, in which the plant anatomy and metabolism is designed to delay or avoid desiccation in hot arid climates. Conversely, lettuce is an example of a plant implementing a tolerance based approach in which its anatomy helps avoid stress to an extent, but the plant metabolism responds to the stress when presented. This is a transient response not a stable inherited one like the cacti (Krasensky and Jonak, 2012). The stress tolerance approach can involve sucrose accumulation for membrane stability and transcriptional changes for further metabolic and biochemical changes in order to tolerate stress levels and are the focus of this thesis.

It is important that responses to cold stress are better understood to possibly genetically alter plants to be more tolerant of this stress. This would allow an expansion of plant growth range, better tolerance within current ranges and increased crop production. Previous research has focused on the major transcriptional components in the cold responsive pathway, but there may have been some downstream transcription factors that have been over-looked or are

understudied. This thesis will examine some of these transcription factors in the signal transduction pathway of cold response in *Arabidopsis thaliana* and *Solanum lycopersicum*.

Literature Review

General Response to Abiotic Stress

When plants encounter an abiotic stress, such as cold, they respond with many metabolic, biochemical, and transcriptional changes. Specifically cold stress responses are reduced growth rates, and sucrose or other solutes accumulating to stabilize the plant membrane, which becomes fluid and leaky in cold stress. Finally, cold stress also causes transcriptional changes that subsequently alter protein activity occur and control some metabolic and biochemical responses. These transcriptional changes may induce hormone signaling to alter plant growth and can also be responsible for changing the plant membrane lipid saturation or accumulating sucrose in the cytoplasm. In general these response to cold stress prepare the plant for further decreasing temperatures.

With the lowering of temperature to freezing or sub-freezing temperatures other stress response pathways can be activated, such as osmotic stress. Osmotic stress occurs in the context of cold stress when ice forms on the outside of the plant cell. Because ice has a lower osmotic potential than water, water rushes from the inside to the outside the cell to equilibrate the potential. This exiting of water from the cell causes the plant cell to collapse. Thus, osmotic stress in the presence of cold can also lead to many changes in the plant, ranging from cell-specific responses to whole plant physiological responses to prevent the cell collapse. These stresses, cold or freezing and osmotic act together to causes strains on the cell either through ice

formation or cell collapse, which in turn can cause irreversible damage to the organism or causes the organism to adapt (Krasensky and Jonak, 2012).

These changes in response to acclimating cold stress induce a variety of cell-specific events in order to cope with the stress and avoid senescence. Changes that occur in the cell when exposed to low temperatures include actin reorganization in order to protect the membrane from possible dehydration-induced concavity or collapse if freezing occurs. This actin reorganization increases membrane viscosity of the cell membrane and induces calcium influx (Orvar *et al.,* 2000). The calcium influx in turn activates signaling pathways, including the Mitogen-Activated Protein Kinase (MAPK) pathway of signal transduction resulting in transcriptional changes including transcription of cold-responsive genes (COR). Calcium is also required for induction of some cold responsive genes (Knight and Knight, 2000; Doherty *et al.,* 2009). Monroy and Dhindsa (1995) found that calcium concentration in the cell was 15 times greater at 4°C than at 25°C and that cold responsive genes depend on calcium to be induced. It was also found that cold acclimation was inhibited when calcium channels were blocked (Monroy and Dhindsa, 1995). Thus, this indicates that calcium influx is important in responding to temperature decreases.

There are also examples of negative cell-specific responses to these stresses include Reactive Oxygen Species (ROS) production (a by-product of metabolism), ionic homeostasis disruption (e.g. calcium) and enzyme deactivation all of which damage the cell, but may conversely promote response and coping mechanisms (Pearce, 2001). Specifically, ROS can inactivate enzymes cause lipid peroxidation, protein degradation, and damage to DNA. ROS can also act as a signaling molecule and promote expression of antioxidant genes (Theocharis *et al.,* 2012).

The pre-osmotic stress pathway of signal transduction that may be activated during cold stress (pre-freezing) may promote a variety of cellular stress sensing and signaling mechanisms, such as calcium signaling. Inositol (1,4,5) Triphosphate (IP_3) signaling, and activation of osmosensors leading to a phosphorelay and eventual activation of MAPKs followed by gene expression changes (Takahashi *et al.,* 2001; Ueda *et al.,* 2004; Hayashi *et al.,* 2006; Kim *et al.,* 2011). All of these transcriptional and signaling responses lead to stress-induced adjustments by the plants in order to survive. Additionally, the metabolic changes induce accumulation of amino acids and solutes and other molecules, such as proline, sucrose, and polyamines (Nanjo *et al.,* 1999; Krasensky and Jonak, 2012). These molecules have been found to work in osmotic adjustment, removing ROS from the cell, and stabilizing cellular structures (Valliyodan *et al.,* 2006; Janska *et al.,* 2010; Alcázar *et al.,* 2011; Husain *et al.,* 2011; Krasensky and Jonak, 2012)**.** These cellular and whole plant responses interact to aid the plant to improve its survival during stress.

Genetic Responses to Cold Stress

Genetic responses initiated by signal transduction pathways of the cold and osmotic stresses activate a variety of genes encoding osmoprotectants and detoxifying enzymes, along with regulatory proteins such as transcription factors, protein kinases, and phosphatases to regulate gene expression (Krasensky and Jonak, 2012). Genes for water channels, dehydrins, antifreeze proteins, chaperones, and proteins involved in the metabolic pathway of sucrose production are also activated to prevent damage to cells at the gross level and protein level (Fowler and Thomashow, 2002; Browse and Xin, 2001; Huang *et al.,* 2012).

The expression of many transcription factors is altered during cold treatments. As an example, 306 genes were found to be cold-responsive from profiling one third of the Arabidopsis

transcriptome following cold treatment at different time points and in plants constitutively expressing transcription factors named C-repeat Binding Factors (CBF1, CBF2, and CBF3), Fowler and Thomashow (2002). Two hundred and eighteen were up regulated, and 88 down regulated. Within this sample of cold responsive genes some show only transient up- or down-regulation while a smaller group long-term up- or down-regulation by cold. Similarly, when profiling the transcriptome of *Arabidopsis* plants in response to cold, salt, and osmotic stress, there are 2,409 unique stress-related genes with changes greater than 2-fold (Kreps *et al.,* 2002). Though, some genes that are regulated during different stresses are not always induced, but become active by phosphorylation, e.g. Inducer of CBF Expression (ICE1), a transcription factor (Miura *et al.,* 2007). So, the number of genes found to be altered in response to these stresses may not accurately reflect the total number involved in the stress response. Regardless, the number of genes affected by cold during the plant response to the stress demonstrates the importance placed by the plant on properly regulating and responding to the abiotic environment.

Solute Accumulation Effect on Plants During Cold Stress

Sucrose and other soluble carbohydrates play a pivotal role in plant survival, the development from germination to leaf greening and the development of leaves, as well as in signaling and membrane stabilization in response to abiotic stresses (Kang *et al.,* 2002). However, if sucrose is too highly concentrated, the effect on the plant may be detrimental (Kang *et al.,* 2002; Yuan and Wysocka-Diller, 2006). In particular, Kang *et al.* (2002) described inhibition of cotyledon greening and true leaf development with sucrose levels greater than 4% concentration on growth medium. In addition to abiotic signaling and response, sugars also play a role in both germination and plant development. Glucose, in particular, has been found to delay germination as exogenous concentration on medium is increased, but if applied after germination

it has a concentration dependent positive effect on root and shoot growth. For example, intermediate levels of glucose concentrations, 1.5-5% supplemented media, increase root and shoot growth. Concentrations greater than 5% in media show decreased plant growth similar to sucrose un-supplemented media (0%) (Yuan and Wysocka-Diller, 2006).

Additionally, many connections have been made between sucrose and cold acclimation and osmotic stress. Tabaei-Aghdaei *et al.* (2003) determined that the mRNA expressed in cells of plants supplied with sucrose resembled that of cold treated cells. Not only does sucrose induce cold acclimation responses in plants, but freezing tolerance of plants is also dependent on sucrose concentration. Freeze tolerance has been shown to increase with increasing sucrose concentration (Tabaei-Aghdaei *et al.,* 2003). The mechanism by which sucrose contributes to freezing tolerance is only partially understood; however, Uemura and Steponkus (2003) described *Arabidopsis* seedlings with increased freeze tolerance when exogenously supplied with different sucrose concentrations. Low concentrations of sucrose decrease expansion-induced lysis while higher concentrations reduced the loss of osmotic responsiveness to the cold. Additionally, solutes, including sucrose, trehalose, and sorbitol, can interact with the phosphates in membrane lipid head groups to decrease membrane leakage by decreasing the phase transition temperature (Hincha and Hagemann, 2004).

In addition to the effects of sucrose on general cold response mechanisms, sucrose also plays a role in changing gene expression during abiotic stress. More specifically, sucrose has been found to act at an early state of cold acclimation by inducing a gene downstream in the cold response pathway Cold Responsive gene (COR), COR78 during 2 hours exposure to cold. Interestingly, the plant does not have to experience cold to induce COR78 expression, since sucrose can induce the expression of COR78. However, the effect of sucrose on the expression

of COR78 is part of a feedback mechanism that suppresses the original response of induction of COR78 if actual cold temperatures are not experienced (Rekarte-Cowie *et al.,* 2008). Therefore, this study demonstrates the ability of sucrose to modulate gene expression of at least one cold responsive gene. Overall these studies suggest that it is important to understand at what levels sucrose is beneficial and harmful to the plant when examining its role in abiotic stress response.

Cold Stress Signal Transduction

Under cold stress conditions multiple signal transduction pathways are induced including an abscisic acid (ABA) dependent and ABA independent signal transduction. ABA accumulates quickly in response to abiotic stresses, and ABA is responsible for many physiological changes (Lang *et al.,* 1994). Specifically, ABA is known to promote stomatal closure and inhibit stomatal opening to prevent further water loss under osmotic stress conditions, when not exposed to cold conditions. ABA has also been shown to promote survival following freezing, promote freeze tolerance, of plants grown under standard lab conditions (not cold) (Lang *et al.,* 1994; Mantyla *et al.,* 1995). Also, when treated with cold acclimating temperatures ABA levels increase 3-fold (Lang *et al.,* 1994; Mantyla *et al.,* 1995). ABA is involved in inducing a variety of gene expression changes (Hubbard *et al.,* 2010; Raghavendra *et al.,* 2010; Krasensky and Jonak, 2012). In fact, ABA is known to activate transcription of genes involved in stress response through two cis-elements, AREB/ABF (ABA-responsive element-binding protein/ABA binding factor) and MYC/MYB (myelocytomatosis oncogene/myoblastosis oncogene) (Yamaguchi-Shinozaki and Shinozaki, 2005). Soluble carbohydrates can also act in a regulatory fashion in connection with ABA signaling during abiotic stress response (Kang *et al.,* 2002). Although cold stress responses are general thought to function primarily through ABA signaling, other signaling pathways also appear to have a role in this stress response. An example is ABA-

insensitive (*abi*) mutants can cold acclimate. Thus, plants have more than one mechanism of cold acclimation.

This ABA independent pathway has been well characterized and specific proteins have been identified in its signal transduction. The main regulator proteins of this pathway are DREB/CBFs (Dehydration responsive element binding/CRT Binding Factor). This pathway is referred to as the ICE-CBF-COR signaling pathway (Fig.1A). ICE1 is a MYC-type basic helix-loop-helix transcription factor that is constitutively expressed in the cell, but is only active following cold stress (Huang *et al.,* 2012). To activate this signal transduction pathway, ICE1 needs to be phosphorylated by High Expression of Osmotically Responsive Genes 1 (HOS1). Doing this HOS1 targets ICE1 for ubiquitination and degradation. Conversely, SUMO E3 ligase (SIZ1)-dependent sumoylation can block ubiquitination of ICE1 and allow ICE1 to actively function during cold stress (Chinnusamy *et al.,* 2003; Dong *et al.,* 2006; Saibo *et al.,* 2009; Huang *et al.,* 2012). The phosphorylation of ICE1 not only activates this transcription factor but also stabilizes it so it can activate one of the next steps of the pathway DREB1A/CBF3 (Miura *et al.,* 2007).

Once activated following cold stress, ICE1 binds to MYC recognition elements and activates DREB/CBF gene expression, specifically DREB1A/CBF3 (Miura *et al.,* 2007). In an ICE1 mutant background, the basal expression levels of many cold-responsive genes are altered (Chinnusamy *et al.,* 2003; Lee *et al.,* 2005; Huang *et al.,* 2012). In addition to gene expression changes, *ice1* mutant plants have a chilling sensitive phenotype, whereas over-expression of ICE1 conveys a freeze tolerant phenotype. Collectively, these results suggest that ICE1 plays a major role in cold stress signal transduction.

CBF genes encode a family of AP2/ERF family transcriptional activators that respond to both cold and osmotic stress and including DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu *et al.,* 1998; Kasuga *et al.,* 1999). The DREB/CBF transcription factors bind to a DRE/CRT (dehydration response elements) sequence in the promoter region of osmotic and cold responsive genes (Fig. 1A). This DRE/CRT promoter sequence is 5'-CCGAC-3' and when bound by DREB/CBFs, activates many genes that convey cold tolerance. One of the groups of genes is COR genes that are critical in plants for cold acclimation and chilling tolerance (Thomashow, 1999; Xiong *et al.,* 2002; Huang *et al.,* 2012). This transcription factor binding was initially found in the RD29A (responsive to dehydration 29A) gene that contained the ABRE sequence in its promoter (5'-ACGTGG/TC-3'). In addition RD29A also has a the DRE/CRT sequence in its promoter, indicating some genes can be involved in response pathways from different stresses such as drought, high-salinity, and cold (Yamaguchi-Shinozaki and Shinozaki, 1994).

Individual members of the DREB/CBF family of transcriptional activators have functional overlap with other family members, yet also have unique functional roles in stress response. The DREB1 (1A, 1B, and 1C) genes have highly similar amino acid sequences, and these genes are found tandemly on *Arabidopsis* chromosome 4 (Gilmour *et al.,* 1998). These DREB1 genes are activated by cold, salt, ABA, and drought stress, whereas the related DREB2 is activated by dehydration, but not cold (Liu *et al.,* 1998; Nakashina *et al.,* 2000). DREB1A/CBF3 is primarily target of ICE1 and thus primarily responsible for transcriptional activation of cold-responsive genes (Lee *et al.,* 2005).

Over expression of DREB1A/CBF3, DREB1B/CBF1, and DREB1C/CBF2 increases plant tolerance to freezing, drought, and high-salt concentrations in addition to reducing growth

effects, such as retardation under these conditions (Liu *et al.,* 1998; Jaglo-Ottosen *et al.,* 1999; Kasuga *et al.,* 1999; Gilmour *et al.,* 2004). DREB1A/CBF3 and DREB1B/CBF1 are known to be positive regulators of cold response whereas DREB1C/CBF2 is a negative regulator (Novillo *et al.,* 2004; Huang *et al.,* 2012). In addition to general tolerance to abiotic stresses DREB1/CBFs when over-expressed also induced biochemical changes such as accumulation of proline and soluble carbohydrates (sucrose, glucose, raffinose, and fructose) (Gilmour *et al.,* 2000, 2004). These biochemical changes have been suggested to result from the activation of COR genes and other cold-responsive genes by the DREB/CBF transcriptional activators. In addition to DREB/CBFs activating cold responsive genes, these transcription factors can also induce expression during drought stress, indicating that an ABRE cis element is not always needed for drought stress regulation (Narusaka *et al.,* 2003). Thus there is possibly cross talk among abiotic stress signaling systems. Haake *et al.* (2002) and Sakuma *et al.* (2002) identified DREB1D/CBF4 as the mediator between cold and drought stress, i.e. between DREB1 and DREB2 genes respectively.

Some of the mechanisms by which COR genes function in cold stress response is understood, specifically COR78 has been shown in the accumulation of soluble carbohydrates, including sucrose, glucose, raffinose, and fructose (Gilmour *et al.,* 2000). Another example of COR gene function is COR15a, which acts to stabilize membranes against damage during freeze conditions (Thomashow *et al.,* 1997). Support for DREB/CBF activation of COR genes was found in studies showing that cold treatment induced CBF accumulation within 15 minutes followed by COR genes accumulation within 2 hours (Gilmour *et al.,* 1998; Huang *et al.,* 2012). These results suggest a direct correlation between DREB/CBF and activation of COR genes.

Other participants of cold signal transduction include induction of Inositol polyphosphate 1-phosphatase (FRY1), which catabolizes IP_3. IP_3 has been implicated in generating calcium oscillations, thus acting during the primary steps of signal transduction, and functions as a second messenger (Viswanathan and Zhu, 2002). This process eventually activates a MAPK cascade of signal transduction, possibly activating ICE1 phosphorylation and the remainder of the pathway. Eskimo 1 (ESK1) is another participant in the cold-response pathway. This protein acts as a negative regulator of freeze tolerance, possibly by controlling proline accumulation through regulation of genes affecting proline synthesis and degradation. Support for ESK1 as a negative regulator was shown in a study of the *esk1* mutants that was constitutively freeze tolerant (Xin and Browse, 1998). Additionally, there is a CBF-independent pathway of cold response that includes activation of HOS9, a constitutively expressed transcription factor, which needs to be examined further (Shinozaki *et al.,* 2003; Zhu *et al.,* 2004; Chinnusamy *et al.,* 2004; Huang *et al.,* 2012).

Cytokinin Two Component Signaling Pathway and Cold Stress

The cytokinin Two Component Signaling pathway (TCS) system is a multistep phosphorelay signaling pathway based on a modified bacterial two component system. It has histidine kinase receptors that are phosphorylated after ligand, cytokinin, binding, followed by relay of the phosphorylation signal to response regulators in the nucleus. In plants this has become a multistep phosphorelay in order allow the signal to move from the site of perception into the nucleus where the response regulators act to output the signal response. This two component bacterial signal transduction pathway informs our understanding of signal transduction in the plant system especially in cytokinin signaling, cold, osmosensing signaling where a similar mechanism is employed (Urao *et al.,* 1999; Hwang and Sheen, 2001;Werner and

Schmülling, 2009; Jeon *et al.,* 2010). The specific components of the TCS include receptors, *Arabidopsis* histidine kinases (AHKs), found on the plasma or ER membrane which when activated auto-phosphorylate the histidine residue, which is subsequently transferred to the aspartic acid residue. The phosphate is then transferred to the signal transducers, *Arabidopsis* histidine phosphotransfer proteins (AHPs). These AHPs relay the signal to the *Arabidopsis* response regulators (ARR's), type-A and type-B. Type-A proteins regulate signal transduction by inhibiting Type-B transcription factors in a protein-protein binding manner or by competitively accepting the phosphate from the AHPs. Type-B transcription factors if phosphorylated act to regulate expression of target genes with type-A ARRs being one of the target genes **(**Hwang and Sheen, 2001; Dortay *et al.,* 2006; Werner and Schmülling, 2009; Fig. 1B). This pathway of signaling is implemented during each type of signaling mentioned above. Among the transcription factors involved in this TCS system in plants are the CRFs (Cytokinin Response Factors). These CRFs interact with the AHPs and are most similar to the type-B ARRs during cytokinin signaling (Rashotte *et al.,* 2006). This family of genes will be examined in further later and is the focus of this research.

The TCS components function in more than just cytokinin response and are also involved in cold signal transduction and response. Some type-A ARR components function by inhibiting ABA signaling during cold stress, linking the cytokinin and ABA pathways (Jeon *et al.,* 2010). ARR7, type-A ARR is induced at 4 hours following initial cold stress. Furthermore, over-expressers of ARR7 were insensitive to ABA and had decreased freeze tolerance. Mutants were overly sensitive to ABA and had enhanced freeze tolerance (Jeon *et al.,* 2010) Thus, a component of the TCS pathway seems to moderate the cold response of plants in an ABA-

dependent manner. These results may indicate other components also playing a role that is yet to be examined.

The TCS system is important for many abiotic stresses signaling. This system provides a possible explanation in how the genes of interest to this paper, CRFs, may be involved in cold stress response in an ABA dependent manner.

Cytokinin Response Factors (CRFs)

Cytokinin Response Factors (CRFs) are a family of proteins found in all land plants that were first identified in *Arabidopsis thaliana*. These proteins act as transcription factors and are members of the large AP2/ERF (apetala-2/ethylene response factors) protein family. These proteins bind to DNA of varied sequences and effect many environmental and developmental responses in plants (Sakuma et al., 2002). Furthermore, these CRF proteins are members of the ERF subfamily of AP2/ERF proteins based on certain amino acid residues within the AP2 DNA binding domain (Rashotte and Goertzen, 2010).

The ERF subfamily has been divided into 6 or more subgroups with CRFs belonging to subgroup VI (Nakano *et al.,* 2006). Further examination of CRFs revealed a group specific domain, the CRF domain, found in all CRFs along with the AP2/ERF DNA binding domain, and a putative MAPK phosphorylation motif (Rashotte and Goertzen, 2010, Zwack *et al.,* unpublished; Fig. 2).

Phylogenetic analysis of the CRFs further dissects this group into 5 clades, with a total of 12 CRF proteins in *Arabidopsis* (Zwack *et al.,* unpublished). Analysis of CRFs across land plant species revealed homologous gene sequences and proteins. Specifically, tomato has been determined to have homologous CRF proteins, i.e. *Solanum lycopersicum* cytokinin response factors (*Sl*CRFs), to those found in *Arabidopsis*. The 11 *Sl*CRFs identified can similarly be

divided into clades; only 1 protein has no direct orthologous clade in *Arabidopsis, Sl*CRF1 (Shi *et al.,* 2012a).

Functional examinations of CRFs have been performed to reveal these proteins act in a side branch of the TCS pathway, described above (Cutcliffe *et al.,* 2011). In fact CRFs interact with the AHPs of the TCS pathway and induce many of the same target genes as the type-B transcription factors (Rashotte *et al.,* 2006, Cutcliffe *et al.,* 2011). Most CRFs are transcriptionally induced by treating plants with cytokinin, a plant hormone responsible for many developmental processes such as cell division (Rashotte *et al.,* 2006, Shi *et al.,* 2012a). However, CRF3 and CRF4, clade II CRFs, are transcriptionally unaffected by cytokinin thus their role in the TCS is also unclear (Rashotte *et al.,* 2006).

Loss-of-function mutants of the receptor and other components of the TCS pathway demonstrated lack of cytokinin response and mutational phenotypes such as reduced cotyledon size, lack of cell expansion, and reduced cell number (Rashotte *et al.,* 2006). The loss-of-function double and triple mutations of CRFs demonstrated similar phenotypes and additionally some phenotypes not seen in TCS component mutations; however, the CRF mutants did not completely lack cytokinin responsiveness (Rashotte *et al.,* 2006), indicating that CRF proteins function as only a side branch in this TCS pathway or are redundant in functionality (Fig. 1A).

More recent functional examination of CRF proteins demonstrated their ability to both homo- and hetero-dimerize (Cutcliffe *et al.,* 2011). Natural variants and partial CRF protein parts were used or created to analyze the CRF protein. The analysis revealed the group specific CRF domain to be necessary for protein-protein interaction. This analysis further revealed dimerization occurring at or near the nucleus, supporting previous claims of CRF relocalizing to the nucleus following cytokinin treatment (Rashotte *et al.,* 2006; Cutcliffe *et al.,* 2011). This

study also indicated that CRFs not only interact with each other but also with AHPs in the TCS pathway much in the same way as other TCS components; however, CRFs did not interact with AHKs, the receptors of the TCS pathway, nor rarely the Type-A or Type-B Response Regulator proteins (Cutcliffe *et al.,* 2011).

CRF proteins may be classified into 5 clades within the CRF family of genes (Zwack *et al.,* unpublished). Preliminary research indicates that these clades may serve different purposes within the plant; specifically not all of the CRF proteins are transcriptionally induced by cytokinin (Rashotte *et al.,* 2006). One apparently unifying characteristic of these proteins is their expression in the vasculature tissue of plants, specifically the phloem, (Zwack *et al.,* unpublished) along with the specific protein domains mentioned above (Rashotte and Goertzen, 2010; Fig. 2). Expression of CRFs in the vascular tissue may be an important mechanism to respond to cold stress. CRF vascular expression may induce transcription of solute accumulating proteins transporting sucrose from the phloem into cells for protective mechanisms during cold stress and possibly photosynthetic reasons under standard conditions. The reverse may be true in order to re-localize sucrose from one tissue to another. CRF proteins may also induce transcription of proteins involved in breaking sucrose down in to the components, glucose and fructose, such as the invertase protein. Thus, this unifying theme of CRF localization may indicate a role in stress conditions. However, we are currently pursuing the specific functions of each gene and each clade.

CRFs in Cold Stress

As previously described the CRFs were first identified as components in the TCS signaling pathway (Rashotte *et al.,* 2006). However, more recently, CRF4 has been found to bind to ABE (ABRE), EBE (GCC), and DRE/CRT (GCCAC) sequences with level of affinity

respectively (Gong *et al.,* 2008). Additionally, through microarray examination of transcription factor induction, Feng *et al.* (2005) showed a minimal induction of CRF3 (signal difference of 5 compared to the control) and no induction of CRF4 during cold stress, but a greater induction during drought (signal difference of 92 and 59 compared to the control). However, the eFP Browser shows microarray induction of CRF3 during cold treatment (Winter *et al.,* 2007). CRF4 is not included on the microarray chip used by the eFP browser, so there is limited information of its induction during stress treatments. Thus, CRF3 and 4 may be responding to the subsequent osmotic stress induced with cold stress through similar DREB/CBF pathway of ABA-independent cold response or through the TCS system, an ABA-dependent pathway. Ultimately, this demonstrates the possibility of CRFs participating in cold response and osmotic signaling through two possible mechanisms, TCS and ICE-CBF-COR, as a side pathway in either system.

The other subgroup of AP2/ERF domain proteins within the ERF subfamily contains DREB/CBF, which have been repeatedly shown to be responsive to cold and osmotic stress or dehydration (Sakuma *et al.,* 2002; Zhuang *et al.,* 2008). These transcription factors activate cold responsive genes by acting on DRE/CRT (dehydration response element) cis-regulatory elements of genes responsible for inducing cold responses in plants (Fig. 1A). These transcription factors can also respond to drought by binding to ABRE cis elements (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu *et al.,* 1998; Sakuma *et al.,* 2002; Kasuga *et al.,* 2005; Mirura *et al.,* 2007; Lata and Prasad, 2011; Fig. 1A).

The DRE/CRT sequence and ABRE, which are found in the promoters of cold and ABA-osmotically inducible genes, are also found with in some CRFs. These cis-elements can be bound by DREB/CBF transcription factors, which are members of the same ERF family as the CRFs (Yamaguchi-Shinozaki and Shinozaki, 1994; Choi *et al.,* 2000; Uno *et al.,* 2000; Nakashima and

Yamaguchi-Shinozaki, 2006). Thus, there may be interactions between the ABA-independent, ICE1-CBF-COR pathway and the ABA-dependent, TCS, through CRF induction and subsequent transcriptional regulation. This may be due to up-stream proteins, DREB/CBFs acting on the DRE/CRT or ABRE sequences within the promoter regions of some CRFs (Fig. 1).

A few of the CRF proteins have been subjects of investigations regarding their specific purpose; however, the two *Arabidopsis* genes within clade II of the CRF family have yet to be studied due to their lack of induction by cytokinin (Rashotte *et al.,* 2006). Though these CRFs are the outliers in terms of hormone response compared to other CRFs, CRF3 and CRF4 are both expressed within the vasculature of plants like the other CRFs within this family (Zwack *et al.,* unpublished) and have a putative MAPK domain that all but two truncated CRFs (CRF7 and 8) possess. Thus, there may be similar functions among CRFs for plant development and also possibly clade specific function in response to other environmental stimuli. For example, Zwack *et al.* (unpublished) has demonstrated that CRF6 may be involved in sucrose re-localization during senescence due to the vascular localization. CRF3 and CRF4 could be involved sucrose re-localization during cold stress due to similar localization.

Cross Talk Among Signaling Systems for Abiotic Stresses

There are some examples of cross talk of pathways within cold stress. ARR7, a type-A ARR, when over expressed leads to the plant being sensitive to freezing. Thus the possible mechanism might through which this could function would be to block cytokinin signaling. Additionally, over expression of ARR7 blocked ABA signaling and response during cold treatment and again, the plants were found to be freeze sensitive (Jeon *et al.,* 2010).

Ethylene also plays a role in cold response by blocking expression of CBF and ARR5, 7, and 15. Plants with negatively regulated CBF and ARR5, 7, and 15 were freeze sensitive (Shi *et*

al., 2012b). This is in contrast to Jeon *et al.* (2010) and Shi *et al.* (2012b) that found higher survival rates for plants over expressing ARR5, 7 or 15. Thus, these previous findings indicate some cross talk among the TCS cytokinin signaling pathway, ABA cold response, and the ABA-independent ICE-CBF-COR response to cold stress.

The MAPK pathway is also involved in development, hormone, and biotic stress signaling in addition to abiotic stress signaling (Jonak *et al.,* 2002). Specifically MPK4 is activated during cold, low humidity, osmotic stress, and wounding by post-translational modifications (Ichimura *et al.,* 2000). This demonstrates some common mechanisms needed for response to different stresses (Sheen, 1996). Perhaps, the cold stress creating osmotic stress leading to similarly described responses.

Even more over-lap between stress signaling systems is found with calcium-dependent protein kinases being involved, in cold, drought, and wounding signaling (Sheen, 1996). Finally, the DREB/CBF pathway can activate genes involved in cold and osmotic stress (Narusaka *et al.,* 2003). The common theme is the osmotic stress being induced in response to other primary stresses that can elicit similar responses using the same proteins. It seems efficient for the plant to use similar mechanism to cope with stress on the system as a whole.

Focus of Present Study

The purpose of this research study is to determine the functional role of CRF4 in *Arabidopsis thaliana*. Despite being a member of the Cytokinin Response Factor family, CRF4 lacks induction by cytokinin as compared to the other CRFs and thus its functional role is generally unknown. This research focuses on cold stress and CRF4 in early developmental. Preliminary results indicated that a key cold signaling mutant, *ice1* altered expression of CRF3, paralog to CRF4, suggesting that CRF4 might function in cold stress response (Ref) . Similar

examinations of related abiotic stress from publically available microarray data also suggest that this pair of CRF genes, CRF3 and 4, might be involved more in cold than general stress response (Chinnusamy *et al.,* 2003; Lee *et al.,* 2005; Winter *et al.,* 2007; Rashotte and Goertzen, 2010; Huang *et al.,* 2012).. Although, Feng *et al.* (2005) did find that that CRF3 and CRF4 were highly induced by drought, it is possible that their drought induction suggest manner that these CRFs function in response to cold stress. Perhaps, these CRFs are being induced in response to the osmotic stress that results from further temperature decreases. These processes are also linked from the identification of DRE/CRT sequence in CRF4 and ABRE sequence in CRF3 revealed during promoter analysis.

Therefore, this research will examine how altered CRF4 expression influences development of different tissues in Arabidopsis under standard laboratory conditions versus cold treatment conditions (4°C) at different developmental time points. This examination over the lifetime of the plant is important because during development comes altered gene expression and protein regulation in response to cold stress. Specifically, changes in expression of representatives from each CRF clade due to cold will be examined at late vegetative and early floral stages to determine if other CRFs might also be involved in cold stress response. Expression level changes of CRF3 and CRF4 following cold treatment over different stages of development, will determine if expression if these genes show differentially developmental regulation in response to cold stress. Additionally, cold tolerance and growth with varying sucrose concentrations will be examined since many studies have found the sucrose aids in stress tolerance (Rekarte-Cowie *et al.,* 2008). Finally, effects of freeze treatment on the survival of CRF4 altered expression plants will be explored along with expression changes in CRF4 and CRF3 in response to this treatment.

Materials and Methods

1. Plant Materials and Growth Conditions

Arabidopsis thaliana (Columbia ecotype, Col-0) plants were used in all experiments, these plants served as Wild type and parental lines for the B-Glucoronidase (GUS) reporter lines (pro^{CRF4}:CRF4:GUS), over-expression lines (35S:CRF4), and loss-of-function mutants, *crf4-1* (Sk_035063). The GUS reporter line contains translational fusion transgenic plants with the GUS promoter fused to the gene of interest (Zwack *et al.,* unpublished). Additionally, over-expression lines contain the Cauliflower Mosaic Virus (CamV 35S) promoter sequence fused to the gene of interest. Loss-of-function mutant was ordered as a T-DNA-insert line from SK-collection (Robinson and Parkin, 2009). Plants with altered genotypes were selected to greater than 2 generations and confirmed using GUS staining or quantitative RT-PCR (qRT-PCR), respectively.

All sterilized *Arabidopsis* seeds were germinated in sterile petri dishes on 0.8% agar medium containing Murashige and Skoog salts (1XMS) (4.8g/L) supplemented with1% sucrose media buffered to pH 5.7. Sucrose supplementation was adjusted to 0% (no supplementation) or 2 % in sucrose experiments as indicated. Seed sterilization was performed following Weigel and Glazebrook (2002) protocol. Seeds were stratified for two days at 4°C at approximately 1 micro-Einstein (μE). Plants were grown under 16/8 hour light: dark photoperiod at approximately 100 μE with 22°C/18°C light/dark temperatures in controlled environmental chambers (standard

conditions). Plants used in extended growth studies were transferred to sterilized magenta boxes containing the same medium.

Cold treatment of *Arabidopsis* plants for the developmental and extended cold and recovery experiments was performed by placing plants in a controlled chamber at 4°C under constant dim light (~1μE). During recovery plants were placed under standard (non-cold) growth conditions. Plants examined in germination experiment at 4°C were maintained at this temperature after sterilization in a controlled chamber at 4°C under constant dim light (~1μE). Freeze treatments were performed in a controlled chamber in the dark between (-8°C and -13°C) on plants after growth at standard conditions for X days.

Tomato dwarf cultivar Microtom was used in tomato experiments. Tomatoes were grown in sunshine mix #8 soil with 16/8 hour light/dark photoperiod at approximately 150μE with 26°C/22°C, light/dark temperature in controlled environmental chambers.

Tomato cold treatments consisted of placing the soil-sown plant in its pot in an ice bath in a 4°C chamber with constant dim light (1μE).

2. Mutant Screening and Expression

CRF4 over-expression (35S:CRF4) and mutant (*crf4*) genotype plant transcription levels were confirmed by qRT-PCR. Samples from 1-2 plants of 35S:CRF4 or *crf4* that were selected to the second generation were used for expression analysis. CRF3 expression in over-expression and mutant CRF4 background was also examined by qRT-PCR (Fig. 3).

3. Promoter Screening

To determine the presence of DRE/CRT and ABRE cis-elements in the promoters of CRFs a search was executed using word/letter search in 2Kb of CRF sequence upstream of the ATG start site (Table 1).

4. Comparative Development

4.1 Germination Study

Seeds of 35S:CRF4, *crf4,* and wt were sterilized and sown on the 0%, 1%, or 2% sucrose supplemented MS plates; the plates were exposed to 22°C (control treatment) or 4°C (cold treatment). Germination of seeds was monitored at 6, 9 and 12 days following a 48h stratification period. Germination was defined as radical emergence. Percent germination was calculated from total seeds of each genotype per plate treated (n=20-52) from 3 replicates.

4.2 Shoot Development Study

Seeds of 35S:CRF4, *crf4*, and wt were sterilized and sown on MS medium 0%, 1% or 2% sucrose. Each plate was marked and divided into sections contained seeds of one genotype per section. Seeds were grown at 22°C until germination when plates were exposed to 4°C or remained at 22°C standard conditions. Shoot development was monitored every 3 days post treatment.

Plant developmental stage characterization for comparative development of transgenic plants versus Wild type plants were categorized as follows: stage 0, seed; stage 1, radical emergence (germination); stage 2 cotyledon emergence; stage 3, yellow cotyledon; stage 4, green cotyledon; stage 5, first set of true leaves forming; stage 6, second set of true leaves forming; stage 7, third set of true leaves forming (Fig 5A). Three experimental replicates per sucrose concentration were performed.

4.3 Root Growth Study

35S:CRF4, *crf4,* and wt seeds were sterilized and placed on separate 1% sucrose supplemented MS medium plates. Seeds were stratified and then exposed to 22°C. Once plants reached the green cotyledons stage, plants were moved to new plates, oriented vertically and initial root growth was marked. Plants were then exposed to 4°C (cold) or 22°C (control) treatment. Root growth was calculated after 12d for control and 33d for cold treatment. Root growth was measured using ImageJ (n= 13-26) from 4 experimental replicates.

5. RNA Isolation, cDNA synthesis, RT-PCR quantitative-RT-PCR

RNA was isolated with Qiagen RNeasy Kit according to the manufacturer's instructions. RNA concentration was determined by a Nanodrop spectrophotometer and was normalized for each tissue type and experimental run. cDNA was synthesized from the RNA templates extracted from samples following the Quanta Biosciences qScript cDNA supermix protocol. The cDNA was then diluted before use in qRT-PCR. The primers used for qRT-PCR are listed in Table 2. QRT-PCR was performed with SYBR-Green chemistry using the Eppendorf Realplex Mastercycler. For *Arabidopsis*, the reaction contained 9µl of SYBR-Green supermix, 0.12µl of 100M, gene-specific forward and reverse primers, 5.76µl of sterile water, and 5µl of cDNA. For tomato, the reaction consisted of 9µl of SYBR-Green supermix, 3µl of 4M gene-specific forward and reverse primers, and 5µl of cDNA.

The qRT-PCR program for *Arabidopsis* CRF1-4, 7, 8, and 10, consisted of one cycle at 95°C followed by 40 cycles of 95°C/15 seconds, 57°C/45 seconds, and 68°C/30 seconds. The qRT-PCR program for *Arabidopsis* CRF5, 6, and 9, consisted of one cycle at 95°C followed by 40 cycles of 95°C/15 seconds, 54.5°C/20 seconds, and 68°C/35 seconds. For tomato, the qRT-PCR program consisted of one cycle at 95°C followed by 40 cycles of 95°C/15 seconds, 57°C/45 seconds, and 68°C/40 seconds. Relative expression per experimental run was normalized to

reflect concentration of initial RNA. The reference gene was elongation factor one-alpha (EF1-alpha) and was selected based on data synthesized from *Arabidopsis* eFP Browser (Winter *et al.,* 2007) indicating stable expression during cold stress and from data reported by Nicot *et al.* (2005) demonstrating that EF1-alpha is the most stable internal control gene for abiotic stress treatments. *Tip41* was used as the reference gene for tomato (Shi *et al.,* 2011). QRT-PCR data are the mean of 2 experimental runs (n=2-4) with 2 technical replicates per sample for each qRT-PCR run. Real-time data was analyzed according to Willems *et al.* (2008) protocol. Fold change values were normalized to control gene values

6. Cold Treatments at Developmental Stages

6.1 Developmental Expression with Cold Stress

Wild type plants were sterilized, stratified, and grown on 1% sucrose supplemented MS medium plates. At 10, 17, 24, and 33 days post germination plants were placed in 4°C under 1μE light. Whole plants were taken at 1 and 6 hours and separated in to root and shoot samples from which RNA was isolated and expression was quantified by qRT-PCR separately as described above.

Samples taken at 10, 17, 24, and 33 days post germination were had the following phenotype features. At 10d plants had 4 emerged leaves and were in the vegetative stage of development. At 17d plants had 8 emerged leaves with inflorescence forming and were at the early stage of floral development. At 24d plants had an inflorescence emerged and flowers were opening. At 33d plants had terminal flower buds open and axillary flower buds present and were at the end stage of floral development.

6.2 Expression with Extended Cold Treatment

Plants 17 days post germination were treated with extended cold (4°C), 7 days. During cold treatment, samples were taken at 1,3,6, 12, and 24 hours. After 7 days of cold treatment, plants were exposed 22°C standard conditions for recovery from cold. Samples were gathered at 1,3,6,12,24, 48, and 96 hours during recovery. All samples were separated in to roots and shoots, from which RNA and expression was quantified by qRT-PCR separately as described above.

7. GUS Selection and Staining

Stable translational fusion CRF4 GUS lines (proCRF4:CRF4:GUS) were confirmed by selection of homozygous BASTA resistance in the T3 generation from which a single line with representative expression patterns was used for further analysis. Plants used for GUS staining were submerged in GUS staining solution (X-gluc buffer) and placed in a vacuum for 20 minutes, then incubated at 37°C for 2 hours (Weigel and Glazebrook, 2002). After staining, tissue was cleared of chlorophyll and other materials using 70% ethanol. Pictures were taken with Nikon QiCam camera at indicated magnifications**.**

8. Freeze treatments

8.1 Freeze Survival

Sterilized seeds of 35S:CRF4, *crf4,* and wt plants were placed on 1% sucrose supplemented MS medium plates (all on same plate ~ 5 plants/section), stratified, then treated with 22°C. At 10 days post germination, plants were treated for 5 hours in a dark freeze chamber, which was cooled to -9°C to -11°C. Percent survival was determined for each genotype.

8.2 Freeze Expression

Wild type plants were treated with -8°C to -13°C for 5 hours in the dark. Shoot samples were taken following freeze treatment from which RNA was extracted and qRT-PCR was performed as described above.

9. Data Analysis

All data was analyzed on Microsoft Excel 2010. Results are shown as the average value from all experimental replicates. Error is calculated as the standard error of the mean (SEM). Significance testing was performed with using a two-tailed student's t-test with $p < 0.05$ being significant.

Results and Discussion

CRF4 Over Expression and Mutant Genotype Transcript Levels

In order to examine whether *crf4* and 35S:CRF4 lines had altered expression levels of CRF3 and 4 compared to Wild type RNA was extracted, cDNA synthesized, and transcript levels examined in these genotypes compared to Wild type using qPCR. CRF4 transcript levels are induced in the 35S:CRF4 genotype with a fold change value of 3.32 compared to Wild type with a fold change of 1 representing no change in expression (Fig. 3). CRF3 transcript levels are similarly induced in 35S:CRF4 plants with a fold change value of 3.94. The transcript levels of CRF4 and CRF3 were also examined in the *crf4* background with fold change values of 0.01 and 0.55, respectively (Fig. 3).

These results indicate that the over expression of CRF4 by the constitutively expressed promoter does indeed induce CRF4 expression greater than that of Wild type (Fig. 3). CRF3 was similarly induced in 35S:CRF4. Interestingly expression levels of both CRF4 and CRF3 were reduced in the *crf4* plants (Fig. 3). These results indicate the possibility of CRF4 and CRF3 co-regulating transcription of each other in a positive feedback mechanism. It may also be inferred that CRF4 or CRF3 activity does not compensate for the lack of the other transcription factor, which might be predicted for homologous genes. It could also be possible that CRF4 and CRF3 proteins must bind in order to promote transcription of other genes as a heterodimer since these protein have been found to interact in a previous study (Cutcliffe *et al.,* 2011).

CRFs have Cold Inducible Promoters

In order to determine if CRFs could be involved in cold response, promoter sequences of these genes were examined for the presence of cold regulated cis-elements. This revealed many of the CRFs genes in Arabidopsis have either DRE/CRT or ABRE cis-elements in their promoter regions 2kb upstream from the ATG start site (Table 1). CRF2,4,6,7, and 11 each have DRE/CRT elements in their promoter regions. CRF2,3,5 and 6 have ABRE elements in their promoter sequences.

These promoter elements are known to be bound by cold induced transcription factors (Theocharis *et al.,* 2012). Thus, these results suggest that CRFs may play a role in cold response through being bound and induced by such transcription factor then potentially acting to further bind and promote transcription of other cold responsive genes. It is also possible that CRFs are transcribed as a result of other stress response pathways such as ABA signaling as suggested by the presence of ABRE elements within their promoter sequences. The actual binding of various transcription factors to these specific promoter elements needs to be examined in future study.

Comparative Development

Cold and Sucrose Affect Germination in Altered CRF4 Expression Genotypes

In order to examine if expression of CRF4 affected germination with sucrose and temperature treatments, seeds of CRF4 altered expression lines were exposed to varying sucrose concentrations on MS medium with standard conditions and cold conditions of 4°C. Cold was found to decrease the percent germination of seeds from each genotype and sucrose concentration (Fig. 4B). For seeds in control conditions of 22°C the percent germination was not significantly affected by sucrose concentrations (Fig. 4A). However, with increased sucrose concentration, percent germination of all genotypes decreased with cold treatment of 4°C (Fig. 4B).

At 22°C there is a similar percent germination between 35S:CRF4 and Wild type seeds, and both genotypes have a greater percent germination compared to *crf4* on 0% and 1% sucrose supplemented medium (SSM). At 4°C there is a negative and additive effect of sucrose and cold on germination. The 35S:CRF4 genotype was the most affected by these treatments with percent germination being significantly less than Wild type at day 9 on 0% sucrose ($p=0.001$). The percent germination of 35S:CRF4 was also significantly less than Wild type at days 9 and 12 on 1% SSM ($p<0.001$ and $p=0.017$, respectively) and at day 6, 9 and 12 on 2% SSM ($p=0.05$, $p=0.01$, and $p=0.03$, respectively).

Additionally, there was a sucrose affect with Wild type on 1% SSM having significantly lower percent germination than Wild type on 0% SSM at day 9 ($p=0.003$). At day 9 *crf4* was also negatively affected to significant levels by the increase in sucrose concentration from 0% to 2% ($p=0.04$). 35S:CRF4 was also negatively affected by the increase of sucrose from 0% to 2% at day 12 ($p=0.05$).

These results suggest an additive affect of sucrose and cold on germination in all genotypes. The results at 22°C suggest that CRF4 expression and activity on germination percentage is not affected by sucrose concentration. However, results from cold treated experiments suggest otherwise with the over expression of CRF4 negatively affecting germination percentages when cold treated at any sucrose concentration. The negative additive affect of cold and sucrose on the 35S:CRF4 genotype seeds suggest that CRF4 negative regulates germination under stress conditions, specifically cold and increased sucrose concentration. The *crf4* seeds were also negatively affected by increased sucrose concentrations whereas Wild type seeds were affected to a lesser extent.

Additionally, these results suggest that a carefully regulated expression of CRF4 may be necessary for germination under stress conditions. The results may also be interpreted as cold having an effect delaying germination and increased sucrose concentration further delaying germination. However, it seems that over expression of CRF4 under stress conditions consistently negatively affects germination percentages.

Cold and Sucrose Additively Affect Shoot Development in Altered CRF4 Expression Genotypes

In order to determine if altered CRF4 expression influences shoot development, plants were grown under standard conditions on various sucrose supplemented media (SSM) until germination and then exposed to cold. The plants exposed to cold treatment compared to the 22°C standard conditions were found to be at earlier developmental stages across sampling time points and sucrose concentrations (Fig. 5B and C). Plants developmental stage was less at greater sucrose levels when exposed to 4°C.

The rate of seedling development measured as stage per day (s/d) was also affected by sucrose supplementation. For example, the rate of development was lower in Wild type and 35S:CRF4 genotypic plants grown on 2% versus 1% SSM (wt rate= 0.362 to 0.292s/d, respectively and 35S:CRF4 rate= 0.279 to 0.244s/d, respectively) exposed to 22°C standard conditions (Fig. 5B). Conversely, the *crf4* genotypic plants increased in rate of development with increasing sucrose concentrations from 1% to 2% SSM (*crf4* rate= 0.214 to 0.238s/d, respectively). The opposite was true with plants exposed to 4°C (Fig. 5C). The rate of development was greater in Wild type and 35S:CRF4 genotypes grown on 2% compared to 1% SSM exposed to 4°C (wt rate=0 to 0.19s/d, respectively and 35S:CRF4 rate= 0.14 to 0.16s/d,

respectively). The *crf4* plant rate of growth was slower in 2% compared to 1% SSM exposed to 4°C (*crf4* rate= 0.245 to 0.236s/d).

There were also significant differences among the genotypes of plants exposed to 22°C within sucrose treatments (Fig. 5B). On 0% SSM at day 9, *crf4* was significantly more developed than Wild type plants (p=0.001), and 35S:CRF4 was significantly less developed (p=0.002). On 1% SSM *crf4* and 35S:CRF4 plants were significantly different than Wild type on day 6 and 12 (Fig. 5B). At day 6, *crf4* was more developed than Wild type and 35S:CRF4 was less developed (p=0.002 and p=0.009, respectively). At day 12, *crf4* and 35S:CRF4 were less developed than Wild type ($p=3.0x10^{-5}$ and $p=1.0x10^{-7}$, respectively). Plants grown on 2% SSM also demonstrated genotypic differences (Fig. 5B). At day 6, shoot development of *crf4* plants was significantly greater than Wild type (p=0.003); whereas 35S:CRF4 plants were less developed than Wild type ($p=3.0x10^{-4}$). At day 12, the 35S:CRF4 plants had significantly less shoot development than Wild type ($p=9.9x10^{-7}$). There were no sucrose effects on genotypes in the 22°C exposed plants.

Both genotype and sucrose affected shoot development differently among plants exposed to a 4°C cold treatment (Fig. 5C). On 0% SSM *crf4* plants were significantly more developed than Wild type plants (p=0.005) at day 6. At day 9, 35S:CRF4 plants had significantly less shoot development than Wild type (p=0.024). At day 6 and 9 on 1% SSM *crf4* was significantly less developed than Wild type (p=0.002 and p=0.002, respectively), and it was significantly more developed than Wild type at 12 days (p=0.017). The 35S:CRF4 plants had significantly less shoot development than Wild type at day 6, 9, and 12 on 2% SSM (p=0.021, p=0.001, and p=0.018, respectively).

Sucrose affected shoot development plants of the same genotype exposed to 4°C (Fig. 5C). The *crf4* plants grown on 1% and 2% SSM were significantly less developed than those grown on 0% sucrose at day 6 ($p=1.42x10^{-8}$ and $p= 1.24x10^{-31}$, respectively). At day 9 and 12, *crf4* plants grown on 2% sucrose were less developed different than those sampled at day 9 and 12 on 1% sucrose ($p=6.96x10^{-5}$ and p=0.004, respectively). When grown on 2% SSM Wild type plant shoot development at days 6 and 9 was significantly less than 0% SSM ($p=7.72x10^{-10}$ and $p=4.0x10^{-4}$, respectively) and 1% SSM ($p= 4.58x10^{-11}$ and p=0.006, respectively). At days 6, 9 and 12, 35S:CRF4 plants grown on 2% SSM had shoot development that was significantly less than those grown on 0% SSM ($p=1.55x10^{-18}$, $p=3.36x10^{-6}$ and p=0.002, respectively) and 1% SSM ($p=1.17x10^{-5}$, p=0.007 and $p=1.49x10^{-5}$, respectively). 35S:CRF4 plant shoot development grown on 1% sucrose was also significantly less than those grown on 0% sucrose when monitored at day 6 ($p=1.0x10^{-4}$). Plants grown on varying SSM exposed to 4°C did not exceed green cotyledon stage of development (stage 4).

The results of these shoot development experiments suggest an opposite affect of increasing sucrose concentrations on shoot development between 22°C and 4°C exposed plants. There is a positive affect of increased sucrose concentration on shoot development in 22°C and a negative effect in 4°C. Thus, with the decreased shoot development effect of 4°C along with the negative sucrose effect, there seems to be an additive affect of sucrose and temperature on shoot development. The reverse is true for plants exposed to 22°C.

Additionally, the rate of seedling development is altered with both sucrose concentration and temperature treatment. The rate of development is greater in plants with normal or excessive CRF4 expression in 22°C; whereas the absence of CRF4 in 4°C promotes a greater growth rate. These results suggest that CRF4 negatively regulates shoot development rate under cold stress

conditions. It is also possible that CRF4 expression is reduced, thus reducing activity during cold stress. This is true for plants on both 1% and 2% SSM exposed to 4°C. However, the final age examined shows the greatest shoot development in *crf4* plants across all SSM. This is not the case in 22°C exposed plants, where *crf4* has varied final stages compared to Wild type that is dependent on the SSM. For example, *crf4* has the greatest shoot development on 0%SSM, less than Wild type on 1% SSM, and equal to WT on 2%. Thus the effect of missing CRF4 expression changes with sucrose treatment.

The Absence of CRF4 Promotes Root Growth in 22°C

In *crf4* plants exposed to 22°C, root growth is significantly greater than Wild type plants under the same conditions ($p=0.003$) (Fig. 6). The root growth is similar between Wild type and 35S:CRF4 plants in 22°C. Conversely, 35S:CRF4 plants had significantly less root growth in 4°C than did Wild type under the same conditions ($p=7.9\times10^{-5}$) (Fig. 6). Root growth between *crf4* and Wild type plants exposed to 4°C is similar. There is significantly less in root growth within the same genotype, *crf4,* Wild type, and 35S:CRF4, when exposed to different temperatures ($p=2.19\times10^{-7}$, $p=0.004$, $p=1.0\times10^{-5}$, respectively) (Fig. 6).

These results suggest that CRF4 negatively regulates root growth at 22°C. The mechanism by which CRF4 regulates root growth might be through inducing transcription of root growth regulators. It may also be inferred that excess CRF4 protein negatively affects root development in plants exposed to 4°C. This could possibly occur by inducing transcription of similar genes that promote root growth in plants under standard conditions. Thus, CRF4 may work in a similar way under both temperature conditions, but it seems to have a greater effect under standard conditions.

CRF4 is Highly Induced in Shoots and Roots

In order to determine if other CRFs were induced to cold stress, Wild type plants in late vegetative stage (10 days post germination:10d) and early floral stage (17 days post germination:17d) were exposed to 4°C for 1 and 6 hours. CRF transcripts levels were quantified and compared to control levels using qPCR. A shift in timing of greatest induction and location can be observed between the two time exposures to cold examined (Fig. 7).

At 10d, CRF4 is the most highly induced CRF in the shoots to a 9.8 fold change increase. CRF3 is also induced to 2.7 fold at 6 hours in the shoots (Fig. 7A). The other CRFs examined had transcript levels at 6 hours below CRF3. However, CRF1,2,5-10 had higher fold change values at 1 hour than the fold change that was seen for each of these genes at 6 hours. CRF8 had the greatest fold change at 1 hour with a value of 3.4. The transcript levels for CRFs in the roots at 6 hours are greater than in at any other time or tissue examined, with CRF3 and 4 being induced to 7.1 and 6.5 fold change, respectively (Fig. 7A). The greatest induction of any CRF was found for CRF9 at 6 hours in the roots induced to 31.1(Fig.7A).

At 17d, the highest induction of CRF3 and 4 in the shoots is found at 1 hour with fold change values of 1.86 and 1.82, respectively. The greatest induction of any CRFs examined at this age was CRF2 with a fold change of 6.3. All CRFs examined in the shoots at 17d show higher induction at 1 hour versus 6 hours (Fig. 7B). In contrast, root expression at 17d shows greatest expression of CRFs at 6 hours. CRF4 had the greatest fold change in the root to 6.7, with CRF3 induced to 2.5 (Fig. 7B).

These results indicate consistent induction of CRF3 and CRF4 in the shoots and roots with 6 hours of exposure to 4°C at 10d. While other CRFs are highly induced in the roots at 6 hours, specifically CRF9, they lack a consistent induction in the shoots as is seen for CRF3 and

4. Interestingly, these results also suggest a shift in timing of expression of CRF3 and CRF4 in the shoots at between 10d and 17d from 6 hours to 1 hour induction but with lower fold change values. This may be due to the shift from vegetative to floral stage between these sampling times or an alternative response to 4°C exposure. CRF4 shows similar induction at 6 hours in the roots at both 10d and 17d suggesting a potential need for CRF4 during cold stress especially during the vegetative state of the plant. Perhaps the levels of CRF4 in the roots that are being maintained at a constant level across developmental stages indicates that CRF4 is involved in the same cold response pathway in the roots, different from that seen in the shoots.

The Maximum Induction of CRF3 and CRF4 Occurs within the First 6 Hours of Cold Treat and is not Affected during Recovery

Next, it was examined whether the difference in high levels of CRF3 and CRF4 induction at 6 hours in the roots at 10d to low expression levels at 17d was due to a shift in developmental timing or a delayed response (Fig. 7). This was done by examining CRF3 and 4 transcript levels in plants exposed to extended cold treatment followed by recovery at 22°C. Shoot transcript levels of CRF3 and CRF4 were similar to no treatment levels throughout the extended cold and recovery with values fluctuating around 1 indicating no change from control. The highest shoot transcript levels were found at 1 hour of cold treatment (fold change=1.8) and the lowest found at 24 hours during recovery (fold change=0.08) (Fig. 8). The root transcript levels for CRF4 peak at 6 hours of cold treatment (6.69 induction) and return to just below 1 (no change) through the rest of the cold treatment and recovery (Fig. 8). Transcript levels for CRF3 reach maximum levels in the root at 3 and 6 hours of cold treatment, and then follow a pattern similar to CRF4 for the rest of the cold treatment and recovery (Fig. 8).

The results of the extended cold and recovery treatment indicate that the first 6 hours of cold treatment are the most important for expression CRF4 and CRF3in the roots. In fact, CRF4 and CRF3appear unlikely to be involved in any step after 6 hours since there is no transcript changes during cold treatment after that point. This suggests that CRF4 and CRF3 are primarily involved in the acclimation stage of cold stress, i.e. preparing the plant for freezing temperature. CRF3 and CRF4 do not appear to be involved in the recovery process the plant undergoes following cold stress as their transcripts are unaltered during that period.

Maximum Transcript Levels of CRF3 and CRF4 Shift from 1 to 6 Hours During Development

To further examine CRF3 and CRF4 transcript level changes in response to cold treatment, samples of Wild type plants exposed to 4°C were examined at different developmental time points post germination, 10d (early vegetative), 17d (early floral stage), 24d (floral stage), and 33d (late floral stage) (Fig. 9). The greatest induction of CRF4 in shoots occurs at 10d with 6 hours of cold treatment (fold change=9.8) (Fig. 9D). After 10d expression levels for both CRF3 and CRF4 are at lower levels with 6 hours of cold treatment. Treatment with 1 hour of cold does not induce CRF3 or CRF4 in the shoots throughout development (Fig. 9B).

Root expression revealed a shift in timing of maximum expression induction of CRF3 and CRF4 that is related to developmental stage (Fig. 9A, C). CRF3 and CRF4 are highly induced at 10d with 6 hours of cold treatment (fold change= 7.1 and 6.5, respectively) and CRF4 continues to be induced at 17d in the roots (fold change=6.7). At 6 hours of cold treatment, both CRF3 and CRF4 transcript levels in the roots decrease following peak level of induction at 10d and 17d, respectively (Fig. 9C). At 24d, transcript levels are now greatest at 1 hour of cold

treatment with fold change values for CRF3 and CRF4 reaching 4.4 and 3.9, respectively (Fig. 9A).

These results suggest a developmentally dependent expression of CRF3 and CRF4. The changes in maximum expression timing from 6 to 1 hours within the roots suggest a possible change in the mechanism by which CRF3 and CRF4 act during cold stress over development. Perhaps in earlier stages of development, CRF3 and 4 function by responding to cold stress in an indirect manner, since maximum expression levels are not reached until 6 hours. In contrast, at 24d CRF3 and 4 appear to work in a more direct manner, since maximum induction is much more rapid, 1 hour versus 6 hours. The shift may be due to the changing needs of the plant as it transitions from vegetative (10 days) to fully reproductive (24 days) stages. Additionally, the similar expression profiles of CRF3 and 4 seen throughout development suggest that CRF3 and CRF4 are likely to be involved in similar mechanisms of response to the cold stress.

Gus Reporter Gene Expression of CRF4 is Induced with Cold Treatment

In order to confirm the transcriptional expression data found previously indicating CRF4 being highly induced in the shoots and roots at 6 hours of cold treatment at 10d (Fig 9), GUS translational fusion reporter line plants were exposed to cold treatment of 4°C for 6 hours. The results show an increase in CRF4 GUS reporter line staining with 6 hours of cold treatment in the leaf (Fig. 10). The spatial expression pattern is unchanged between control and 6 hours of cold treatment, only abundance is increase. The same is true for CRF4 GUS expression in the roots after cold treatment, though to a lesser extent than that seen in the shoots (Fig. 10).

These results support the transcript level data by demonstrating that not only are transcripts highly induced at 10 days but also the protein levels. These results also suggest that the transcript level data is a good indicator of CRF4 protein presence during cold treatment. The

GUS results also provide a good visual indication of protein level changes and localization of the protein that occur during cold treatment.

Orthologous CRF Genes in Tomato are Induced with Cold Treatment

Tomato plants were exposed to 1 and 6 hours of 4°C treatment in order to examine if the genes orthologous to CRF3 and 4 in *Arabidopsis* are also induced by cold. Plants examined were at a young developmental stage with only 2 true leaves present. This stage was similar to 10d Arabidopsis plants. RNA was isolated from the entire plant after cold treatment and was used to determine transcript expression levels by qPCR. The orthologous CRF3 and 4 genes in tomato, *Sl*CRF4 and *Sl*CRF6, were found to be highly induced at 6 hours of cold treatment with fold change values of 7.3 and 12.4, respectively (Fig. 11).

These results suggest that the expression changes seen in *Arabidopsis* CRFs in response to cold may be similarly found in the orthologous CRFs in other plants. It also indicates that tomato, a fruiting and cold sensitive plant, may implement similar strategies as *Arabidopsis* when responding to cold. This data also supports the use of *Arabidopsis* as a model plant system for understanding other plants.

Survival and Expression During Freeze Treatment

In order to examine whether CRF4 was involved in freezing response, the survival of CRF4 altered expression plants were examined following freeze treatment, 5h at -8 to-13°C (Fig. 12). The survival percentage of 35S:CRF4 was equal to 100% at 9 days post treatment (n=4). The percent survival of *crf4* and Wild type were 50% and 46.2% (n=2 and 13), respectively (Fig. 12A). This suggests that over expression of CRF4 aids survival of plants following freeze treatment to subzero temperatures.

In order to examine the transcript level changes in response to freeze treatment, Wild type plant shoots were gathered following freeze treatment and expression levels were examined using qPCR. CRF3 and CRF4 were both found to be highly repressed with fold change values of 0.035 or -28 and 0.036 or -27, respectively (Fig. 12B).

The results of both the survival and expression experiments together suggest that CRF4 is not induced, but is actually highly repressed in response to freeze stress. Wild type levels of CRF4, very down regulated under these conditions, likely resemble those of *crf4* when stressed with freezing temperature, resulting in low survival levels. The CRF4 levels of the 35S:CRF4 although likely also reduced with freeze treatment should be at levels above both the Wild type and *crf4* and could be responsible for its greater survival rate. These results suggest that if CRF4 expression could be maintained at high levels, that CRF4 would be able to promote survival of plants exposed to freeze treatment. However, the mechanism by which CRF4 promotes survival needs to be explored further.

Conclusions

The results of this thesis suggest that CRF4 and CRF3 are involved in cold and freezing stress response. It also appears that CRF4 has a different mechanism of response to cold stress in the shoots and roots of plants that can be seen in expression differences throughout development.

Additionally, despite the homology between CRF3 and CRF4 they do not seem to compensate for the loss of each other, but actually function in a transcriptional feed-forward mechanism. This suggests that CRF3 and CRF4 are either working on similar processes and require the activity each other or are both equally needed for development or responses to stresses for different purposes. CRF4 may also be important in promoting germination even in adverse conditions, i.e. the stress of cold and increased sucrose concentration. In shoot development, CRF4 seems to negatively regulate shoot growth during similar adverse conditions; however, the effect of CRF4 on roots is the opposite. The normal or lack of CRF4 expression does not seem to affect root growth, yet excess of CRF4 decreases root growth compared to Wild type exposed to cold conditions. Under standard conditions, CRF4 negatively regulates root growth as seen by the significant difference in root growth between the *crf4* plants and Wild type. This further suggests that CRF4 plays different roles in development of shoots and roots and throughout development. CRF4 negatively affects growth at earlier stages of development, but only affects root growth at later stages when expressed beyond Wild type levels.

Additional developmental expression changes were seen with Wild type plants exposed to cold treatment further supporting the involvement of CRF4 in a developmentally derived cold response. CRF4 seems to be primarily involved in earlier developmental stage cold response. It appears that there is a shift in the mechanism by which CRF4 responds to cold during development from an indirect response at 10d to a direct response at 24d. The same expression profile is seen for CRF3 suggesting either that the transcription factors, CRF3 and CRF4, are working together or are both needed for similar responses. Additionally, expression of CRF3 and CRF4 is only induced during the first 6 hours of cold treatment suggesting that these transcription factors play a role in inducing transcription of genes that are involved in the acclimation process of cold response.

Finally, freeze treatment of CRF4 altered expression plants indicate that greater than Wild type levels of CRF4 can actually increase percent survival. The similarity between Wild type and *crf4* survival may be understood in light of the transcript levels of CRF3 and CRF4 being drastically reduced with freezing treatment. Thus, the expression of CRF3 and 4 in Wild type are likely similar to *crf4* levels before freeze treatment. Thus CRF4 if expression is maintained or increased in the presence of freezing it may promote freeze tolerance and survival.

Overall this thesis shows that CRF4 and CRF3 are involved in cold response and possibly freeze response, although the mechanism by which these transcription factors are induced needs further examination. It is likely that the transcription of CRF4 with the DRE/CRT promoter sequence is induced by DREB/CBF transcription factor binding following cold stress that in turn may activate transcription of CRF3. These transcription factors either together or separately, seem to be involved in inducing a cold response. Downstream CRF3 and 4 transcription factors binding targets should be a future avenue of investigation and that examination during cold

treatment might also prove to be enlightening. Furthermore, the interaction of the AHPs and CRFs during cold stress might also indicate specific genes targets as a result of cold stress and provide insight as to whether the TCS system is involved in cold response. Finally, there should be further examination of the CRF family of proteins in cold stress beyond *Arabidopsis* in plants such as tomato. The results of such studies might reveal knowledge to enlighten agricultural practices and decrease economic loss from cold and freezing stress.

References

Alcázar R, Cuevas JC, Planas J, Zarza X, Bortolotti C, Carrasco P, Salinas J, Tiburcio AF, Altabella T. 2011. Integration of polyamines in the cold acclimation response. Plant Science **180**, 31-38.

Alcázar R, Planas J, Saxena T, Zarza X, Bortolotti C, Cuevas J, Bitrián M, Tiburcio AF, Altabella T. 2010. Putrescine accumulation confers drought tolerance in transgenic *Arabdiopsis* plants over-expressing the homologous *Arginine decarboxylase 2* gene. Plant Physiology and Biochemistry **48**, 547-552.

Alet AI, Sanchez DH, Cuevas JC, del Valle S, Altabella T, Tiburcio AF, Marco F, Ferrando A, Espasandín FD, González ME, Carrasco P, Ruiz OA. 2011. Putrescine accumulation in *Arabidopsis thaliana* transgenic lines enhances tolerance to dehydration and freezing stress. Plant Signaling & Behavior **6**, 278-286.

Beck EH, Fettig S, Knake C, Hartig K, Bhattarai, T. 2007. Specific and unspecific responses of plants to cold and drought stress. Journal of Biosciences **32**, 501-510.

Browse J, Xin Z. 2001. Temperature sensing and cold acclimation. Current Opinion in Plant Biology **4**, 241-246.

Capell T, Bassie L, Christou P. 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. PNAS **101**, 9909-9914.

Chen THH, Murata N. 2011. Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. Plant, Cell and Environment **34**, 1-20.

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes and Development **17**, 1043-1054.

Chinnusamy V, Schumaker K, Zhu JK. 2004. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. Journal of Experimental Botany **55**, 225-236.

Choi Hi, Hong JH, Ha JO, Kang JY, Kim SY. 2000. ABFs, a family of ABA-responsive element binding factors. Journal of Biological Chemistry **275**, 1723-1730.

Cutcliffe JW, Hellmann E, Heyl A, Rashotte AM. 2011. CRFs form protein-protein interactions among each other and with members of the cytokinin-signaling pathway in *Arabidopsis* via the CRF domain. Journal of Experimental Botany **62**, 4995-5002.

Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF. 2009. Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. The Plant Cell **21**, 972-984.

Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu JK. 2006. The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. PNAS **103**, 8281-8286.

Dortay H, Mehnert N, Bükle L, Schmülling T, Heyl A. 2006. Analysis of protein interactions within the cytokinin-signaling pathway of *Arabidopsis thaliana.* FEBS Journal **273**, 4631-4644.

Draper SR. 1972. Amino acid changes associated with low temperature treatment of *Lolium perenee*. Phytochemistry **11**, 639-641.

Feng JX, Liu D, Pan Y, Gong W, Ma LG, Luo JC, Deng XW, Zhu YX. 2005. An annotation update via cDNA sequence analysis and comprehensive profiling of developmental, hormonal or

environmental responsiveness of the *Arabidopsis* AP2/EREBP transcription factor gene family. Plant Molecular Biology **59**, 853-868.

Fowler S, Thomashow MF. 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. The Plant Cell **14**, 1675-1690.

Gilmour SJ, Thomashow MF. 1991. Cold acclimation and cold-regulated gene expression in ABA mutants of Arabidopsis thaliana. Plant Molecular Biology **17**, 1233-1240.

Gilmour SJ, Fowler SG, Thomashow MF. 2004. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Molecular Biology **54**, 767-781.

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology **124**, 1854-1865.

Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF. 1998. Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. The Plant Journal **16**, 433-442.

Gong W, He K, Covington M, Dinesh-Kumar SP, Snyder M, Harmer SL, Zhu YX, Deng XW. 2008. The development of protein microarrays and their applications in DNA-protein and protein-protein interaction analyses of *Arabidopsis* transcription factors. Molecular Plant **1**, 27-41.

Haake V, Cook, D, Riechmann JL, Pineda O, Thomashow MF, Zhang JZ. 2002. Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiology **130**, 639-648.

Hayashi T, Harada A, Sakai T, Takagi S. 2006. Ca^{2+} transient induced by extracellular changes in osmotic pressure in *Arabidopsis* leaves: differential involvement of cell wall-plasma membrane adhesion. Plant, Cell and Environment **29**, 661-672.

Hincha DK, Hagemann M. 2004. Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. Biochemistry Journal **383**, 277-283.

Huang GT, Ma SL, Bai LP, Zhang L, Ma H, Jia P, Liu J, Zhong M, Guo ZF. 2012. Signal transduction during cold, salt, and drought stresses in plants. Molecular Biology Reports **39**, 969-987.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI. 2010. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Genes and Development **24**, 1695-1708.

Hussain SS, Ali M, Ahmad M, Siddique KHM. 2011. Polyamines: Natural and engineered abiotic and biotic stress tolerance in plants. Biotechnology Advances **29**, 300-311.

Hwang I, Sheen, J. 2001. Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature **413**, 383-389.

Ichimura K, Mizoguchi T, Yoshida R, Yuasa T, Shinozaki K. 2000. Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. The Plant Journal **24**, 655-665.

Jaglo-Ottosen, KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF. 1998. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280**, 104-106.

Janská A, Marsík P, Zelenková S, Ovensná J. 2010. Cold stress and acclimation – what is important for metabolic adjustment? Plant Biology **12**, 395-405.

Jeon J, Kim NY, Kim S, Kang NY, Novák O, Ku SJ, Cho C, Lee DJ, Lee EJ, Strnad M, Kim J. 2010. A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in *Arabidopsis*. Journal of Biological Chemistry **285**, 23371-23386.

Kang JY, Choi HI, Im MY, Kim SY. 2002. Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. The Plant Cell **14**, 343-357.

Kaplan F, Guy CL. 2004. *ß*-Amylase induction and the protective role of maltose during temperature shock. Plant Physiology **135**, 1674-1684.

Kaplan F, Kopka J, Sung DY, Zhao W, Popp M, Porat R, Guy CL. 2007. Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. The Plant Journal **50**, 967-981.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology **17**, 287-291.

Kim SH, Woo DH, Kim JM, Lee SY, Chung WS, Moon YH. 2011. *Arabidopsis* MKK4 mediates osmotic-stress responsive via its regulation of MPK3 activity. Biochemical and Biophysical Research Communications **412**, 150-154.

Knight H, Knight MR. 2000. Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in *Arabdiopsis*. Journal of Experimental Botany **51**, 1679-1686.

Krasensky J, Jonak C. 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of Experimental Botany **63**, 1593-1608.

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiology **130**, 2129-2141.

Kunkel KE, Pielke RA, Changnon SA. 1999. Temporal fluctuations in weather and climate extremes that cause economic and human health impacts: A review. Bulletin of the American Meteorlogical Society, **80**, 1077-1098.

Lång V, Mäntylä E, Welin B, Sundberg B, Palva ET. 1994. Alterations in water status, endogenous abscisic acid content, and expression of *rab18* gene during the development of freeze tolerance in *Arabidopsis thaliana.* Plant Physiology **104**, 1341-1349.

Lata C, Prasad M. 2011. Role of DREBs in regulation of abiotic stress responses in plants. Journal of Experimental Botany **62**, 4731-4748.

Lee BH, Henderson DA, Zhu JK. 2005. The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. The Plant Cell **17**, 3155-3175.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively in Arabidopsis. The Plant Cell **10**, 1391-1406.

Mäntylä E, Lång V, Palva T. 1995. Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LT178 and RAB18 proteins in *Arabidopsis thaliana*. Plant Physiology **107**, 141-148.

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ, Hasegawa PM. 2007. SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freeze tolerance in *Arabiopsis.* The Plant Cell **19**, 1403-1414.

Monroy AF, Dhindsa RS. 1995. Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. The Plant Cell **7**, 321-331.

Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiology **140**, 411-432.

Nakashima K, Shinwari ZK, Sakuma Y, Seki M, Miura S, Shinozaki K, Yamaguchi-Shinozaki, K. 2000. Organization and expression of two *Arabidopsis DREB2* genes encoding DRE-binding proteins involved in dehydration- and high-salinity-responsive gene expression. Plant Molecular Biology **42**, 657-665.

Nanjo T, Kobayashi M, Yoshiba Y, Sanada Y, Wada K, Tsukaya H, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Biological function of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana.* The Plant Journal **18**, 185-193.

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe, H, Narusaka M, Shinozaki K, Yamaguchi-Shinozaki, K. 2003. Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis* rd29A gene in response to dehydration and high-salinity stresses. The Plant Journal **34**, 137-148.

Nicot N, Hausman JF, Hoffmann L, Evers D. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Journal of Experimental Botany **56**, 2901-2914.

Novillo F, Medina J, Rodriguez-Franco M, Neuhaus G, Salinas J. 2011. Genetic analysis reveals a complex regulatory network modulating *CBF* gene expression and *Arabidopsis* response to abiotic stress. Journal of Experimental Botany **63**, 293-304.

Örvar BL, Sangwan V, Omann F, Dhindsa RS. 2000. Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. The Plant Journal **23**, 785-794.

Pearce RS. 2001. Plant Freezing and Damage. Annals of Botany **87**, 417-424.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. 2010. ABA perception and signaling. Trends in Plant Science **15**, 395-401.

Rain and Hail. 2012. U.S. crop insurance Snapshot. RMA Summary of Business and NASS Principle Crop Acreage.

Rashotte AM, Goertzen LR. 2010. The CRF domain defines cytokinin response factor proteins in plants. BMC Plant Biology **10**, 74-84.

Rashotte AM, Mason MM, Hutchison CE, Ferreria FJ, Schaller GE, Kieber JJ. 2006. A subset of Arabidopsis AP2 transcription factors mediate cytokinin responses in concert with a two-component pathway. PNAS **103**, 11081-11085.

Rekarte-Cowie I, Ebshish OS, Mohamed KS, Pearce RS. 2008. Sucrose helps regulate cold acclimation of *Arabidopsis thaliana*. Journal of Experimental Botany **59**, 4205-4217.

Saibo NJM, Lourenço T, Oliveira MM. 2009. Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Annals of Botany **103**, 609-623.

Robinson, SJ, Parkin IAP. 2009. Bridging the gene-to-function knowledge gap through functional genomics. Methods in Molecular Biology **513,** 153-73

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki, K. 2002. DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochemical and Biophysical Research Communication **290**, 998-1009.

Sheen J. 1996. Ca^{2+} dependent protein kinases and stress signal transduction in plants. Science **274**, 1900-1902.

Shi X, Gupta S, Rashotte AM. 2012a. *Solanum lycopersicum cytokinin response factor* (*SlCRF*) genes: characterization of CRF domain-containing ERF genes in tomato. Journal of Experimental Biology **63**, 973-982.

Shi Y, Tian S, Hou L, Huang X, Zhang X, Guo H, Yang S. 2012b. Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-a ARR genes in *Arabidopsis*. The Plant Cell doi: 10.1105/tpc.112.098640

Shinozaki K, Yamaguchi-Shinozaki K, Seki, M. 2003. Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology **6**, 410-417.

Tabaei-Aghdaei SR, Pearce RS, Harrison P. 2003. Sugars regulate cold-induced gene expression and freezing-tolerance in barley cell cultures. Journal of Experimental Botany **54**, 1565-1575.

Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K. 2001. Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in arabidopsis cell culture. Plant Cell Physiology **42**, 214-222.

Theocharis A, Clément C, Barka EA. 2012. Physiological and molecular changes in plants grown at low temperatures. Planta **235**, 1091-1105.

Thomashow MF, Stockinger EJ, Jaglo-Ottosen KR, Gilmour SJ, Zarka DG. 1997. Function and regulation of *Arabidopsis thaliana COR* (cold-regulated) genes. Acta Physioogiae Plantarum **19**, 497-504.

Thomashow MF. 1999. Plant cold acclimation freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology **50**, 571-599.

Ueda A, Kathiresan A, Inada M, Narita Y, Nakamura T, Shi W, Takabe T, Bennett J. 2004. Osmotic stress in barley regulates expression of a different set of genes than salt stress does. Journal of Experimental Botany **55**, 2213-2218.

Uemura M, Steponkus PL. 2003. Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplast isolated from *Arabidopsis thaliana* leaves. Plant, Cell and Environment **26**, 1083-1096.

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. 2000. *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. PNAS **97**, 11632-11637.

Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K. 1999. The A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. The Plant Cell **11**, 1743-1754.

Valliyodan B, Nguyen HT. 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Current Opinion in Plant Biology **9**, 189-195.

Viswanathan C, Zhu JK. 2002. Molecular genetic analysis of cold-regulated gene transcription. Philosophical Transactions: Biological Sciences **357**, 877-886.

Weigel D, Glazebrook J. 2002. Arabidopsis : A laboratory manual. Cold Spring Harbor Laboratory Press: NY**.**

Werner T, Schmülling T. 2009. Cytokinin action in plant development. Current Opinion in Plant Biology **12**, 527-538.

Willems E, Leyns L, Vandesompele J. 2008. Standardization of real-time PCR gene expression data from independent biological replicates. Analytical Biochemistry **379**, 127-129.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. eFP Browser. PLoS One **2**, e718.

Xin Z, Browse J. 1998. *eskimo1*mutants of *Arabidopsis* are constitutively freezing-tolerant. Proceedings of the National Academy of Science **95**, 7799-7804.

Xiong L, Schumaker KS, Zhu JK. 2002. Cell signaling during cold, drought, and salt stress. The Plant Cell doi:10.1105/tpc.000596

Yamaguchi-Shinozaki K, Shinozaki K. 1994. A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell **6**, 251-264.

Yamaguchi-Shinozaki K, Shinozaki K. 2005. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. TRENDS in Plant Science **10**, 88-94.

Yuan K, Wysocka-Diller J. 2006. Phytohormone signaling pathways interact with sugars during seed germination and seedling development. Journal of Experimental Botany **57**, 3359-3367.

Zhu J, Shi, H, Lee, BH, Damsz B, Cheng S, Stirm V, Zhu JK, Hasegawa PM, Bressan RA. 2004. An *Arabidopsis* homeodomain transcription factor gene, *HOS9*, mediates cold tolerance through a CBF-independent pathway. PNAS **101**, 9873-9878.

Zhuang J, Cai B, Peng RH, Zhu B, Jin XF, Xue Y, Gao F, Fu XY, Tian YS, Zhao W, Qiao YS, Zhang Z, Xiong AS, Yao QH. 2008. Genome-wide analysis of the AP2/ERF gene family in *Populus trichocarpa*. Biochemical and Biophysical Research Communications **371**, 468-474.

Figure Legends

Figure 1. CRFs involved in cold response and hormone signaling pathways. (A) Cold stress pathway adapted from Novillo *et al.* (2011). Possible mechanism by which CRF3 and CRF4 are involved in the one of the main cold response pathways. A role that CRF 3 and CRF 4 may play in this pathway is to function on secondary responses to cold, possibly osmotic stress or as target of the DREB/CBFs. ICE (Inducer of CBF Expression) a constitutively expressed transcription factor which is activated upon cold stress binds to the promoter region of the DREB/CBF (Dehydration Response Element Binding protein/C-repeat Binding Factor) and induced transcription of this transcription factor (TF). This TF binds to the promoter cis-element DRE/CRT (Dehydration Response Element/C-Repeat sequence) of cold induced genes of proteins that are involved in cold responses. ABRE (ABA-Responsive Element) is a promoter cis-element to which osmotically induced ABA signal transduction TFs bind inducing transcription of cold response genes of proteins involved in osmotic response. (B) Two-component signaling system (TCS) adapted from Rashotte *et al.* (2006). Pathway is activated through Cytokinin, a plant hormone, to elicit response for development or stress. Cytokinin Response Factor (CRFs) interact with the *Arabidopsis* Histidine Phosphotransfer proteins (AHPs) of the TCS system and were first identified as a side component of this pathway. CRFs, transcription factors that can also act in protein-protein binding and relay the signal by binding to the promoter regions of the same genes of Type-B transcription factors. CRFs may also target genes that may function as proteins which aide the plant in coping with cold stress. *Arabidopsis*

Histidine Kinase protein (AHKs) act as the receptors of Cytokinin signaling. *Arabidopsis* Histidine Phosphotransfer protein (AHPs) act to relay the signal to Type-B *Arabidopsis* Response Regulators (ARRs) (transcription factors) or Type-A *Arabidopsis* Response Regulators (ARRs) (regulator proteins). Type-B ARRs induce transcription of Cytokinin responsive genes.

Figure 2. Schematic of CRF proteins in *Arabidopsis thaliana.* Adapted from Rashotte and Goertzen (2010), individual regions of the proteins are separated by bold black lines. From the N (amino terminus) to C (carboxylic acid terminus) the domains or motifs are as follows The CRF domain distinguishes the CRFs from the rest of the AP2/ERF family of proteins and works in protein-protein interaction. The AP2/ERF domain is the DNA binding domain and classifies CRFS as an AP2/ERF transcription factor. The putative MAP Kinase (Mitogen Activated Protein Kinase) motif acts as a phosphorylation site. The C-terminal Clade Specific region further classifies the CRFs as similar proteins that are subdivided from the larger CRF family of proteins.

Figure 3. CRF4 over expression and mutant genotypes transcript levels. Transcript expression levels of CRF3 and CRF4 were examined using qPCR at in the CRF4 altered genotype with an EF1 alpha control. The fold change values are presented relative to Wild type levels with exact values indicated above each bar.

Figure 4. Cold and sucrose affect germination in altered CRF4 expression genotypes. Seeds of genotypes crf4, Wild type, and 35S:CRF4 were sown on MS medium plates of varying sucrose level, stratified, and placed at 4°C for germination. Germination, defined as radical emergence, was determined at 6, 9, and 12 days after sowing from top to bottom at 0%, 1%, and 2% sucrose at 22°C (A) and 4°C (B). The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) between samples taken at specific days after sowing are

noted by * for comparisons to WT within sucrose treatment levels and as † for comparisons within genotypes to 0% sucrose levels.

Figure 5. Cold and sucrose affect shoot development additively in altered CRF4 expression genotypes. (A) Schematic of developmental stages of Arabidopsis growth used for classification of plant growth. Development, defined as by specific stage in (A), was determined for crf4, Wild type, and 35S:CRF4 plants at 6, 9, and 12 days after treatment from top to bottom at 0%, 1%, and 2% sucrose at 22°C (B) and 4°C (C). The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) between samples taken at specific days after sowing are noted by * for comparisons to WT within sucrose treatment levels, † for comparisons within genotypes to 0% sucrose levels, ‡ for comparisons between 1% and 2% sucrose levels.

Figure 6. The absence of CRF4 promotes root growth at 22°C. crf4, Wild type, and 35S:CRF4 seeds were sown on 1% sucrose media and grown vertically on plates at 22°C until they had reached green cotyledon stage of development (Stage 4). Root growth was then examined on after 12d at 22°C or after 33d at 4°C. The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) are noted by * for comparisons to WT root length.

Figure 7. CRF4 is induced highly in the shoots and roots by cold. WT plants were grown and treated with cold for 1, 6 hours at 10d and 17d after germination. Transcript expression levels of CRF1-10 were examined using qPCR at each of these time points. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment at each age. Clade notations show closely related CRF proteins based on CRF domain. The Average and SEM is shown for shoots and roots for 10d (A) and 17d plants (B).

Figure 8. Cold induction of CRF3 and CRF4 is greatest in the first 6 hours of treatment and unaffected during recovery. WT plants were grown and treated with cold (4°C) at 17d for 7 days and the moved to 22°C and allowed to recover. Transcript expression levels of CRF3, 4 were examined using qPCR at 1, 3, 6, 12, and 24h after treatment and 1, 3, 6, 12, 24, 48, 96h after recovery. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment plants. The Average and SEM is shown for shoots (A) and roots (B).

Figure 9. Expression levels of CRF3 and CRF4 transcripts at both 1 and 6 hours shift during development. WT plants were grown and treated with cold for 1, 6 hours at 10, 17, 24, and 33d after germination. Transcript expression levels of CRF3 and CRF4 were examined using qPCR at each of these time points. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment at each age. The Average and SEM is shown for roots (1 and 6h) (A, C) and shoot tissues (1 and 6h) (B, D).

Figure 10. GUS reporter gene expression of CRF4 increases with cold treatment. GUS expression in pro^{CRF4}:CRF4:GUS translational fusion protein in *Arabidopsis* plant 10d post germination were exposed to 4°C for 6 hours (A, B), compared to a 22°C control treatment at 0 hour (C, D). Shown are leaves (A, C) and roots (B, D) of GUS stained plants.

Figure 11. Orthologous CRF genes in Tomato respond to cold. Tomato plants were grown and treated with cold (4°C) for 1, 6h at 20d after germination. Transcript expression levels of *Sl*CRF4 and *Sl*CRF6 (orthologous tomato CRF clade II members to CRF3 and CRF4 in Arabidopsis) were examined using qPCR at 1, 6h after treatment. Fold change values were normalized to TIP41 and are expressed relative to non-cold treatment plants. The Average and SEM is shown for whole plants.

Figure 12. Expression and survival during freezing of CRF4. crf4, Wild type, and 35S:CRF4 plants were grown on 1% sucrose for 10d at 22°C and then exposed to 5h of freezing (-8 to -13°C). (A) Survival of plants was examined in genotypes after freezing that were moved to 22°C. Plants were monitored for percent survival 9d after freezing indicated by the presence of green tissue. (B) Transcript expression levels of CRF3, 4 were examined using qPCR after treatment. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-freeze treatment plants. The Average and SEM is shown.

Table Legends

Table 1. CRFs have cold inducible promoter elements. A promoter screen of CRF 1-10 promoter sequences revealed some genes with cis-promoter elements, DRE/CRT (Dehydration Response Element/C-Repeat sequence) or ABRE (ABA-Responsive Element), to which cold or osmotically active transcription factors bind. X indicates presence of promoter element in the promoter sequence of the gene.

Table 2. QRT-PCR primers of CRFs. Primers used in qRT-PCR SYBR-green chemistry are found in this table. Primers are 5' to 3' for forward and reverse stands.

Figures

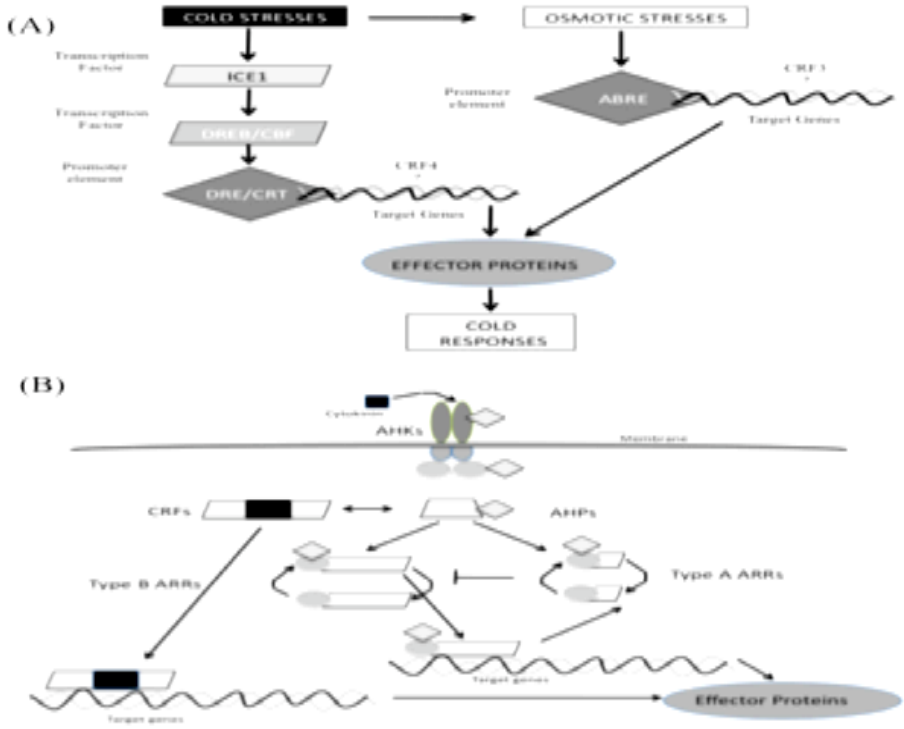

Figure 1. CRFs involved in cold response and hormone signaling pathways. (A) Cold stress pathway adapted from Novillo *et al.* (2011). Possible mechanism by which CRF3 and CRF4 are involved in the one of the main cold response pathways. A role that CRF 3 and CRF 4 may play in this pathway is to function on secondary responses to cold, possibly osmotic stress or as target of the DREB/CBFs. ICE (Inducer of CBF Expression) a constitutively expressed transcription factor which is activated upon cold stress binds to the promoter region of the DREB/CBF (Dehydration Response Element Binding protein/C-repeat Binding Factor) and induced transcription of this transcription factor (TF). This TF binds to the promoter cis-element DRE/CRT (Dehydration Response Element/C-Repeat sequence) of cold induced genes of proteins that are involved in cold responses. ABRE (ABA-Responsive Element) is a promoter cis-element to which osmotically induced ABA signal transduction TFs bind inducing transcription of cold response genes of proteins involved in osmotic response. (B) Two-component signaling system (TCS) adapted from Rashotte *et al.* (2006). Pathway is activated through Cytokinin, a plant hormone, to elicit response for development or stress. Cytokinin Response Factor (CRFs) interact with the *Arabidopsis* Histidine Phosphotransfer proteins (AHPs) of the TCS system and were first identified as a side component of this pathway. CRFs, transcription factors that can also act in protein-protein binding and relay the signal by binding to the promoter regions of the same genes of Type-B transcription factors. CRFs may also target genes that may function as proteins which aide the plant in coping with cold stress. *Arabidopsis* Histidine Kinase protein (AHKs) act as the receptors of Cytokinin signaling. *Arabidopsis* Histidine Phosphotransfer protein (AHPs) act to relay the signal to Type-B *Arabidopsis* Response Regulators (ARRs) (transcription factors) or Type-A *Arabidopsis* Response Regulators (ARRs) (regulator proteins). Type-B ARRs induce transcription of Cytokinin responsive genes

Figure 2. Schematic of CRF proteins in *Arabidopsis thaliana.* Adapted from Rashotte and Goertzen (2010), individual regions of the proteins are separated by bold black lines. From the N (amino terminus) to C (carboxylic acid terminus) the domains or motifs are as follows The CRF domain distinguishes the CRFs from the rest of the AP2/ERF family of proteins and works in protein-protein interaction. The AP2/ERF domain is the DNA binding domain and classifies CRFS as an AP2/ERF transcription factor. The putative MAP Kinase (Mitogen Activated Protein Kinase) motif acts as a phosphorylation site. The C-terminal Clade Specific region further classifies the CRFs as similar proteins that are subdivided from the larger CRF family of proteins.

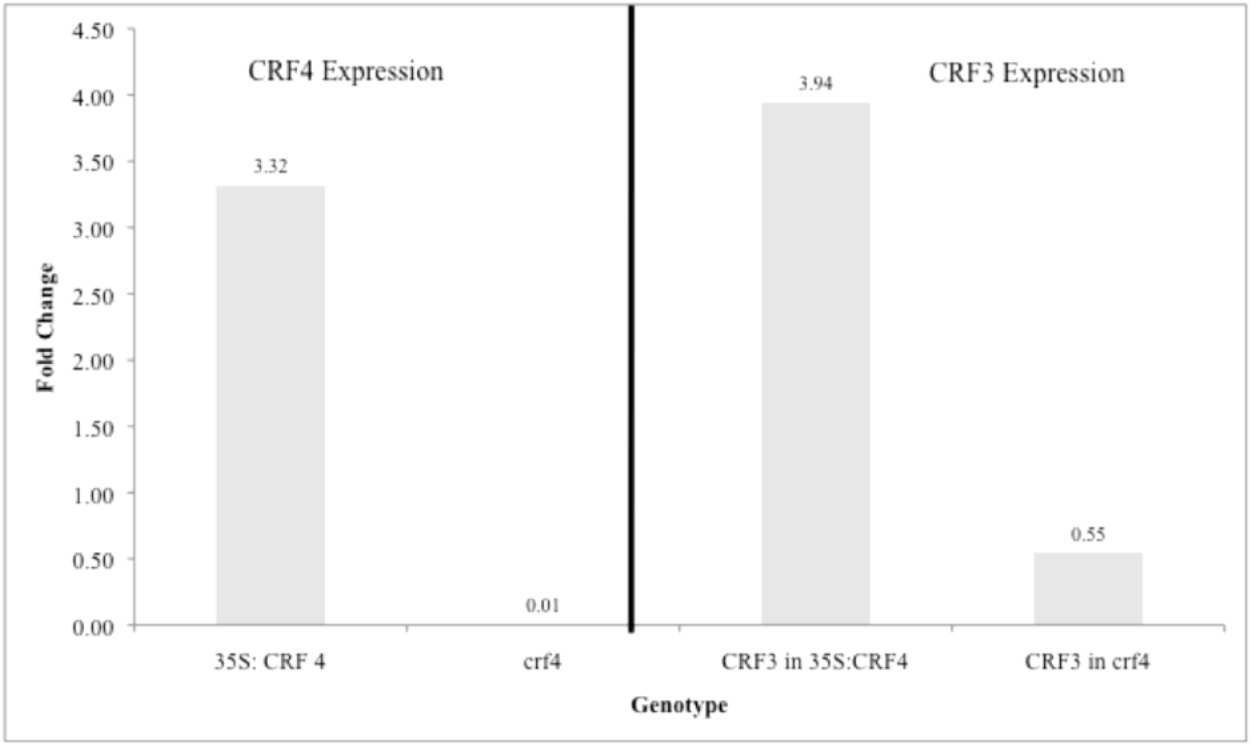

Figure 3. CRF4 over expression and mutant genotypes transcript levels. Transcript expression levels of CRF3 and CRF4 were examined using qPCR at in the CRF4 altered genotype with an EF1 alpha control. The fold change values are presented relative to Wild type levels with exact values indicated above each bar.

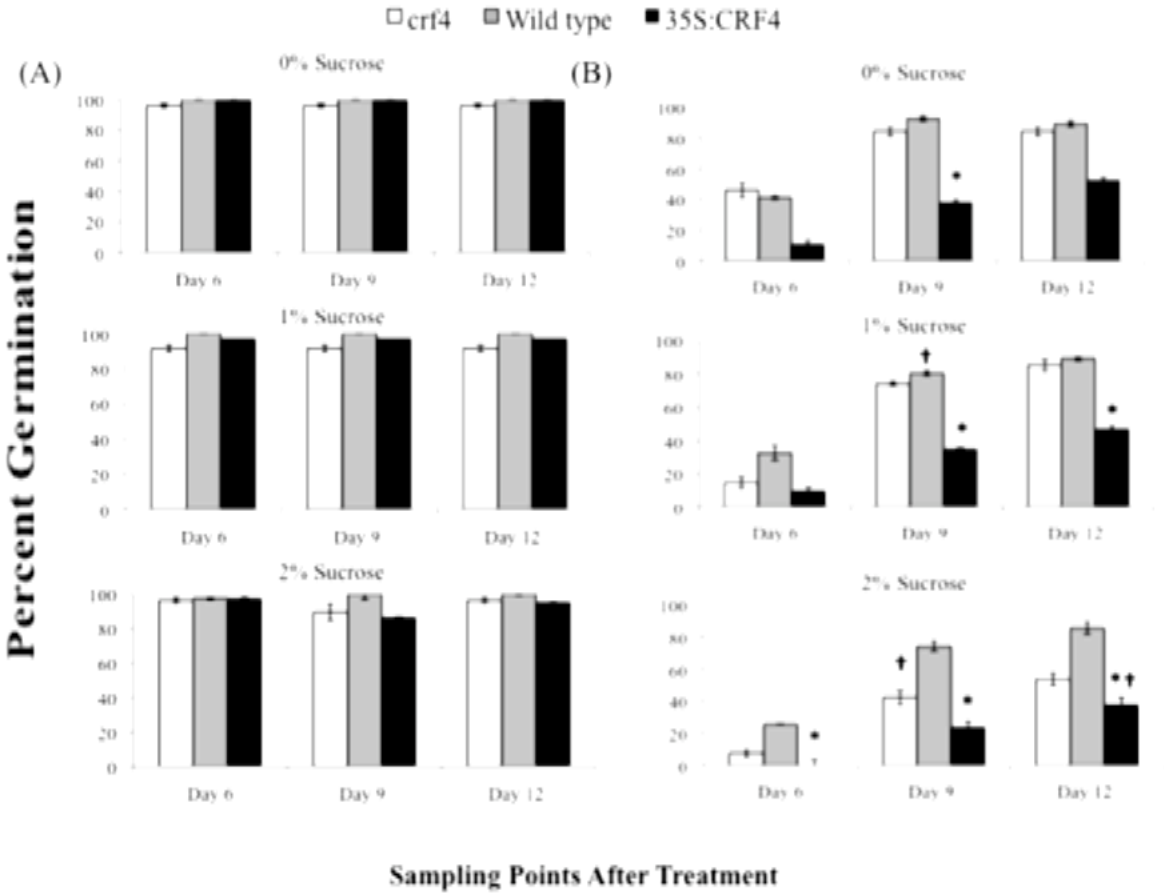

Figure 4. Cold and sucrose affect germination in altered CRF4 expression genotypes. Seeds of genotypes crf4, Wild type, and 35S:CRF4 were sown on MS medium plates of varying sucrose level, stratified, and placed at 4°C for germination. Germination, defined as radical emergence, was determined at 6, 9, and 12 days after sowing from top to bottom at 0%, 1%, and 2% sucrose at 22°C (A) and 4°C (B). The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) between samples taken at specific days after sowing are noted by * for comparisons to WT within sucrose treatment levels and as † for comparisons within genotypes to 0% sucrose levels.

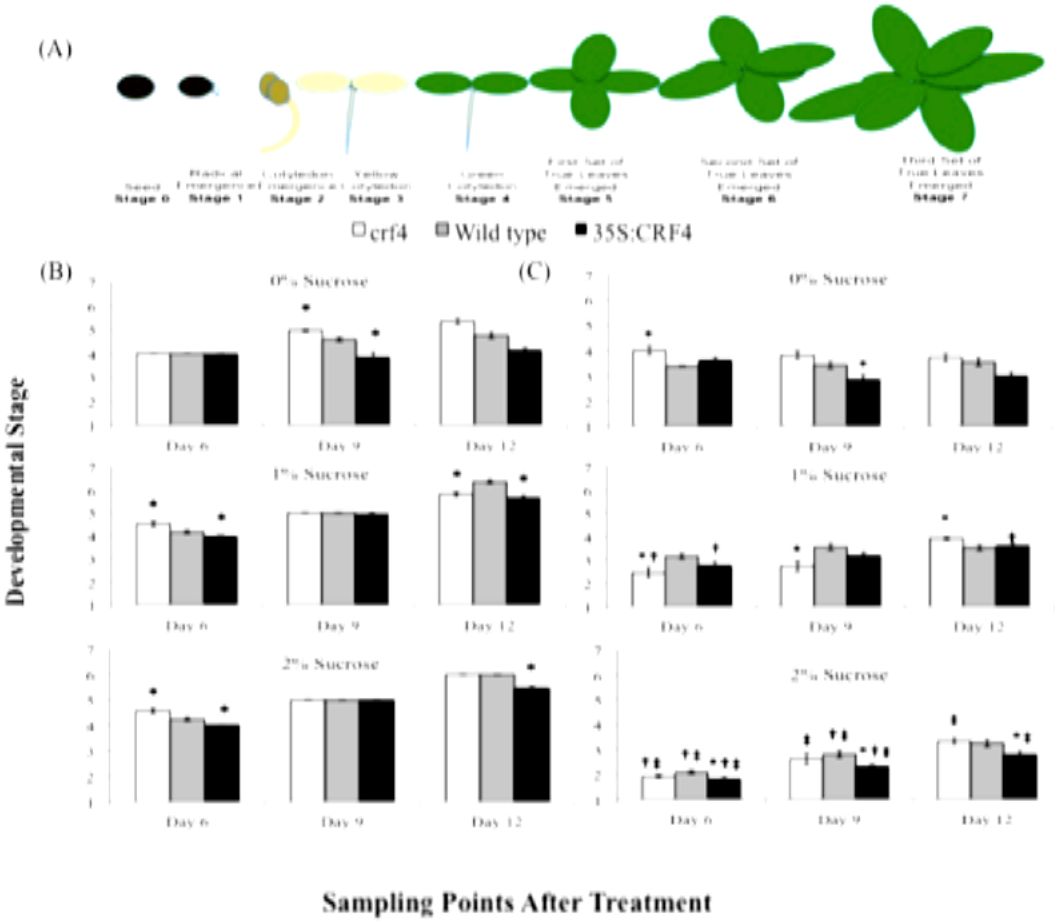

Figure 5. Cold and sucrose affect shoot development additively in altered CRF4 expression genotypes. (A) Schematic of developmental stages of Arabidopsis growth used for classification of plant growth. Development, defined as by specific stage in (A), was determined for crf4, Wild type, and 35S:CRF4 plants at 6, 9, and 12 days after treatment from top to bottom at 0%, 1%, and 2% sucrose at 22°C (B) and 4°C (C). The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) between samples taken at specific days after sowing are noted by * for comparisons to WT within sucrose treatment levels, † for comparisons within genotypes to 0% sucrose levels, ‡ for comparisons between 1% and 2% sucrose levels.

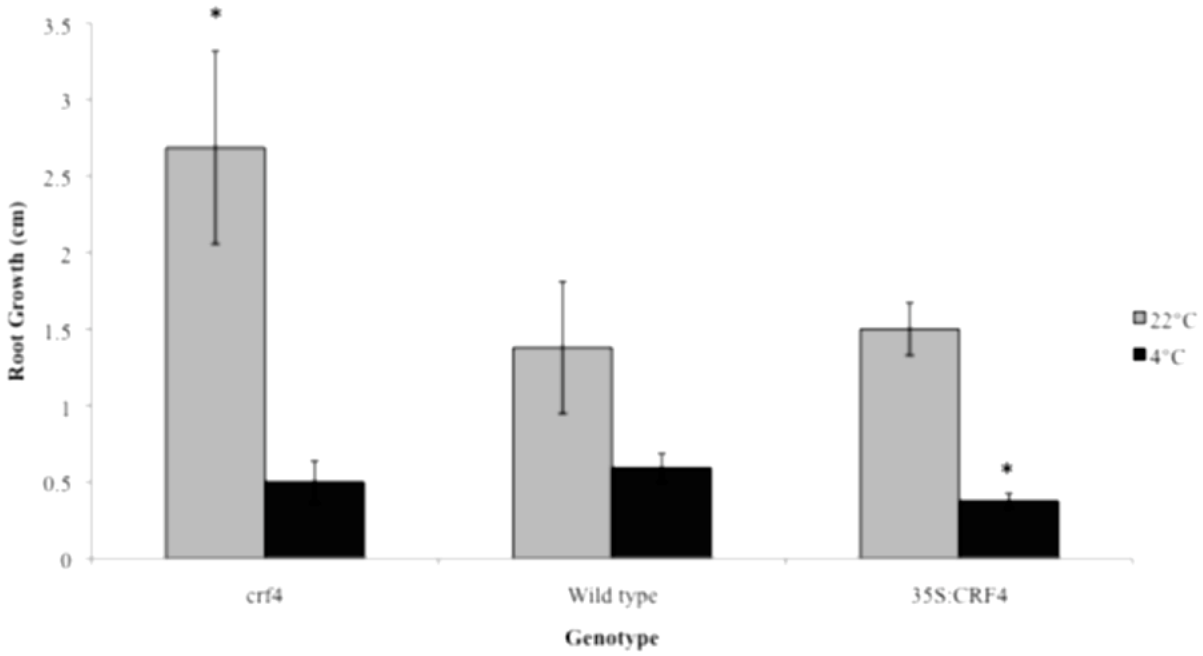

Figure 6. The absence of CRF4 promotes root growth at 22°C. crf4, Wild type, and 35S:CRF4 seeds were sown on 1% sucrose media and grown vertically on plates at 22°C until they had reached green cotyledon stage of development (Stage 4). Root growth was then examined on after 12d at 22°C or after 33d at 4°C. The Average and SEM is shown. Significance differences ($p<0.05$ Two-tailed Student's T-test) are noted by * for comparisons to WT root length.

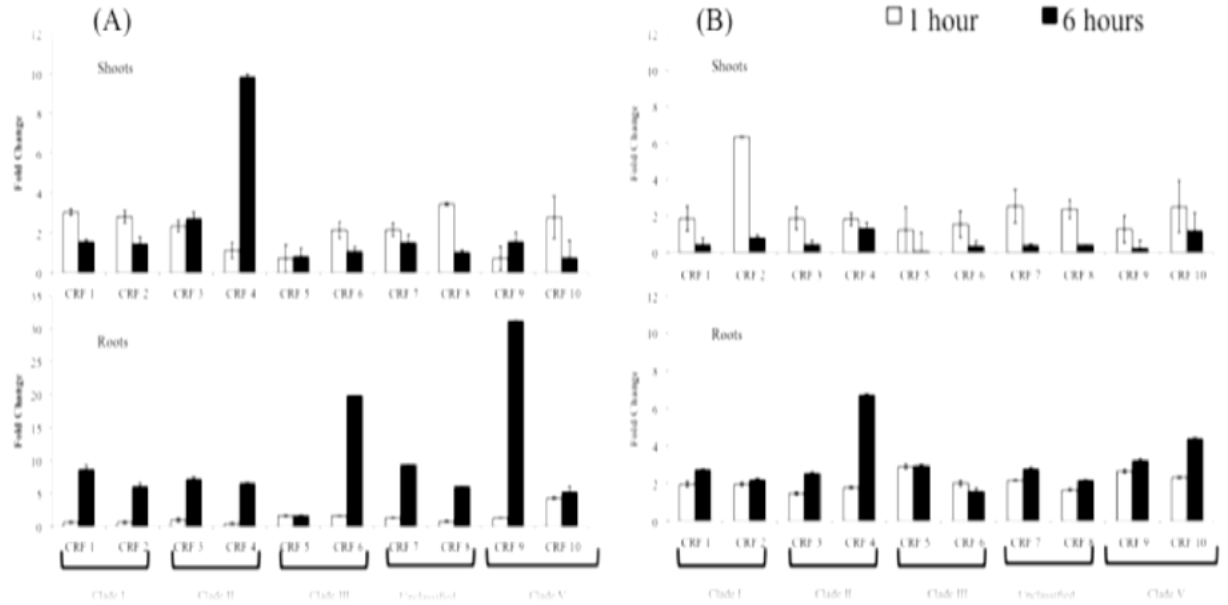

Figure 7. CRF4 is induced highly in the shoots and roots by cold. WT plants were grown and treated with cold for 1, 6 hours at 10d and 17d after germination. Transcript expression levels of CRF1-10 were examined using qPCR at each of these time points. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment at each age. Clade notations show closely related CRF proteins based on CRF domain. The Average and SEM is shown for shoots and roots for 10d (A) and 17d plants (B).

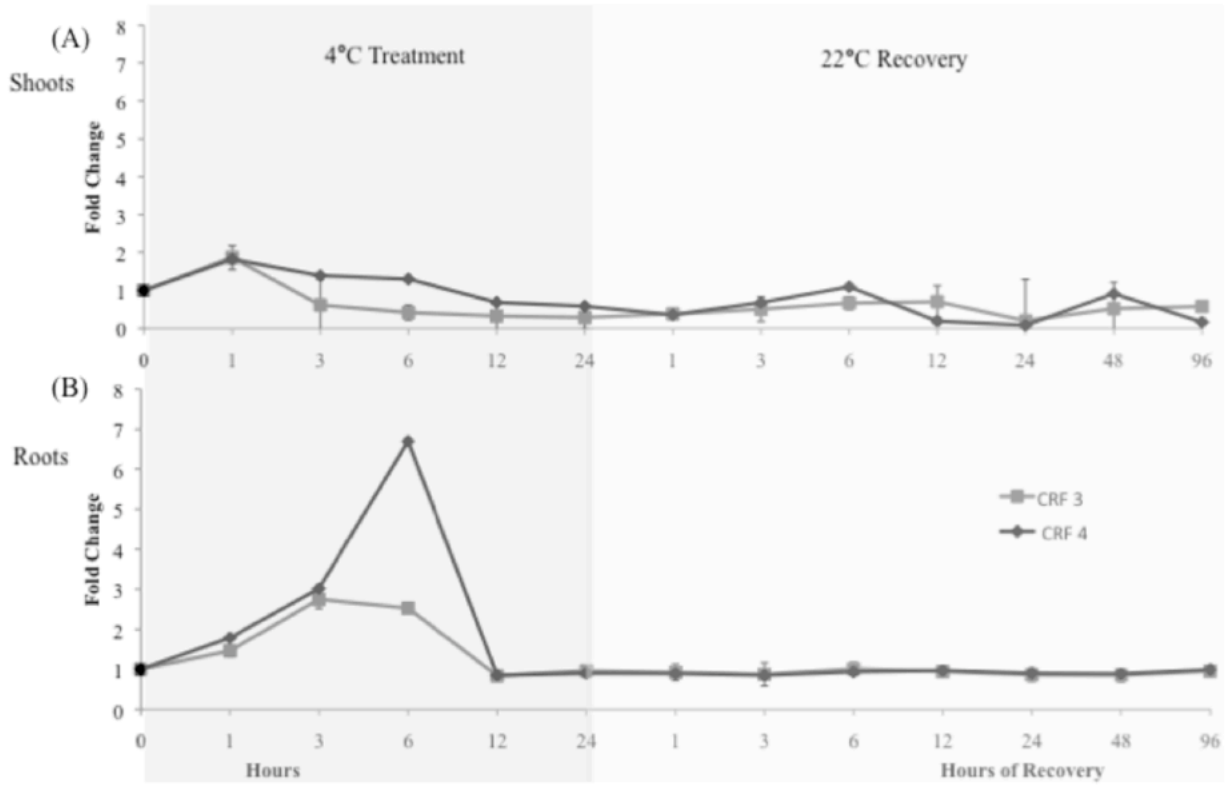

Figure 8. Cold induction of CRF3 and CRF4 is greatest in the first 6 hours of treatment and unaffected during recovery. WT plants were grown and treated with cold (4°C) at 17d for 7 days and the moved to 22°C and allowed to recover. Transcript expression levels of CRF3, 4 were examined using qPCR at 1, 3, 6, 12, and 24h after treatment and 1, 3, 6, 12, 24, 48, 96h after recovery. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment plants. The Average and SEM is shown for shoots (A) and roots (B).

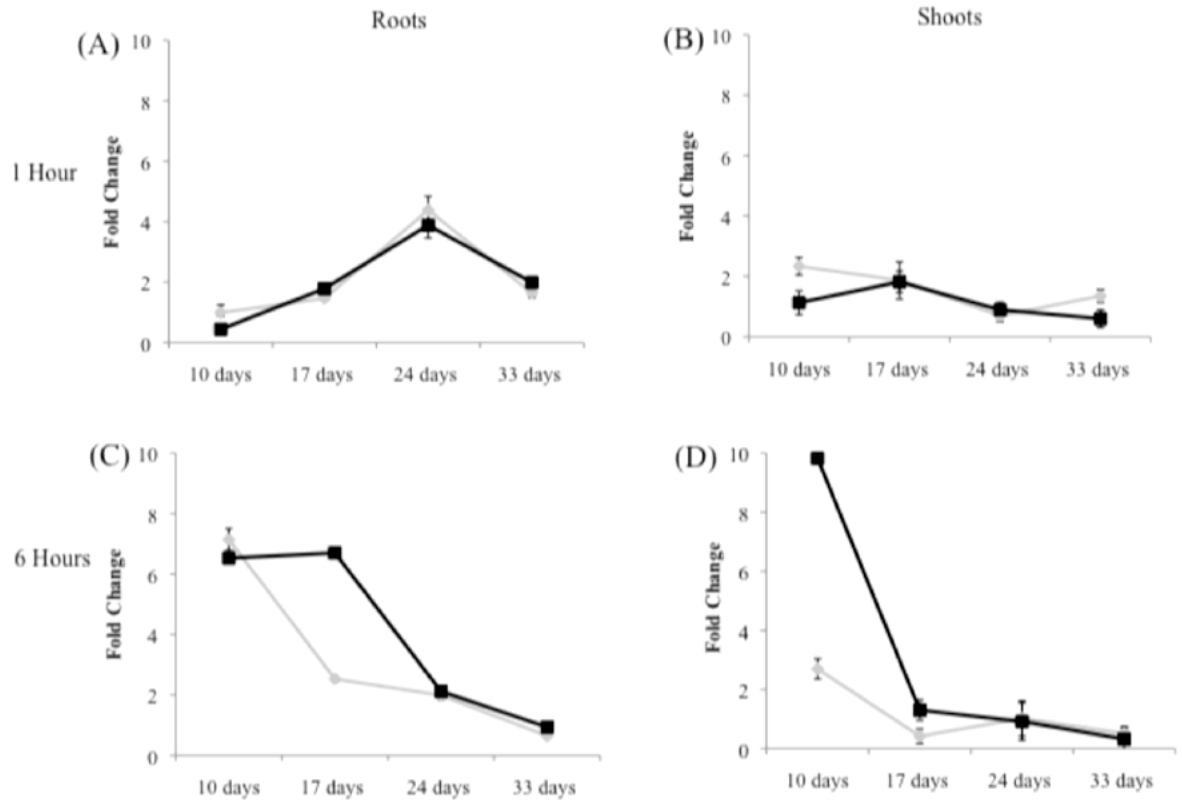

Figure 9. Expression levels of CRF3 and CRF4 transcripts at both 1 and 6 hours shift during development. WT plants were grown and treated with cold for 1, 6 hours at 10, 17, 24, and 33d after germination. Transcript expression levels of CRF3 and CRF4 were examined using qPCR at each of these time points. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-cold treatment at each age. The Average and SEM is shown for roots (1 and 6h) (A, C) and shoot tissues (1 and 6h) (B, D).

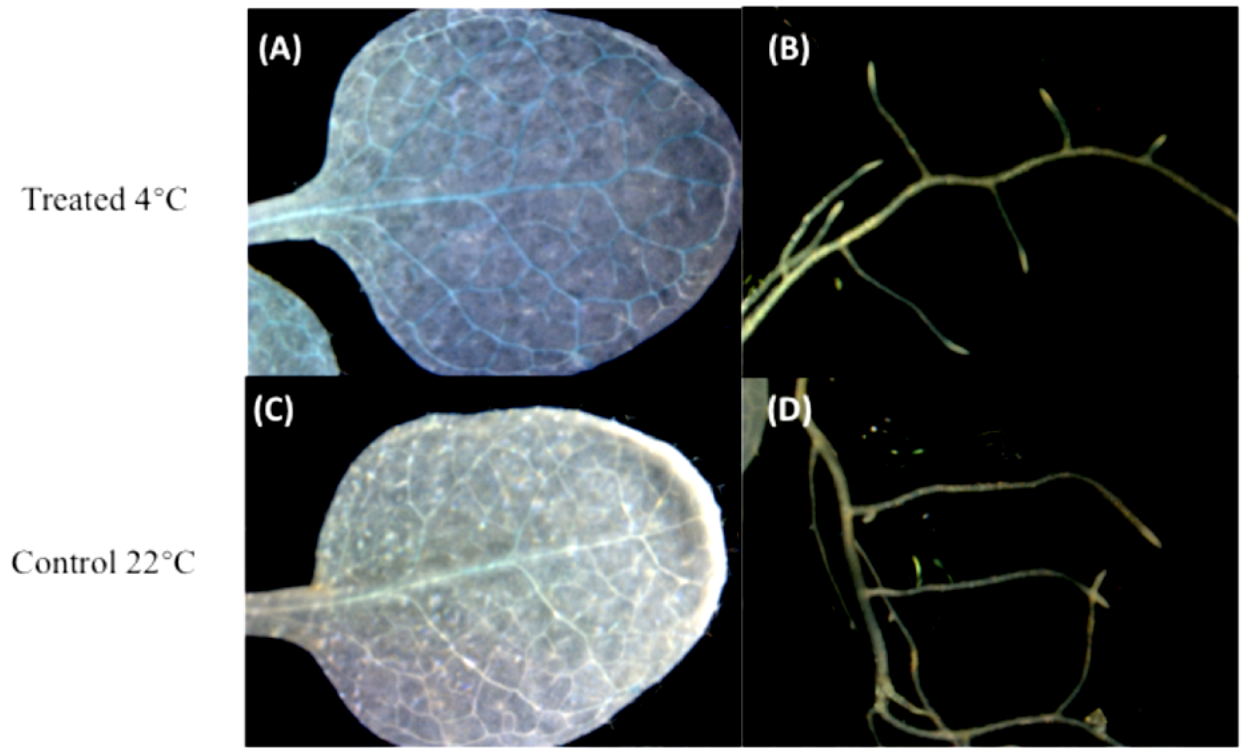

Figure 10. GUS reporter gene expression of CRF4 increases with cold treatment. GUS expression in pro^{CRF4}:CRF4:GUS translational fusion protein in *Arabidopsis* plant 10d post germination were exposed to 4°C for 6 hours (A, B), compared to a 22°C control treatment at 0 hour (C, D). Shown are leaves (A, C) and roots (B, D) of GUS stained plants.

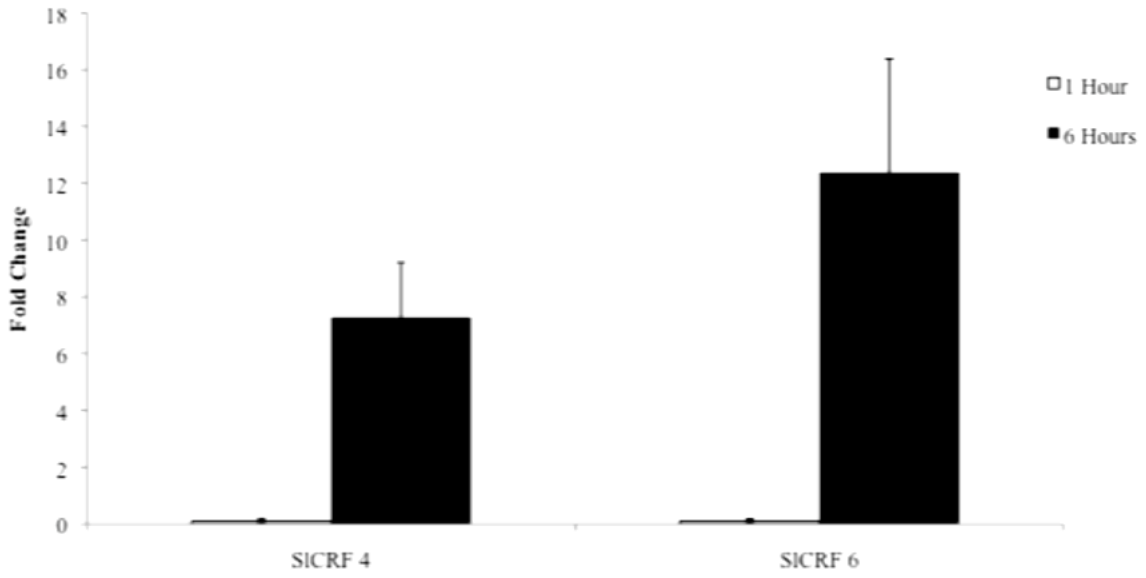

Figure 11. Orthologous CRF genes in Tomato respond to cold. Tomato plants were grown and treated with cold (4°C) for 1, 6h at 20d after germination. Transcript expression levels of *Sl*CRF4 and *Sl*CRF6 (orthologous tomato CRF clade II members to CRF3 and CRF4 in Arabidopsis) were examined using qPCR at 1, 6h after treatment. Fold change values were normalized to TIP41 and are expressed relative to non-cold treatment plants. The Average and SEM is shown for whole plants.

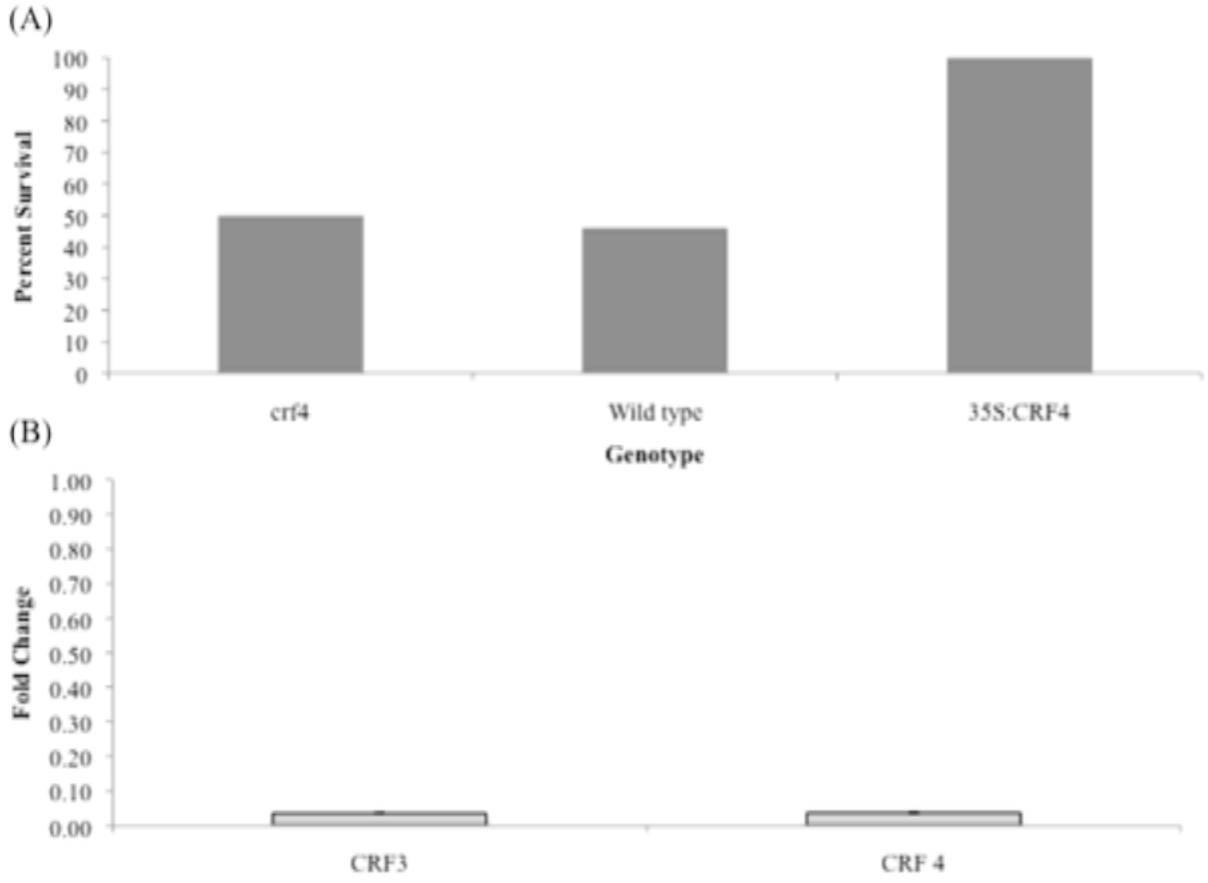

Figure 12. Expression and survival during freezing of CRF4. crf4, Wild type, and 35S:CRF4 plants were grown on 1% sucrose for 10d at 22°C and then exposed to 5h of freezing (-8 to -13°C). (A) Survival of plants was examined in genotypes after freezing that were moved to 22°C. Plants were monitored for percent survival 9d after freezing indicated by the presence of green tissue. (B) Transcript expression levels of CRF3, 4 were examined using qPCR after treatment. Fold change values were normalized to EF1 alpha controls and are expressed relative to non-freeze treatment plants. The Average and SEM is shown.

Tables

Table 1. CRFs have cold inducible promoter elements. A promoter screen of CRF 1-10 promoter sequences revealed some genes with cis-promoter elements, DRE/CRT (Dehydration Response Element/C-Repeat sequence) or ABRE (ABA-Responsive Element), to which cold or osmotically active transcription factors bind. X indicates presence of promoter element in the promoter sequence of the gene.

GENE	ABRE 5'-ACGTGG/TC-3'	DRE/CRT 5'-CCGAC-3'
CRF1		
CRF 2	X	X
CRF 3	X	
CRF 4		X
CRF 5	X	
CRF 6	X	X
CRF 7		X
CRF 8		
CRF9		
CRF10		
CRF 11		X
CRF 12		

Table 2. QRT-PCR primers of CRFs. Primers used in qRT-PCR SYBR-green chemistry are found in this table. Primers are 5' to 3' for forward and reverse stands.

GENE	FORWARD 5'-3'	REVERSE 5'-3'
Arabidopsis		
CRF1	GAATCTTACGAATCTCTGTTACTG	CACCGAATCAAGCACCAC
CRF2	AACCAGGATTCTTCGTATCTC	CACAACTACCAGTAACAACAG
CRF3	ACGCAACAGATTCAT CAAGTG	CATCGGAGGAAGAAGAGAGTC
CRF4	TCTACGACAACGCAGCAATC	TGGCTCTTGTTCTGGTTCAA
CRF5	TTTGGTAAAGTCTGTGGTG	ATTCGTCTGAGCGTTATG
CRF6	GAAGTTCACAGAGAATCG	CCTTGGCAGAATCTAATC
CRF7	CGACGGCGAATAAGAAGAAG	CGCAGCGTTATCATAAGCAA
CRF8	TCGTCTCTGGCTGGGTACTT	AGCAACCTCAGAAGCTCCAA
CRF9	TTTTTGGTGATTCGGCTGTT	TGATCCCCAATCTAAATCCA
CRF10	TTTTGGTTTCTTGGCAGAGG	ACATCCGCAAAGTCGAAATC
EF1-alpha	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA
Tomato		
SlCRF4	TGAATCCCTCTGTTCC AAGG	GTTTTGCCATTTCCACTGCT
SlCRF6	AGATGAGCTTTTTGG GCGTA	TCGCTTCTTCCCATTACCAC
TIP41	ATGGAGTTTTTGAGTCT	GCTGCGTT TCTGGCTTA