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IDENTIFICATION OF eQTLs REGULATING EXPRESSION OF EARLY LOW TEMPERATURE RESPONSIVE GENES IN ARABIDOPSIS

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Ritu Sharma

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of the requirements for the

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Major Professor's Signature

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IDENTIFICATION OF eQTLs REGULATING EXPRESSION OF EARLY LOW TEMPERATURE RESPONSIVE GENES IN ARABIDOPSIS

By

Ritu Sharma

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology

2008

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Abstract

IDENTIFICATION OF eQTLs REGULATING EXPRESSION OF EARLY LOW TEMPERATURE RESPONSIVE GENES IN ARABIDOPSIS

By

Ritu Sharma

Many plants have acquired the ability to enhance their tolerance to freezing temperatures through the process of cold acclimation. Cold acclimation involves a multitude of temporal transcriptional changes that serve as a central step to developing freezing tolerance. Several hundred low temperature responsive genes have been identified, however, the regulation of the gene networks remains poorly understood.

In this study, natural genetic variation was used to identify regulators of early changes in gene expression occurring at low temperatures. Comparison of *Arabidopsis* accessions from different geo-climatic regions identified quantitative variation in the expression of some early responsive genes. Measurement of transcript levels as a quantitative trait in a mapping population derived from a cross between Cvi-0 X L*er* revealed transgressive segregation, suggesting allelic variation at multiple regulatory loci. Further analysis determined that the heritable component underlying this variation in gene expression was contributed by several expression quantitative trait loci (eQTLs). Many transcripts were found to be regulated by multiple eQTLs. Most of these eQTLs were *trans*-acting with the exception of two genes, *CBF2* and 3, which showed *cis*-acting eQTLs. Thus, the variation in gene expression

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observed was not merely due to sequence variation in the promoters of the genes themselves. Furthermore, for some eQTLs, Cvi-0 contributed the positive allele, suggesting that these may arise from variation in negative regulators of transcription for which weaker alleles in the accession not regularly exposed to low temperature may have resulted in higher expression of their target genes. Some of these eQTLs were identified to commonly regulate the expression of multiple genes, suggesting a common regulatory pathway, whereas others regulated unique genes. These findings allowed the construction of a regulatory network for the genes analyzed with the regulatory positions of the different eQTLs. Two adjacent eQTLs on chromosome 1 were identified as the master regulators of seven of the nine genes analyzed. Underlying these eQTLs could be early regulators of the low temperature response, such as the elusive thermo-sensor(s). Significantly, this study has served to narrow down the regions of the genome where further research should be focused to identify the genes underlying the regulatory eQTLs, which will facilitate our understanding of the regulation of gene networks at low temperatures.

This thesis is dedicated to the loving memory of my mother,

Late Mrs. Sushila Sharma.

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I would like to thank Michael F. Thomashow for accepting me to his lab after Pam Green moved to Delaware. I'm thankful to him for allowing me to work on this project and trusting my instincts when technical limitations were blurring the picture. I am also grateful to the members of my Guidance Committee, Ken Keegstra, Steve Triezenberg, Barb Sears and Gregg Howe, for all their compassion, guidance and support.

The period in which this study was completed proved to be extremely challenging for several reasons and without the help of some, it would not have been possible. I want to express my deep gratitude to family, friends, and faculty who helped me to reach the end of this project.

The decision to not move with Pam to Delaware after having started a sound project in her lab was a tough one. Starting afresh in a new lab was a challenging period and if it were not for the support of my friends Kristin Mcnally, Dan Appledorn, Gauri Jawdekar and Sarah Fowler I would literally have not survived it. I can never thank them enough for their support.

I traveled halfway across the globe for my PhD in the hopes of finding an enriching scientific experience. I would like to thank the Plant Research Lab as a whole for providing me that experience through interaction with some distinguished scientists, both internal and invited.

I am grateful to Jan Zeevaart for sharing his time and wisdom for some very motivating discussions of my data. All throughout my graduate study motivation and guidance has come to me from Steve Triezenberg. I am extremely grateful to him for helping me grow as a scientist. I also thank Ken

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Keegstra for his patience and guidance during times I felt completely lost in the program.

I want to thank the members of Thomashow lab, especially Donatella Canella, Sarah Gilmour, and Marcella Carvallo, for their support. I would like to thank Annette Thelen at the Research Technology Support Facility for initiating me into the world of real time RT-PCR and then letting me take over her workspace to set up my experiments. She understood my frustration with the technical problems I was facing and helped me keep faith in my ability to overcome them. I will never forget her compassion. She and Jeff Landgraf helped me feel welcome at the RTSF during the long hours I spent there and their support was invaluable. Thanks are also due to Dr. Curt Wilkerson, Dr. Chang-Bao, and Dr. Vinod Prabhu for their helpful suggestions during data analysis.

Finally I would like to thank my family who believed in me until the very end when I had almost quit. My father, my brother, my husband and my parents-in-law have been the ones who pulled me through the last stage of this PhD.

Table of Contents

1. Table 2
2. Table 3
3. Table 3

List of Tables

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

15.

16.

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List of Figures

18. Fi

19. Fi
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CBF	C
ERF	E
TF	T
LOD	L
CIM	C
RIL	F
NIL	N
QTL	C
eQTL	e

List of Abbreviations

CBF	CRT/DRE Binding Factor
ERF	Ethylene Responsive Factor
TF	Transcription Factor
LOD	Log of Odds
CIM	Composite Interval Mapping
RIL	Recombinant Inbred Line
NIL	Near Isogenic Line
QTL	Quantitative Trait Locus
eQTL	expression Quantitative Trait Locus

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Chapter 1

Literature Review

1.1 Introduction

Plants are constantly exposed to numerous abiotic stresses in the environment such as drought, salinity, chilling, freezing, high temperature, high light intensity, anoxia, and nutrient imbalances. Being sessile organisms, plants are unable to escape these stresses and have developed various coping mechanisms (Korner and Larcher, 1988; Tran et al., 2007). Low temperature constitutes a significant environmental stress that limits crop yields in large parts of the world, often leading to significant economic losses. It is a physical stress that can influence basic aspects of cell survival, such as solute diffusion rates, enzyme kinetics, macromolecular interactions, membrane fluidity, and overall integrity of the cell (Bugbee, 1995).

Most plants of tropical and subtropical origin are sensitive to low temperatures and are injured by chilling temperatures ranging from 0°C to 12°C. Tomato, maize, and rice are some examples of chilling sensitive plants (Taylor et al., 1974). Other species, such as potato, can tolerate chilling temperatures but are injured or killed at freezing temperatures (Sukumaran and Weiser, 1972). Plants sensitive to low temperatures suffer physiological alterations including changes in cell membrane structure, consequent leakage of ions from cells (Nagao et al., 2008), and changes in photosynthesis and respiration rates (Taylor et al., 1972). On the other hand, cold tolerant plants are able to prevent the physiological damage associated with exposure to low

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temperatures. Many temperate plants such as Arabidopsis, wheat, rye, and canola, show a wide range of cold tolerance ranging from -5°C to -30°C (Gilmour et al., 1988; Kaye and Guy, 1995; Guy, 1999). This ability to survive freezing temperatures is not a constitutive property as sudden transfer of these plants from warm temperatures to freezing conditions causes death. However, a pre-exposure to low non-freezing temperatures conditions them to tolerate freezing temperatures. This induction of freezing tolerance is achieved through the process known as cold acclimation (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow, 1999; Zhu, 2001).

Cold acclimation involves specific changes in cellular biochemistry and gene expression that are rapidly triggered in response to low temperature. The process can be divided into sequential stages that collectively serve to prevent low temperature induced injury (Guy et al., 1985; Thomashow, 1998). The mechanisms by which plants perceive low temperature and develop tolerance to the stress remain far from understood. Elucidation of these mechanisms will ultimately aid in the development of strategies for improving crop productivity.

1.2 Low temperature stress

Low temperature causes several kinds of injuries to plants, the type and severity of which vary with temperature (Shinozaki and Yamaguchi-Shinozaki, 1996, 2000). Plants are broadly classified according to their level of tolerance as being chilling sensitive, freezing sensitive, or freezing tolerant. Those unable to withstand temperatures in the range of 0°C to 15°C are

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regarded as chilling sensitive whereas those susceptible to subzero temperatures, freezing sensitive (Paull, 1981).

Exposure of chilling sensitive plants to temperatures below 15°C causes chlorosis, wilting, necrosis and subsequent death (Garber, 1977). These may largely be a consequence of photoinhibition and dehydration, the two major adverse effects of chilling (Hetherington et al., 1989). Low temperature stress in these plants manifests as defects in germination, growth, and development.

Freezing sensitive plants, although able to tolerate the adverse effects of chilling temperatures, however, are unable to survive sub-zero temperatures. This is partly due to further injury caused by the formation of ice crystals at sub-zero temperatures (Yamada et al., 2002; Nagao et al., 2008). Ice crystals are initiated in the extracellular space due to the lower solute concentration in this environment. Ice crystal formation physically damages cells by rupturing the cell membranes and thus leads to leakage of cell contents and subsequent cell death (Webb et al., 1993; Webb and Steponkus, 1993; Webb et al., 1994). Another damaging effect of ice formation is cellular dehydration resulting from decreased water potential in the extracellular space and subsequent movement of water out of cells (Webb et al., 1993; Webb and Steponkus, 1993; Webb et al., 1994).

Freezing injury has been observed to occur primarily at the level of cell and organellar membranes. Three types of freezing injury have been well documented: expansion-induced lysis, hexagonal II lipid formation, and fracture jump lesions (Webb et al., 1994; Uemura et al., 1995; Uemura et al., 1996). Expansion-induced lysis typically occurs at -2°C to -4°C and is caused

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by repeated freeze/thaw cycles. The rapid movement of water into the cells following thawing leads to cell lysis. Another phenomenon resulting from freezing is the endocytic vesiculation of plasma membrane that causes reduction in cell surface area, increasing the probability of cell lysis when the water re-enters the cells (Uemura et al., 1995).

The hexagonal II lesions observed at freezing temperatures appear adjacent to the membrane bilayer and are composed of hexagonally packed arrays of monolayer tubes (Webb et al., 1994). In non-acclimated oat protoplasts, these lesions start to appear below -3°C and increase in abundance at -10°C. The transition of membrane bilayers (gel-phase lamellae) to the hexagonal II phase (non-lamellar) structures occurs over a wide range of temperatures depending on the lipid composition of the membranes. They are usually formed at the interbilayer junction of the chloroplast and the plasma membrane where the two membranes are in apposition. When the membranes are returned to warm temperatures, the hexagonal II phase structures are thought to form inverted micelles within the membrane bilayer, resulting in the loss of permeability barrier of membranes (Webb et al., 1994).

Fracture-jump lesions are aparticulate regions in the membranes observed at temperatures below -10°C (Uemura et al., 1995). Although it is unclear how they arise, they are suggested to result from inter lamellar associations and fusion of the plasma membrane with membranes of other organelles.

Hexagonal II phase lipids or fracture-jump lesions arise from a common intermediate phase (Uemura et al., 1995). Upon dehydration that occurs at

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freezing temperatures, the head groups of lipids lose the stabilizing water molecules that maintain the lipid bilayer structure. As a result, there is excessive pressure in the acyl groups of the lipids as compared to the head groups and it exerts a bending force. This bending energy causes either the formation of hexagonal II phase lipids or fracture-jump lesions. The outcome is determined by the intrinsic curvature of the monolayers undergoing the destabilization. Hexagonal phase II lipids are formed when the intrinsic curvature of the monolayers is high, whereas fracture-jump lesions are formed when the curvature is low (Uemura et al., 1995).

In addition to the effects on cell membranes, the reduced water content inside the cell that results from ice formation in the extracellular space leads to protein denaturation and precipitation of other macromolecules inside the cells.

1.3 Cold Acclimation

Biochemical and physiological changes during cold acclimation are achieved through changes in gene expression that begin shortly after exposure to low temperatures (Thomashow, 1999; Zhu, 2001; Zhu et al., 2007). The promoters of specific genes respond to the low temperature signal resulting in transcriptional activation of these genes. Many such early-induced genes encode transcriptional factors that recognize specific sequence elements in promoters of other genes to activate/repress these targets. Additionally, early-induced genes encode common cell signaling components such as serine/threonine protein kinases, phosphatases, phospholipases, response regulators, calmodulin related proteins, calcium-binding proteins etc

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(Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu et al., 2007). Induction of these signaling genes also results in upregulation of genes whose products are enzymes in specific metabolic pathways, transporters, chaperones, membrane stabilizing cryoprotectants, or RNA binding proteins. The cumulative effects of this cascade are biochemical changes that result in protecting cells and establishing homeostasis in the altered environment.

1.3.1 Biochemical Changes Accompanying Cold Acclimation

Many plants accumulate osmolytes, which correlate with their freezing tolerance (Garg et al., 2002; Sakamoto and Murata, 2002; Beck et al., 2007). Metabolome studies revealed a significant change in the overall metabolite composition of cells during cold acclimation. Simultaneous assay of 434 metabolites in Arabidopsis accession Ws-2 revealed changes in 325 in response to low temperature (Cook et al., 2004). These included carbohydrates, amines, organic acids etc. Increase in soluble sugar levels has been demonstrated to correlate with enhanced freezing tolerance, which may be achieved by regulating osmotic balance of cells or affording protection to specific macromolecules (Stitt and Hurry, 2002).

Comparison of two accessions of Arabidopsis (Ws-2 and Cvi-0) that essentially differed in freezing tolerance revealed quantitative differences in their metabolomes at low temperatures, suggesting that metabolic changes achieved during cold acclimation may play a significant role in low temperature tolerance (Cook et al., 2004).

In addition to osmoprotective solutes, plants also accumulate proteins with cryoprotective properties. Proteins accumulated by cabbage and spinach

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during cold acclimation have been shown to protect isolated thylakoid membranes against freeze-thaw induced damage (Volger and Heber, 1975; Volger et al., 1978; Hincha et al., 1993). Arabidopsis seedlings accumulate hydrophilic polypeptides, such as COR15 and COR6.6, in response to low temperatures. In vitro, these polypeptides demonstrate the ability to decrease freeze-induced fusion of liposomes but are unable to prevent their freeze-induced leakage (Uemura et al., 1995). In vivo, over-expression of *COR15* improves the freezing tolerance of chloroplasts by 2°C, without affecting whole plant tolerance. Localization studies revealed COR15 to be targeted to chloroplasts, which was suggested to be the site of action (Uemura et al., 1996). COR15 is thought to defer the formation of deleterious hexagonal II phase lesions in the membrane to lower freezing temperatures (Uemura et al., 1996).

In order to maintain the functional roles of cellular membranes at low temperatures, changes in lipid profiles are made during cold acclimation resulting in homeoviscous/homeophasic adaptation (Wallis and Browse, 2002). These changes compensate for the lipid rigidification and resultant decrease in membrane fluidity observed at low temperatures (Wallis and Browse, 2002). Membrane desaturation is increased by introduction of double bonds in the acyl chains, which results in increased level of molecular motion or fluidity of the membranes. Both transcriptional (delta-9 desaturase gene in Arabidopsis) and post-transcriptional mechanisms were found to be involved in this process (Wada et al., 1990; Kodama et al., 1994; Upchurch, 2008). Such alterations of the chloroplast membranes increase the turnover of components of the photosynthetic electron transport chain, resulting in

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Photosynthetic suppression observed at low temperatures is accompanied by increase in soluble sugars (Strand et al., 1997). This occurs due to transcriptional down-regulation of photosynthetic genes, such as the chlorophyll a/b binding protein (*CAB*) and ribulose-1,5-bisphosphate carboxylase (*RBCS*). During cold acclimation several genes involved in the Calvin cycle are induced and some enzymes are post-translationally modified (Strand et al., 1997; Strand et al., 1999; Hurry et al., 2000; Stitt and Hurry, 2002).

1.3.2 Changes in Gene Expression Associated with Cold Acclimation

Studies elucidating the total transcriptome after different periods of exposure to low temperature showed hundreds of genes to be induced or repressed (Fowler and Thomashow, 2002; Vogel et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). Extensive gene regulatory networks appear to be involved in adaptation to low temperature stress. In response to low temperature, 302 genes were found to be upregulated in Arabidopsis seedlings (Vogel et al., 2005). These differentially expressed genes were clustered based on their temporal expression profiles. A few genes are transiently expressed with peak transcript levels 2-4h after a low temperature stimulus and subsequently decrease to background warm levels. These genes are thought to be general shock genes, although they may also contribute to cold acclimation. Another cluster of genes is highly induced early in response to low temperature followed by a gradual decrease in levels.

However, the expression of this gene cluster is maintained at levels above background warm levels. This cluster includes several transcription factors genes such as *CBF*, *Rav1*, *Zat12*, MYB, etc. Genes are also induced later during cold treatment, remaining elevated throughout exposure to low temperature. In addition to clusters of genes upregulated, 212 genes are down regulated during cold acclimation. The down-regulation of these genes may be significant for enhancing the freezing tolerance of plants (Fowler and Thomashow, 2002; Vogel et al., 2005).

As mentioned earlier, freezing reduces the amount of water available through formation of ice crystals resulting in an osmotic imbalance inside the cells, a characteristic shared with drought and salinity stress (Beck et al., 2007). In addition, considerable overlap is observed in the resulting transcriptional responses to these stresses (Shinozaki and Yamaguchi-Shinozaki, 1996, 2000; Zhu, 2001; Shinozaki et al., 2003). Greater overlap is observed between drought and salinity stress where more than half of the genes induced in response to drought are also induced in response to salinity stress. In contrast, only 10% of the genes induced by drought are also induced by low temperature. Extensive cross talk between the gene networks is suggested for these stresses (Knight, 2002).

1.3.3 The *CBF* Gene Regulatory Pathway

Studies of low temperature responsive genes resulted in the identification of *CBF* (C-repeat binding factor) genes in Arabidopsis *thaliana* (Stockinger et al., 1997). The *CBF* genes comprise a gene family with six members (*CBF1-CBF6*) encoding transcriptional activators belonging to the

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AP2/EREBP (Apetella2/Ethylene Responsive Element Binding Protein) family of DNA-binding proteins (Stockinger et al., 1997; Haake et al., 2002). Low temperature activates rapid induction of *CBF* promoters resulting in detection of the *CBF* transcript within 15 min of cold treatment. *CBF* transcript levels peak 2-4h after exposure to low temperature and subsequently decrease to levels above background (Gilmour et al., 1998). The mechanism of low temperature induction of *CBF*s appears to perceive and respond to absolute temperature of the environment (Zarka et al., 2003). The accumulation of *CBF* transcripts increases with decreasing temperatures (Zarka et al., 2003).

CBF proteins play an important role in the process of cold acclimation. The CBF protein binds to the CRT/DRE (C-Repeat/Dehydration response Element) element in promoters of target genes to up-regulate their expression. Over-expression of *CBF* in Arabidopsis plants leads to activation of the *COR* (Cold Responsive) genes regardless of a low temperature stimulus and as a result these transgenic plants display constitutive freezing tolerance (Jaglo-Ottosen et al., 1998).

Transcriptome profiling of Arabidopsis plants over-expressing *CBF*1, 2 or 3 identified more than 90 genes showing constitutive expression (Fowler and Thomashow, 2002). These 90 genes comprise the *CBF* regulon and include genes encoding cryoprotective polypeptides such as COR15a, enzymes involved in synthesis of solutes with cryoprotective properties, transcriptional activators, etc., which may act in concert to confer cold tolerance to the plant.

1.3.4 Transcriptional Regulation of *CBF* Genes

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The mechanism by which the low temperature stimulus is relayed to *CBF* and other concurrently expressed genes is not known. Promoter analysis using reporter gene fusions have identified a 125 bp region in the *CBF* promoter that is sufficient for low temperature induction (Zarka et al., 2003). Within this segment, two regions named ICEr1 and ICEr2 (induction of *CBF* expression region 1 or 2) are found to act in concert to impart cold responsiveness (Zarka et al., 2003). These sites have not yet been shown to bind any transcription factors, though the ICEr2 region contains the consensus recognition site for a family of transcription factors, the bHLH (basic Helix-Loop-Helix) proteins (Massari and Murre, 2000).

Mutational analysis has led to the identification of some genes involved in *CBF* expression. The *LOS4* gene encoding a DEAD-box RNA helicase appears to have a positive role in *CBF* expression as the *los4* mutant shows reduced or delayed induction of *CBF* genes (Gong et al., 2002). *ICE1* was identified in another mutant screen as a regulator of *CBF*3 expression (Chinnusamy et al., 2003). The *ICE1* gene encodes a MYC-like bHLH transcription factor that binds to MYC DNA binding sites. Over-expression of *ICE1* increases the freezing tolerance of Arabidopsis plants (Chinnusamy et al., 2003). Finally, *FRY2* (Xiong et al., 2002) and *HOS1* (Lee et al., 2001), encoding a novel transcriptional repressor and a novel RING-finger protein respectively, are thought to play a role in *CBF* expression through down-regulation of *CBF* genes.

In addition to mutants affecting the expression of *CBF* genes, mutants altered in freezing tolerance have been found that are unaffected in *CBF* expression. Sensitive to freezing-6 (*sfr6*) mutant, showing lower freezing

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tolerance than the wild type Arabidopsis, shows normal induction of *CBF* genes in response to low temperature but is unable to induce known targets of *CBF* genes, such as the *COR* genes (Knight et al., 1999). This indicates that the *SFR6* encoded protein acts in conjunction with or downstream of *CBF* to induce transcription of *COR* genes. The *eskimo1* mutant, showing constitutively enhanced freezing tolerance, is unaltered in *CBF* and *COR* gene expression and shows increased accumulation of proline, an amino acid known to correlate with freezing tolerance in plants. The *ESK1* gene has been suggested to be a novel negative regulator of cold acclimation, which is functioning independent of the *CBF* regulon (Xin and Browse, 1998; Xin et al., 2007).

In addition to low temperature, *CBF* genes are also induced in response to mechanical agitation and cycloheximide treatment (Zarka et al., 2003). Studies of the *CBF2* promoter have shown that the elements responsible for induction of this promoter by low temperature and mechanical agitation are tightly linked. The ICEr1 and ICEr2 regions described before are responsive to low temperature, mechanical agitation, and cycloheximide (Zarka et al., 2003). However, it is not yet known if these stimuli are perceived and relayed to the *CBF2* promoter through a common regulatory pathway.

1.3.5 Conservation of *CBF* pathway in higher plants

There is considerable evidence to suggest that the *CBF* cold-responsive pathway is conserved in a wide variety of plant species. Orthologs of *CBF* genes have been identified in cold tolerant and sensitive plants, including *Brassica napus*, wheat, rye, rice, barley, tomato, and maize (Jaglo et

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al., 2001; Choi et al., 2002; Dubouzet et al., 2003; Lee et al., 2004; Qin et al., 2004). The CBF orthologs display highest conservation in the DNA-binding domain and its two flanking "signature sequences" (PKK/RPAGRxKFxETRHP and DSAWR) in *Arabidopsis thaliana* (Jaglo et al., 2001; Choi et al., 2002; Dubouzet et al., 2003; Lee et al., 2004; Qin et al., 2004).

The two *CBF* orthologs in *Brassica napus*, identified through cDNA library screening, are induced within 30 mins of exposure to low temperature, like their Arabidopsis counterparts. Furthermore, two CBF target genes *Bn115* and *Bn28*, orthologs of *COR15* and *COR6.6* respectively, also contain the CRT/DRE element in their promoters that is characteristic of the *CBF* target promoters in Arabidopsis (Jaglo et al., 2001). *Hordeum vulgare* (barley) contains 20 *CBF* orthologs, 11 of which form two tandem clusters on chromosome 5H-L (Skinner et al., 2006). In wheat (*Triticum monococcum L*), QTL (Quantitative Trait Loci) mapping for frost tolerance identified the Fr-A(m)2 locus on chromosome 5A(m), which contains 11 *CBF* genes (Miller et al., 2006). Two additional *CBF* genes in wheat are located on chromosomes 7A(m) and 6A(m) (Miller et al., 2006). QTL mapping for frost tolerance in several other plant species is leading to identification of additional *CBF* orthologs. *Solanum lycopersicum* (tomato), a chilling sensitive plant, contains three *CBF* orthologs, only one of which is induced by low temperatures (Zhang et al., 2004).

1.4 Perception of the low temperature stimulus

The mechanisms of low temperature perception are unknown. However, observations in cyanobacteria and plants point to the role of certain

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signaling components. Change in membrane fluidity is thought to initiate low-temperature responsive signaling in the cyanobaterium *Synechocystis*. Exposure of *Synechocystis* to low temperature leads to induction of the *desA* gene, which encodes a fatty acid desaturase. Reduction in membrane desaturation by Pd-catalyzed hydrogenation also leads to the induction of this gene in a manner similar to its low temperature induction (Vigh et al., 1993).

Analysis of low temperature response in *Synechocystis* also identified two histidine kinases, Hik33 and Hik19, and a response regulator, Rer1, as components of the signaling pathway functioning at low temperature (Suzuki et al., 2000). It is suggested that low temperature induced rigidification of the plasma membrane leads to the phosphorylation of Hik33, a membrane spanning histidine kinase. The phosphate group is transferred to Hik19 and subsequently to the response regulator (Rer1), thereby relaying the change in physical state of the membrane to cell nucleus (Suzuki et al., 2000). In plants histidine kinases have been shown to play roles in ethylene signaling, cytokinin signaling and osmosensing (Urao et al., 2000). Their role in cold signaling has yet to be established.

Studies in Arabidopsis show a rapid increase in cytoplasmic Ca^{+2} concentrations following exposure to low temperatures (Knight, 2002; Kaplan et al., 2006). This Ca^{+2} influx appears to provide the secondary messenger required for expression of certain cold-regulated genes, including *COR* and other *CBF*-targeted genes. Similarly, pharmacologically induced increases in intracellular Ca^{+2} levels at warm temperatures also induced *COR* gene expression (Tahtiharju et al., 1997). The flux signature (the amplitude and duration of Ca^{+2} peak) in response to low temperature is different for different

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cell types. The flux also varies through the stages of cold acclimation, becoming diminished in amplitude and prolonged in duration. This change in the signature of Ca^{+2} flux during cold acclimation is suggested to play a role in either developing a cold memory or serving as a cold memory (Gao et al., 2004).

Ca^{+2} influx is also observed in response to mechanical agitation of plants although it is not yet clear if the increases in Ca^{+2} brought on by cold and mechanical stress are mediated through the same mechanism. However, it is noteworthy that the activity of an anion mechano-sensitive calcium-selective channel has been shown to increase as temperature is lowered to 6°C (Ding and Pickard, 1993).

Arabidopsis suspension cells display an increase in the levels of phosphatidic acid within a few minutes of exposure to low temperatures through the activation of phospholipases C and D (Vergnolle et al., 2005). Further analysis of the transcriptome of suspension cell cultures at low temperatures, in the absence and presence of phospholipase inhibitors identified 58 genes to be regulated by phospholipase C and 87 by phospholipase D, suggesting involvement of both signaling pathways at low temperatures (Vergnolle et al., 2005).

1.5 Naturally occurring genetic variation

Variation is an essential component of natural populations that is discernible both in and between populations (intra- and inter-species) (Farrall, 2004). Natural selection of variation, determined by the existent environmental conditions, has given rise to diverse phenotypic traits in organisms.

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Adaptation of species to their environment is thought to be a result of genome evolution (Farrall, 2004). The genomes of a population are continually undergoing change, mostly through spontaneous mutations. The selective pressures acting on a population are important in determining which polymorphisms will be maintained. Environmental conditions provide significant selective pressures that develop and maintain genetic variation in nature (Hoffmann and Weeks, 2007). Over the course of time, genetic changes that provide a selective advantage under local conditions are accumulated with resultant increase in survival rate of a population. Spatial and temporal changes in environment drive the diversity of survival strategies observed between populations. However, it is not always the case that the variation in a phenotype arises from selection of genetic changes affecting the phenotype directly, in some instances phenotypic evolution results from linkage or pleiotropic effects (Cork and Purugganan, 2004). Although natural selection can have a negative effect on variation as it may lead to fixation of the most adapted genotype in a population, variation in selective pressures over space and time enhance genetic variation (Gebhardt and Stearns, 1992; Byers, 2005).

Fisher in 1930 proposed a random mutation model for evolution, wherein the changes in genome were suggested to be random with respect to their impact on the phenotypes (Farrall, 2004). This was based on the premise that large effect mutations would produce deleterious effects and will be selected against (Farrall, 2004). However, Quantitative Trait Locus (QTL) analysis showed that, in fact, differences in genomes were localized to a few regions, which can have impacts as high as 20% on the variation in

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phenotype (Farrall, 2004). The findings of QTLs lead others, such as Kimura and Orr, to propose modifications to Fisher's adaptation model. Kimura proposed that during the process of changes in the genome, random changes of large effects would have a high probability of fixation if they were favorable (Kimura, 1969; Ota and Kimura, 1971). These favorable changes in the genome can then be identified through QTL analysis. Orr further modified the model to allow the prediction that quantitative variation is determined by few QTLs of large effect and more QTLs with progressively smaller effects (Farrall, 2004). Therefore predicting that the distribution of effect sizes of QTLs is exponential in nature.

1.6 Understanding Complex Traits using Natural Variation

Several independent studies examining different aspects of plant growth, development, and interaction with the biotic and abiotic environment have established the vast genetic diversity in nature (Alonso-Blanco and Koornneef, 2000; Mackay, 2002; Koornneef et al., 2004; Alonso-Blanco et al., 2005; Shindo and Bernasconi, 2007). This variation proves helpful in understanding the regulation of complex plant traits that are under polygenic regulation (Alonso-Blanco and Koornneef, 2000; Mackay, 2002; Koornneef et al., 2004; Alonso-Blanco et al., 2005; Shindo and Bernasconi, 2007). When extended to the study of plants inhabiting diverse environments, this genetic variation would allow identification of genes that help plants adapt to different environments. Furthermore, it would allow quantification of the effects of these genes on the variability in the trait (Alonso-Blanco et al., 2006).

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Variability of a trait that can be measured on a continuum rather than in discrete classes is called continuous variation (Alonso-Blanco et al., 2006). Continuous variation makes possible the gradual changes underlying adaptive change in nature. For a population showing such variation in a trait, the underlying genotypic variation can be identified through crossing experiments. The genetic loci contributing to the variation are termed QTL (Alonso-Blanco et al., 2006). In addition to identifying genetic loci controlling a quantitative trait, statistical analysis can be performed to understand the behavior pattern of the individual loci.

To understand the regulation of a quantitative trait, traditional quantitative genetics defines the trait in terms of variances. The continuous variation for a phenotypic character is measured on a quantitative scale. The total phenotypic variance observed in the population is first partitioned into genetic and environmental variances. The genetic variance is further divided into additive, dominant and epistatic effects. Analysis of this information allows estimation of heritability of the trait (Kearsey and Farquhar, 1998).

Additive genetic effects are the sum of mostly small effects of alleles of many genes influencing the phenotype. The selection for additive genetic effects can accumulate over time, as and when mutations contributing small selective advantages occur. Dominance genetic effects result from the interaction of alleles at the same locus, where one allele exhibits a dominant influence on the phenotype by masking the contribution of the recessive allele at that locus (Frascaroli et al., 2007; Melchinger et al., 2007; Melchinger et al., 2007). Dominance effects are the basis of hybrid vigour (heterosis) in breeding programs (Melchinger et al., 2007). Epistasis refers to the

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nonadditive interactions occurring between alleles at different loci, which lead to an effect on the phenotype that is different from what would be expected if the loci were influencing it independently. This makes it difficult to separate the influence of a single locus on the phenotype (Juenger et al., 2005). Epistatic interactions can be synergistic (greater than that expected from individual loci) (Bonhoeffer et al., 2004) or antagonistic (lower than that expected from individual loci) (Sanjuan and Elena, 2006).

1.7 QTL Mapping

The availability of a large number of molecular markers such as RFLPs (Restriction Fragment Length Polymorphism), AFLPs (Amplified Fragment Length Polymorphism), RAPDs (Random Amplified Polymorphic DNA), and SSLPs (Single Strand Length Polymorphism) dispersed all along the Arabidopsis genome has made it possible to map genes that contribute to quantitative variation. These markers segregate as single genes, and are unaffected by the environment. The genes are identified indirectly as chromosomal regions contributing to the phenotypic variation between ecotypes, i.e. the QTLs.

Major techniques of QTL mapping are based on the same basic principle, which is to establish statistical association between two characters based on co-segregation in a segregating population (Loudet et al., 2002; Perez-Perez et al., 2002; Harada et al., 2004; Keurentjes et al., 2006). In this mapping the statistical association of a phenotype is tested with the molecular marker derived chromosomal maps of the individuals. The power of QTL detection depends on several factors including the population-size and -type,

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the accuracy of trait measurement, and the statistical methods used for the correlation analysis (Knapp and Bridges, 1990; Mackinnon and Weller, 1995; Rebai et al., 1995; Belknap et al., 1996; Zou et al., 2005). The resolution of QTL analysis is affected by few major factors, namely, the number of recombinant events that are being represented in the segregating population and the number of markers examined (Knapp and Bridges, 1990; Mackinnon and Weller, 1995; Rebai et al., 1995; Belknap et al., 1996; Zou et al., 2005).

The general approach for mapping QTLs is to select two lines that display contrasting phenotypes for a quantitative trait and interbreed these lines to generate homozygous descendants (Recombinant Inbred Lines) that contain a small number of segments from one line in the background of the other (Kearsey and Farquhar). The combination of genomes in the descendants is a random process and several lines are chosen to increase chances of finding maximum combinations of the two parent genomes in the segregants. Such hybrid individuals are then assessed for the quantitative phenotype being studied, and estimates are made of the contributions (or lack thereof) of specific segments to the observed variation. Statistical analysis is used to determine which genetic loci are segregating with the phenotype with high significance, i.e. the QTLs (Kearsey and Farquhar, 1998). QTL analysis of several complex traits has revealed their polygenic regulation (Gora-Maslak et al., 1991; Cardon et al., 1994; Plomin et al., 1994; Frankel et al., 1995; Rodriguez et al., 1995; Fijneman et al., 1996; Young, 1996; Fisler and Warden, 1997; Li et al., 1997; Pomp, 1997; Yano and Sasaki, 1997; Vallejo et al., 1998; Page et al., 2000; Consoli et al., 2002).

QTL analysis requires genotypic and phenotypic information of individuals in a given population. Although these populations are usually derived through an experimental cross, natural populations may also be used in such studies. The experimental populations used include backcross, advanced backcross, F2, Recombinant Inbred Lines (RILs), Intermated Recombinant Inbred (IRI) populations, Advanced Intercross (AI) populations, Double Haploid (DH) populations, Introgression Lines (ILs), Near Isogenic Lines (NILs), etc. (Comstock and Robinson, 1948; Stuber et al., 1992; Beavis et al., 1994; Chen et al., 2007; Keurentjes et al., 2007). These populations differ in the characteristics that define population structure, such as homozygosity, genotypic frequencies, and linkage disequilibrium.

To better understand the population structure of RILs, consider two parental inbred lines, P1 and P2, which differ in the quantitative trait of interest (Kao, 2006). A cross between these lines would result in an F1 population in which every individual contains the same heterozygous genotype. The F1 individuals are selfed to produce an F2 population, in which the genotypic frequencies of the P1 homozygote, the P1P2 heterozygote, and P2 homozygote at a single locus are 1/4, 1/2, and 1/4, respectively and the heterozygosity of this population is 1/2. The frequency of recombinants between two loci is equivalent to the recombination fraction. Individuals from F2 can be selfed for several generations to derive an RIL population. An RIL population generated by selfing the F2 individuals for t-2 generations is called an F_t RIL population. The F_t population contains P1 homozygote, P1P2 heterozygote, and P2 homozygote genotypes with the expected frequencies of (1/2)-(1/2t), (1/2t-1), and (1/2)-(1/2t), respectively and the heterozygosity of

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the population is (1/2t-1) (Kao, 2006). Therefore, the homozygosity of such a population increases with higher number of generations to which the population is selfed.

It is generally assumed that the data for genotype and phenotype are obtained from the same population. However this is not always the case when using RILs. Phenotypic trait values are often obtained for the progeny of the genotyped population. This is possible because of the high homozygosity as it increases the correlation of t generation with t + 1 generation. The use of such a design in QTL mapping has several benefits. It reduces the overall cost of the experiment as it eliminates the need to generate genotypic data every time the phenotypic data is collected (Kao, 2006). It also improves the statistical power of the experiment through sampling several progeny in biological repeats for each genotyped individual (Knapp and Bridges, 1990; Kao, 2006).

For the purpose of obtaining maximum information it is helpful to use different segregating populations as they display differences in the population structure. Different aspects of the population structure provide distinct advantages. For instance, the increase of homozygosity may help in the estimation of additive effects due to accumulation of homozygotes, whereas a higher number of heterozygotes allow estimation of the dominance effects. Introgression Lines, derived through repeated backcrossing of RILs, contain a small number of specific genomic regions from the donor parent in a nearly isogenic background of the other parent.

The number of crossover events (pairing and synapsis) that occur during meiosis determines the recombination frequency in a population. In

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case of Arabidopsis, a maximum of 2 chiasmas are formed on any one chromosome, thus allowing only two recombination events, at the most, per chromosome (Sanchez-Moran et al., 2002). Therefore, an RIL population represents a low number of recombination events. To increase the recombination frequency within the population, the lines can be intercrossed further before inbreeding them to achieve homozygous populations (Zou et al., 2005). Populations derived in this manner are called Advanced Intercross Lines (AILs).

The advantage of using RIL populations is that in such a population, an individual contains several introgression fragments from the donor parent (50%), which allows analysis of more genomic regions using a smaller population and detection of inter-allelic interactions like epistasis. Moreover, both parental genotypes are almost equally represented in the population. However, the RIL population also presents some limitations, such as diverse developmental timings of different lines. This poses a significant challenge in analysis of traits requiring phenotype information of individuals at the same stage of growth and development. Furthermore, simultaneous segregation of multiple loci in the population may hinder QTL detection because of inter-allelic interactions, making it important to test for epistatsis.

In the absence of epistasis, the detection of small-effect QTLs can be masked in an RIL population by the presence of large-effect QTLs. Detection of small effect QTLs can be improved by using a different population, such as the Near Isogenic Lines (NILs) or Introgression lines (ILs), because individuals in these populations contain a single introgression of the donor parent. The NIL population also shows more homogeneous growth and

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developmental characteristics since most of the genetic background in the population is identical (Swarup et al., 1999; Keurentjes et al., 2007).

Keurentjes et al compared populations of RILs and NILs derived from Cvi and Ler and reported discovery of additional QTLs using the NIL population compared to the RIL population (Keurentjes et al., 2007). In total 12 small-effect QTLs were detected for different aspects of plant growth and development (flowering time, length at first silique, total plant length and basal branch number) that were masked by presence of large-effect QTLs in the RIL population (Keurentjes et al., 2007).

1.8 Statistical methods of QTL Mapping

QTL mapping procedures such as Interval Mapping (Jansen, 1993; Choi et al.) and Composite Interval Mapping (CIM) (Jiang and Zeng, 1995) test the null hypothesis that a QTL is absent for the given trait based on the data obtained from a given population. A putative QTL is assumed to exist at every position throughout the genome and multiple tests are performed across the whole genome by analyzing small portions at a time, thus eliminating regions that lack statistical support for containing a QTL. To control the error rate for this genome-wide analysis and improve the confidence of results, the critical threshold value of the test statistic is selected. The methods used today fall mainly into four classes: Nonparametric methods, Least-squares based regression methods, Likelihood based methods and Bayesian mapping. These analyses are computationally demanding due to many repetitive tests during the search procedure. Therefore, several computer softwares such as MAPQTL, BQTL

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and QTL Cartographer have been developed for performing QTL analysis (a list can be found at www.stat.wisc.edu/biosci/linkage.html).

1.9 Genetical Genomics: QTL Mapping for Regulators of Transcription

The molecular complexity of quantitative variation has been analyzed in many studies. The analysis of alcohol dehydrogenase (Adh) enzyme activity in Drosophila revealed a complicated molecular basis of allelic variation (Stam and Laurie, 1996). Allelic variation was found to arise both due to differences in the coding as well as non-coding regions of the gene. Furthermore, it was noted that several allelic effects were combining to appear as "super-alleles" which were segregating as a single major QTL.

Although QTL analysis has been primarily employed in plant breeding to identify regulators of commercially interesting traits, it has also helped understand the mechanics of evolution. QTL mapping is now being extended to gain insights into regulation of differential gene expression, thus bringing this methodology to the genotype-phenotype interface that is the transcriptome. Variation in gene expression can result from a DNA sequence variation existing in the gene, for example in its promoter region, that directly influences expression of its transcript. Variation in gene expression can also result from variation in the DNA sequence of another gene that is involved in its expression. Therefore it is reasonable to imagine that eQTLs could arise from sequence variation of the gene itself, termed as cis-acting eQTLs or from sequence variations that would affect levels or functional efficacy of a different gene, termed trans-acting eQTLs. In the recent past, several studies were performed to understand the heritability of differential gene expression.

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Microarrays were used in most of these studies to compare genome-wide expression profiles of different ecotypes/strains of organisms to determine the proportion of genes that show genotype specific variation in expression. Studies in yeast, Drosophila, mice, and humans show a strong heritable component of differential gene expression. Doss et al used microarrays to analyze genome-wide expression profiles of F2 mice generated from a cross between two common inbred strains of mice and identified eQTLs contributing to the variation in several thousand genes (Doss et al., 2005). They found approximately one-third of these eQTLs to lie within 10 cM of the gene being used as the trait. They classified these eQTLs as *cis*-acting and through further analysis reported at least 64% of these *cis*-acting eQTLs as true positives, whereas the remaining 36% could not be confirmed as truly *cis*-acting (Doss et al., 2005). They also noted that the *cis*-acting eQTLs showed higher linkage statistics. Interestingly, several other groups also reported cis-acting eQTLs to be supported with higher linkage statistics than *trans*-acting eQTLs (Bystrykh et al., 2005).

Trans-acting eQTLs identified in yeast were shown to map to several different classes of genes besides transcription factors (Yvert et al., 2003). Genes that were clustered based on similar function often revealed regulation by the same eQTL. Therefore, this approach can be used to identify regulators of differential gene expression that lie further upstream of the process of transcription. In fact, in the recent past this approach of genetical genomics has helped dissect several complex traits (Brem et al., 2002; Wayne and McIntyre, 2002; Yvert et al., 2003; Ron et al., 2007; Ronald and Akey, 2007).

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1.10 Main Objectives of Current Study

In the present study, a QTL mapping approach was chosen to understand the regulation of low temperature responsive transcription in plants. As mentioned above, the transcriptome of *Arabidopsis thaliana* undergoes extensive change at low temperature (Vogel et al., 2005). The *CBF* regulon, although significant for low temperature tolerance, is not the only set of genes induced in response to low temperature. In fact, the *CBF* regulon genes comprise only about 33% of genes found to change in response to low temperature. In an attempt to determine how many early regulators of low temperature induced gene expression exist, change in gene expression at low temperature was treated as a molecular trait. The goal was to identify QTLs regulating expression (eQTLs) of genes induced early in response to low temperature. Regulators of early responsive genes would include those that encode the thermosensor(s) and protein(s) that relay the signal from the thermosensor to the upregulated genes. Therefore this approach would potentially allow identification of the thermosensor(s) and early signal transduction components of low temperature signaling in addition to providing an insight into the complexity of low temperature responsive gene regulation.

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Chapter 2

eQTLs Regulating Low Temperature Responsive Expression of *CBF1-3*

2.1 Introduction

Cold acclimation involves several changes in the biochemistry and physiology of plants (Thomashow, 1998; Zhu, 2001; Yamaguchi-Shinozaki and Shinozaki, 2006). Changes in gene expression have been established as a central step translating into the biochemical and physiological adaptations observed in cold acclimated plants (Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu et al., 2007). Transcriptome studies using microarrays have identified nearly 700 genes that were differentially expressed during cold acclimation, at least some of which may function in a concerted manner to generate cold tolerance (Fowler and Thomashow, 2002). These differentially expressed genes were grouped into distinct classes based on their temporal expression profiles (Vogel et al., 2005).

Among the genes rapidly upregulated by low temperature are the *CBF* family of transcription factors, which recognize the CRT/DRE sequence element in the promoters of *COR* genes, resulting in the induction of these targets (Zarka et al., 2003). *CBF* genes play an important role during cold acclimation as over-expression of *CBF1*, *2*, or *3* confers constitutive freezing tolerance to transgenic Arabidopsis (Jaglo-Ottosen et al., 1998). Nearly 90 low temperature-induced genes are found to be constitutively expressed in

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plants over-expressing *CBF1*, *2*, or *3* and are classified as the CBF regulon (Fowler and Thomashow, 2002).

Studies indicate complex regulation of the *CBF* genes at low temperatures involving positive and negative regulators (Zarka et al., 2003). Sequence elements required for low temperature responsiveness of the *CBF2* promoter have been identified, however, the transcription factors interacting with these elements remain unknown (Zarka et al., 2003). Several genes that appear to play a role in the regulation of *CBF* expression at low temperatures have been identified through mutant screens. *LOS4* and *ICE1*, encoding a DEAD-box RNA helicase (Gong et al., 2002) and a MYC-like bHLH transcription factor (Chinnusamy et al., 2003), respectively, appear to positively regulate *CBF* expression. The *los4* mutant displays decreased levels of *CBF1*, *2*, and *3* transcripts whereas the *ice1* mutant demonstrates lower levels of *CBF3* transcripts only (Chinnusamy et al., 2003), suggesting possible differences in the regulation of individual members of the *CBF* family.

In addition to positive regulators of *CBF* expression, genes showing evidence for a role in negative regulation of *CBF*s have also been identified. These genes include *FRY2* and *HOS1* that encode a novel transcriptional repressor and a novel RING-finger protein, respectively (Xiong et al., 2004), (Lee et al., 2001). HOS1 has been proposed to function by targeting certain positive regulator(s) of *CBF*s for ubiquitination and subsequent degradation (Lee et al., 2001).

Additionally, there is evidence to suggest *CBF* gene regulation by a feedback repression mechanism, either involving the CBF proteins or a downstream target. The *los1* mutant, disrupted in low temperature specific

protein synthesis, shows super-induction of *CBF* genes (Guo et al., 2002). It is proposed that inhibition of protein synthesis in this mutant would prevent accumulation of CBF and downstream target proteins, rendering them incapable of feedback repression, leading to super-induction of *CBF* transcripts (Guo et al., 2002).

However, the mechanism by which *CBF* genes are regulated at low temperatures remains poorly understood. Several approaches can be employed to identify regulators of *CBF* gene expression; biochemical approaches designed to identify proteins binding to promoters of these genes (Baker et al., 1994), genetic approaches involving mutational analysis (Ishitani et al., 1998; Xin and Browse, 1998; Knight et al., 1999; Lee et al., 2001), or molecular approaches studying the effect of over-expressing these genes in planta (Jaglo-Ottosen et al., 1998). Such approaches, however, are limiting when studying complex traits, which involve polygenic regulation and gene X environment interactions, as they generally focus on individual genes or genotypes.

An alternate approach to understand the regulation of complex traits involves the use of naturally occurring genetic variation, which is widespread in nature (Alonso-Blanco and Koornneef, 2000). Gradual genetic change, occurring over long periods of time, contributes to the ability of populations to adapt to their environment (Farrall, 2004). Genetic variation, when coupled with environmental heterogeneity, results in the fixation of different genotypes in different environments. Ecotypes of plants growing in diverse geographical regions that experience different ambient temperatures may contain genetic

variation that allowed them to adapt to local temperatures. Comparison of such ecotypes may reveal the genetic differences that led to this adaptation.

Complex traits, both in plants and animals, tend to show continuous variation, which can be contributed by heritable and/or non-heritable factors (Orr, 1999). The non-heritable component results from the effect of the environment on trait development. The heritable component, on the other hand, arises due to the effect of several genes on trait development (Orr, 1999). These genes can be identified through QTL mapping, which narrows down a chromosomal region that may contain one or more genes affecting trait development (Kearsey and Farquhar, 1998). This approach is limited to detecting only those genes that show allelic differences among the parents.

Use of QTL mapping has recently been extended to provide insights in to the regulatory mechanism of transcription in yeast, *C. elegans*, plants, mice, and humans (Bing and Hoeschele, 2005; Deutsch et al., 2005; Kirst et al., 2005; MacLaren and Sikela, 2005; Li et al., 2006). In these studies transcript levels of genes were considered as a molecular trait in segregating populations to identify QTLs, which were termed eQTLs (expression QTLs) (Zou et al., 2007). Comparison of genome-wide expression profiles in various systems revealed a strong heritable component of differential gene expression (Bing and Hoeschele, 2005; de Koning et al., 2005; Deutsch et al., 2005; Yamashita et al., 2005). Alterations in transcript levels were reported to result in tighter regulation of genes than mutations in gene sequence (Wittkopp et al., 2004). Microarray analysis of two ecotypes of Arabidopsis, Bay-0 and Sha, revealed that 32.2% genes demonstrate heritable differences

in expression (Vuylsteke et al., 2005). Variation in gene expression was found to occur due to *cis*- and *trans*-acting eQTLs (Vuylsteke et al., 2005).

The present study was undertaken to explore the potential of using natural variation in *CBF* gene expression to identify regulators of low temperature associated transcription of these genes. *CBF* transcript abundance at low temperature was considered as a molecular trait to identify eQTLs controlling *CBF* expression. Identification of common eQTLs for *CBF1*, *2*, and *3* genes would indicate that these genes are induced through a common pathway, whereas identification of different eQTLs would suggest involvement of distinct pathways. Characterization of the genomic region underlying such eQTLs would lead to identification of genes regulating *CBF* expression at low temperatures. These regulators could potentially include genes encoding the thermosensor and downstream signal transduction components, amongst others.

2.2 Results

2.2.1 Natural variation in *CBF* gene expression

In addition to the availability of vast genetic and genomic resources, Arabidopsis *thaliana* grows across a wide range of geographical regions with considerably diverse environmental conditions, thus making this species an ideal candidate for the current study (Shindo and Bernasconi, 2007). To identify eQTLs regulating low temperature responsive expression of *CBF1*, *2*, and *3* genes, Arabidopsis accessions collected from warm and cold climates were compared to identify variants in low temperature responsive *CBF* expression. The accessions Col-0, Ct-1 (CS1094), Cvi-0 (CS902), No-0 (CS1394), Stw-0 (CS1538), and Tsu-1 (CS1640) were compared for differences in *CBF* transcript accumulation at low temperatures. These accessions differed with respect to the temperatures (and other factors) they were regularly exposed to in their natural habitats (Figure 2.1).

As described earlier, exposure to low temperature leads to the rapid induction of *CBF1-3* genes (Gilmour et al., 1998). In seedlings exposed to 4°C, *CBF* transcripts can be detected as early as 15 min, with highest expression levels observed at around 2h followed by decrease to levels about three-fold above those in control plants (Gilmour et al.). In a preliminary screen for natural variation in low temperature responsive *CBF* expression, seedlings of each accession were subjected to a gradual decrease in ambient temperature (starting from 21°C, with decrease in set temperature point by 2°C every 4h) and total RNA of each accession, incubated for 1h or 4h at 17°C, 15°C, 13°C,

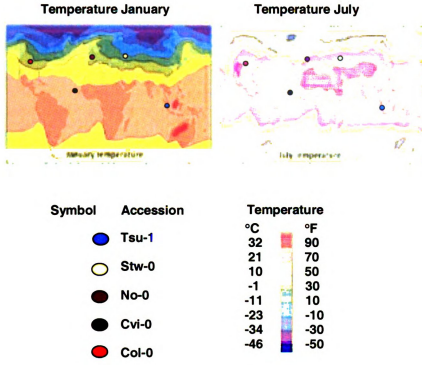


Figure 2.1. **Geographical distribution of Arabidopsis accessions selected for analysis.** Colours indicated in the legend mark the original habitats of the different Arabidopsis accessions. The heat map represents average temperatures across different geographical regions during winter and summer months with the colour scheme depicted in the legend (The Reader's Digest World Atlas, 1991;ISBN 0-89577-388-0;pg 11).

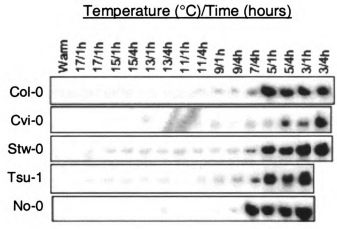


Figure 2.2. **Expression of *CBF* genes in Arabidopsis accessions exposed to gradual reduction in ambient temperature.** Northern blot showing total *CBF* transcript accumulation in seedlings of Col-0, Cvi-0, Stw-0, Tsu-1, and No-0 after reaching indicated temperature for varying periods of time (1h or 4h). A probe that hybridized to *CBF1*, *2*, and *3* was used for detection of total *CBF* transcripts.

11°C, 9°C, 7°C, 5°C, 3°C, and 1°C, was analyzed using Northern analysis (Figure 2.2). Although most accessions demonstrated low-level induction of *CBF* transcription at temperatures as high as 15°C, there was a marked increase at 5°C. Of the accessions tested, Cvi-0 exhibited lowest levels of *CBF* transcript at 5°C. Collectively, these results indicated natural variation in *CBF* expression amongst different accessions of Arabidopsis.

2.2.2 Analysis of low temperature responsive *CBF* expression in L*er* and Cvi-0

Analysis of a segregating population derived from a cross between two parents showing quantitative variation in *CBF* transcript accumulation could be performed for the identification of loci regulating *CBF* expression. Since a segregating population for the Cvi-0 X L*er* cross was available (Alonso-Blanco *et al.*, 1998), quantitative variation in *CBF* transcript accumulation was tested in Cvi-0 and L*er*.

Considering the marked difference in *CBF* expression at 5°C (Figure 2.3), it was assumed that a single temperature point would be sufficient for comparison of *CBF* expression. To confirm this and select a temperature for comparison, seedlings growing in 12h photoperiod at 22°C were exposed to different temperatures for 2h. Northern analysis was performed to compare the response of accessions Ws (previously known to accumulate high levels of *CBF* transcripts at 4°C (Cook et al., 2004)), Cvi-0, and L*er*, following a reduction in ambient temperature from 22°C to 10°C, 8°C, 6°C, 4°C, or 2°C for 2h (Figure 2.4). Although both Cvi-0 and L*er* induced *CBF* expression at

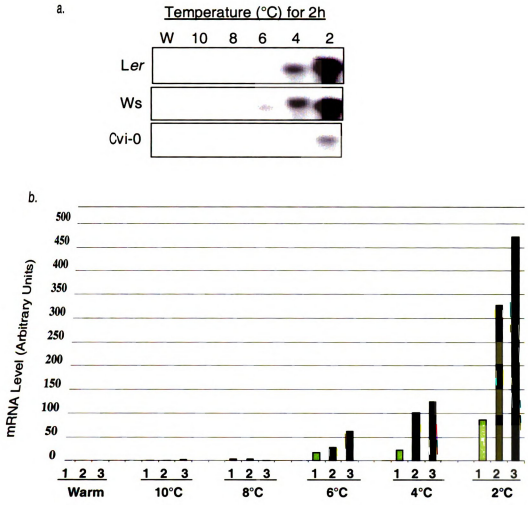


Figure 2.3. **Expression of *CBF* genes in Cvi-0, Ler, and Ws following a sudden drop in ambient temperature.** a) Northern blot showing total *CBF* transcript in short-day photoperiod grown seedlings following 2h incubation at the indicated temperature. A common probe was used for detection of *CBF1*, *2, and 3.* b) Histogram showing *CBF* transcript levels normalized with 18S *rRNA*. The y-axis depicts transcript level in arbitrary units and the x-axis shows the accessions Cvi-0, L*er*, and Ws (1, 2, and 3 respectively) after 2h at depicted temperatures.

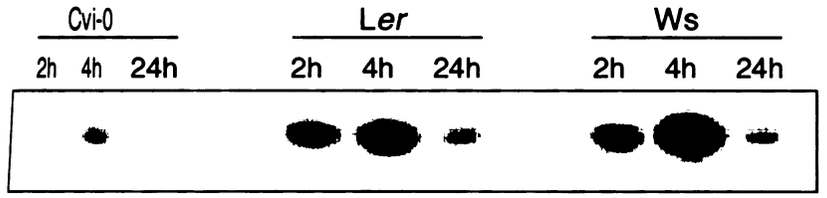
the same temperature, Cvi-0 accumulated lower levels of *CBF* transcript, thus confirming quantitative variation between the two accessions. Furthermore, the quantitative difference in transcript levels among the accessions increased with decrease in temperatures. The accession Ws showed highest levels of *CBF* transcript.

To determine if Cvi-0 was able to accumulate *CBF* transcripts to levels comparable with L*er* following an extended period of exposure to low temperature, a time course experiment was performed at 4°C. As shown in Figure 4, both Cvi-0 and L*er* showed similar kinetics of *CBF* induction, with the transcript peaking at 4h and decreasing to lower levels by 24h.

The *CBF* genes have been shown to be influenced by the circadian clock; basal levels of *CBF3* were found to undergo circadian cycling (Harmer et al., 2000) and the induction levels of *CBF1*, *2*, and *3* were dependent on the time of day at which they were subjected to the low temperature stimulus (Fowler et al., 2005). To determine if the exclusion of circadian effects would abolish the variation in *CBF* transcript accumulation, Cvi-0 and L*er* seedlings grown under continuous light were analyzed after 2h incubations at 12°C, 10°C, 8°C, 6°C, 4°C, 2°C, and 0°C (Figure 2.5). Cvi-0 was found to accumulate lower levels of *CBF* transcript compared to L*er* even in the absence of a photoperiod.

Further, to determine if quantitative variation in *CBF* expression existed in a segregating population derived from Cvi-0 and L*er*, an RIL population derived from the Cvi-0 X L*er* cross was analyzed for *CBF* transcript accumulation after 2h at 4°C in the absence of a photoperiod. The use of

a.



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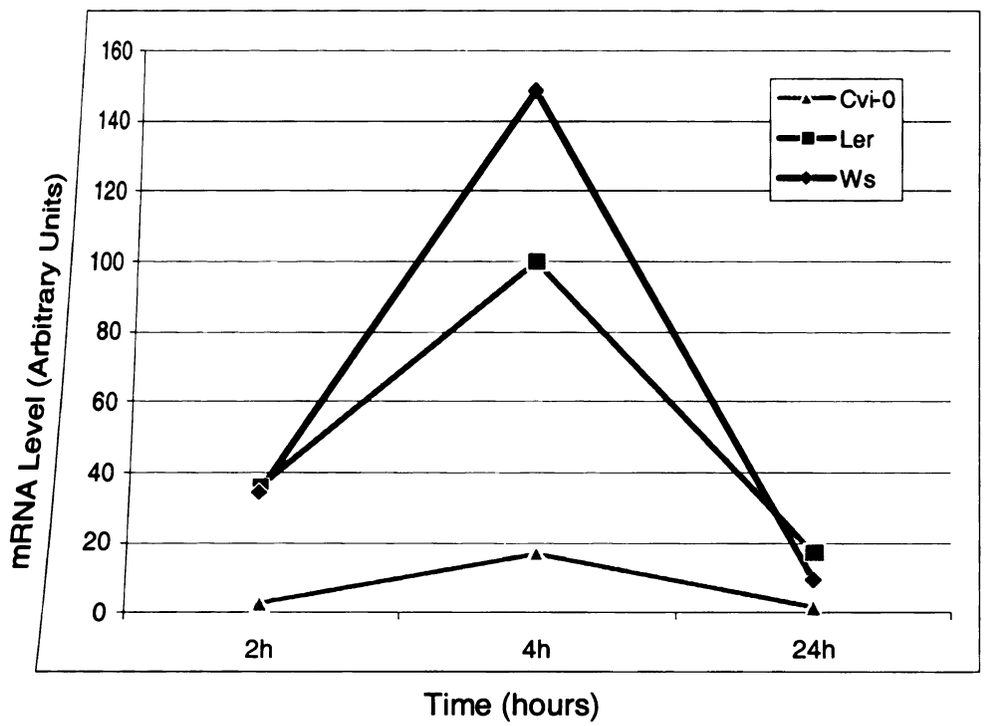


Figure 2.4. **Expression of *CBF* genes in a time course of Cvi-0, L*er*, and *Ws*, at 4°C.** a) Northern blot showing total *CBF* transcript accumulation with a common probe for detection of *CBF1*, *2*, and *3*, in short-day photoperiod grown seedlings after 2h, 4h, or 24h at 4°C. b) Graph showing *CBF* transcript levels normalized with 18S rRNA levels. The y-axis depicts transcript level in arbitrary units and the x-axis depicts periods of 2h, 4h, or 24h at 4°C for the accession mentioned.

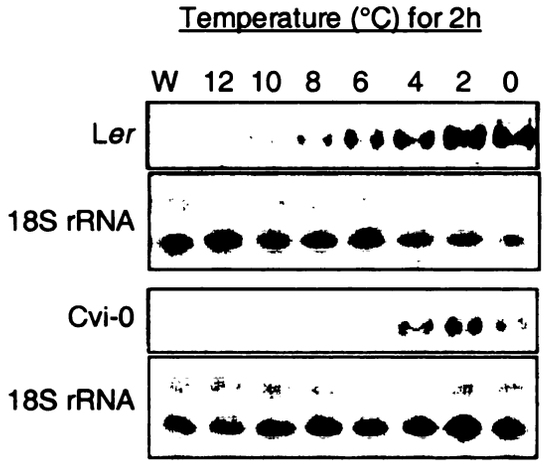


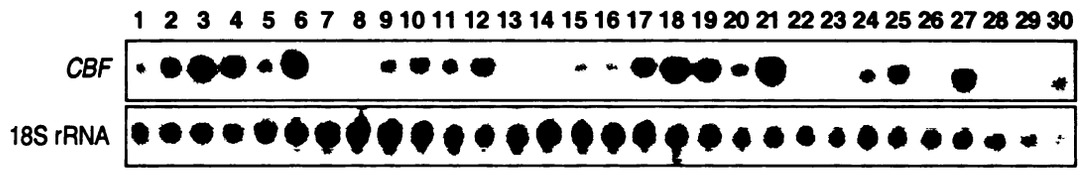
Figure 2.5. Comparison of *CBF* gene expression in Cvi-0 and L*er* in response to sudden change in temperature. Northern blot showing total *CBF* accumulation in continuous-light grown seedlings after 2h at indicated temperatures, using a common probe for *CBF1*, *2*, and *3* detection. 18S rRNA levels in the samples are shown below.

continuous light allowed identification of eQTLs associated with variation in low temperature associated transcription of the *CBF* genes, eliminating eQTLs that may be associated with photoperiod mediated regulation of these genes. Additionally, continuous light conditions simplified the experimental design for simultaneous comparison of large number of lines. The RIL population comprises 162 lines inbred to F_8 generation that have been comprehensively characterized for their genotypes using AFLP and CAPs markers distributed across all five chromosomes (Alonso-Blanco et al., 1998). The results of Northern analysis indicated considerable variation in *CBF* transcript accumulation among the RILs (Figure 2.6). Furthermore, many RILs expressed the *CBF* transcript to significantly higher levels than L*er*, demonstrating transgressive segregation of this molecular trait. This suggested allelic variation in genes involved in low temperature-regulation of *CBF* in the parents.

2.2.3 eQTL Mapping for Regulators of Low Temperature Responsive *CBF* Expression

As mentioned previously (see Section 1.8), accuracy of trait measurement significantly impacts the power and resolution of QTL detection thereby necessitating minimal experimental variation in *CBF* transcript quantification. The high level of homozygosity of the RIL population used in this study allowed biological replicates for trait measurement, which provided greater statistical reliability. Initially, Northern analysis was used for transcript quantification; however, significant experimental variability was observed

a.



b.

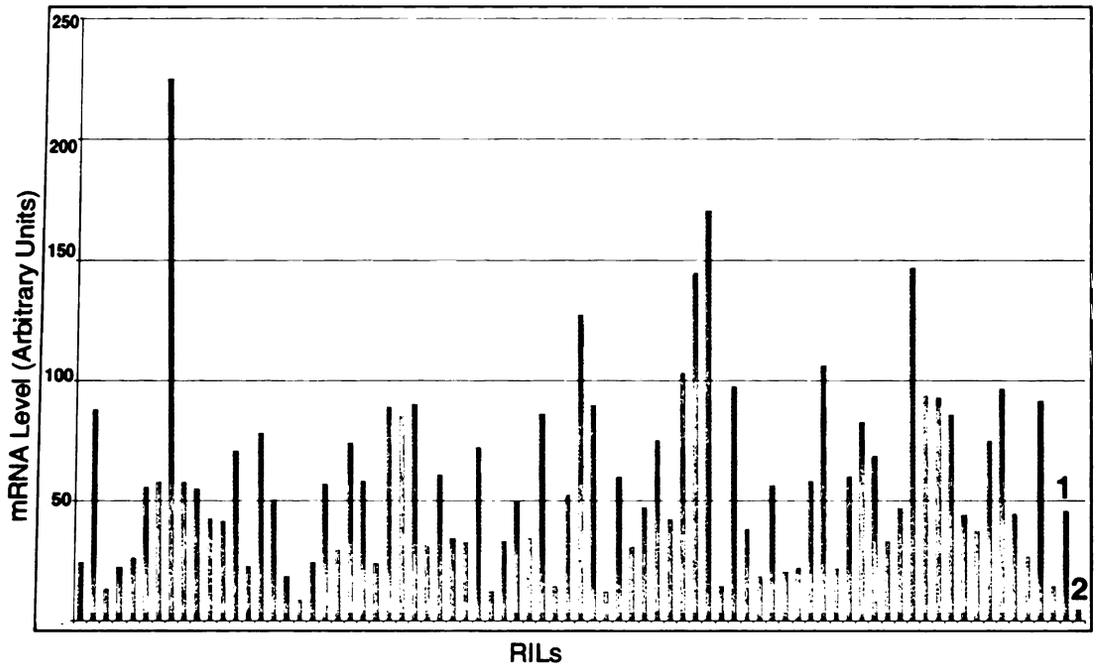


Figure 2.6. a) **Expression of *CBF* genes in Cvi-0 and L*er* following sudden change in ambient temperature.** Northern blot showing *CBF* mRNA levels in continuous-light grown seedlings incubated for 2h at 4°C, using a common probe for detection of *CBF1*, *2*, and *3* transcripts. 18S rRNA levels in the samples are shown below.

b) **Histogram showing *CBF* transcript levels in RILs.** *CBF* levels normalized with 18S rRNA levels in 77 randomly selected RILs and the parental lines Cvi-0 and L*er*. The y-axis depicts transcript level in arbitrary units and the x-axis shows different RILs. *CBF* transcript levels in L*er* and Cvi-0 are numbered 1 and 2 respectively.

when analyzing larger sample sets by this approach, which was not suitable for further analysis. Hence, to improve the accuracy of trait measurement, Taqman® real-time RT-PCR (Applied Biosystems Inc., ABI) was chosen for transcript quantification. This method was optimized for quantification of *CBF* and 18S rRNA transcripts (see Chapter 4, section 4.2). The 18S rRNA gene was selected as the internal reference to normalize expression data for *CBF* genes. Two-step Taqman® real-time RT-PCR was performed for estimation of transcript levels in 155 RILs exposed to 4°C for 2h in two independent experiments. Transcript levels for *CBF 1*, *2*, *3*, and 18S rRNA genes were derived from the C_t values determined from real-time amplification plots of the samples (Figure 2.7). To achieve accuracy and reproducibility of transcript measures, C_t values were determined at the exponential stage of amplification. The amount of transcript present in each sample was estimated as $2^{-\Delta Ct}$ values and outliers were removed before further analysis (see section 2.4 Material and Methods).

Average expression levels from the two biological replicates were used for Composite Interval Mapping (CIM) to identify eQTLs for *CBF1, 2,* or *3* expression at low temperature. Data analysis was performed using the QTL Cartographer program available at www.statgen.ncsu.edu/qtlcart/. The critical threshold level for identifying the eQTLs was determined to be at a LOD score of 2.5 (see section 2.4 Materials and Methods). Mapping of phenotype data obtained using real-time RT-PCR led to identification of eQTLs for *CBF1*, *2* and *3* expression (Figures 2.8, 2.9, and 2.10). The eQTLs were named on the basis of their chromosomal location, for instance eQ C1-3 denotes the third eQTL present on chromosome 1 (Table 2.1).

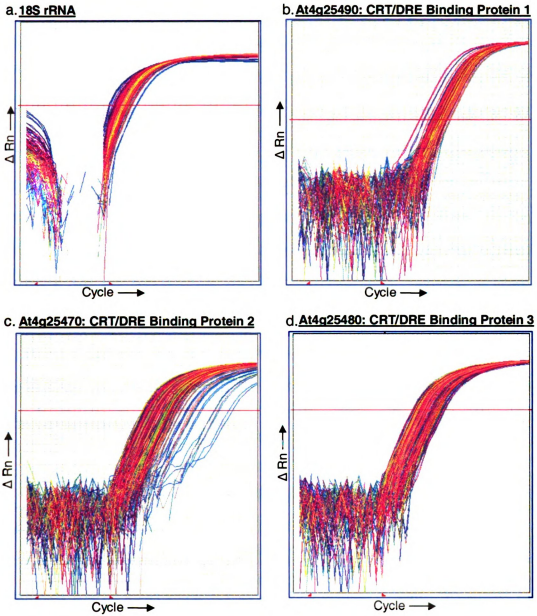


Figure 2.7. **Taqman real-time RT-PCR amplification profiles for** a. **18S rRNA**, b. ***CBF1***, c. ***CBF2***, and d. ***CBF3*** transcripts. The y-axis represents fluorescence emission and the x-axis represents cycle numbers. Each amplification curve represents a single well, and every RIL is measured in triplicate wells. The horizontal red line depicts the threshold at which the C_t value for each amplification curve was determined.

2.2.3.1 Identification of a Common Large-effect eQTL Regulating *CBF2* and *3* Expression at Low Temperature

This analysis identified large-effect eQTLs for differential expression of *CBF2* and *3* genes co-localizing on chromosome 4, which were supported by LOD scores of 19.5 and 17.8, respectively (Figures 2.8 and 2.9). The positive allele leading to higher *CBF2* and *3* accumulation was contributed by L*er* for both genes. Additionally, this eQTL, named eQ C4, was coincident with the location of *CBF* genes in the genome. The overlap with the genomic location of *CBF*s suggested that this might be a *cis*-acting eQTL. However, it might also be a *trans*-acting eQTL, located in physical proximity to the *CBF* genes, overlying a common regulator of *CBF2* and *3* transcription. In contrast, low temperature responsive expression of *CBF1* gene did not identify a regulatory eQTL at this location (Figure 2.10).

2.2.3.2 Identification of Common Small-effect eQTLs for Expression of *CBF1, 2* and *3* Genes

The low temperature responsive transcription of *CBF1*, *2,* and *3* genes identified shared small-effect eQTLs on chromosome 5, with LOD scores of 2.7, 4.4, and 5.5, respectively (Figures 2.10, 2.8 and 2.9). An additional set of eQTLs on this chromosome was identified in common for *CBF2* and *3* expression (3.9 and 2.7 LODs, respectively), which were contributed by L*er* for both genes (Figures 2.8 and 2.9). *CBF1* expression did not reveal an eQTL at this location through this analysis.

Figure 2.8. **eQTLs for *CBF2* expression.** CIM analysis of 18S rRNA normalized *CB21* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

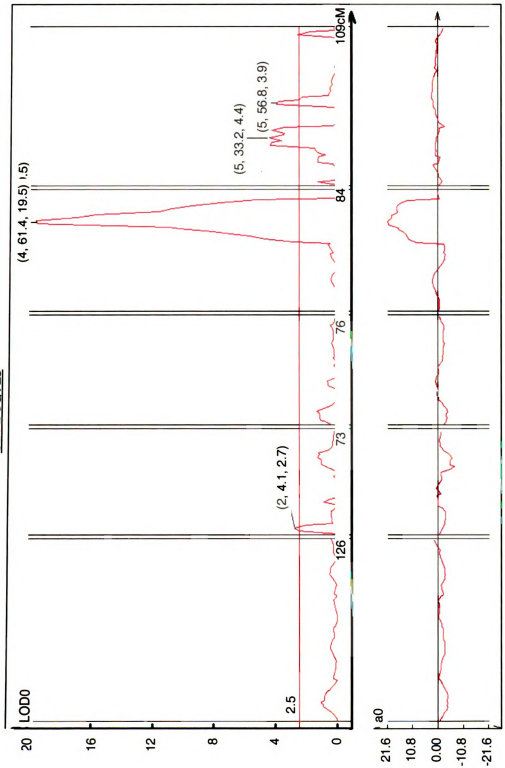


Figure 2.8. **eQTLs for *CBF2* expression.**

Figure 2.9. **eQTLs for *CBF3* expression.** CIM analysis of 18S rRNA normalized *CBF1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

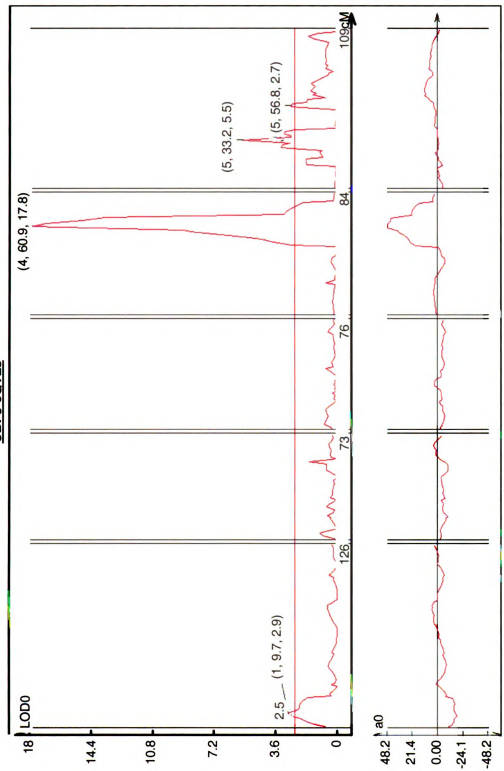


Figure 2.9. **eQTLs for *CBF3* expression.**

Figure 2.10. **eQTLs for *CBF1* expression.** CIM analysis of 18S rRNA normalized *CBF1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

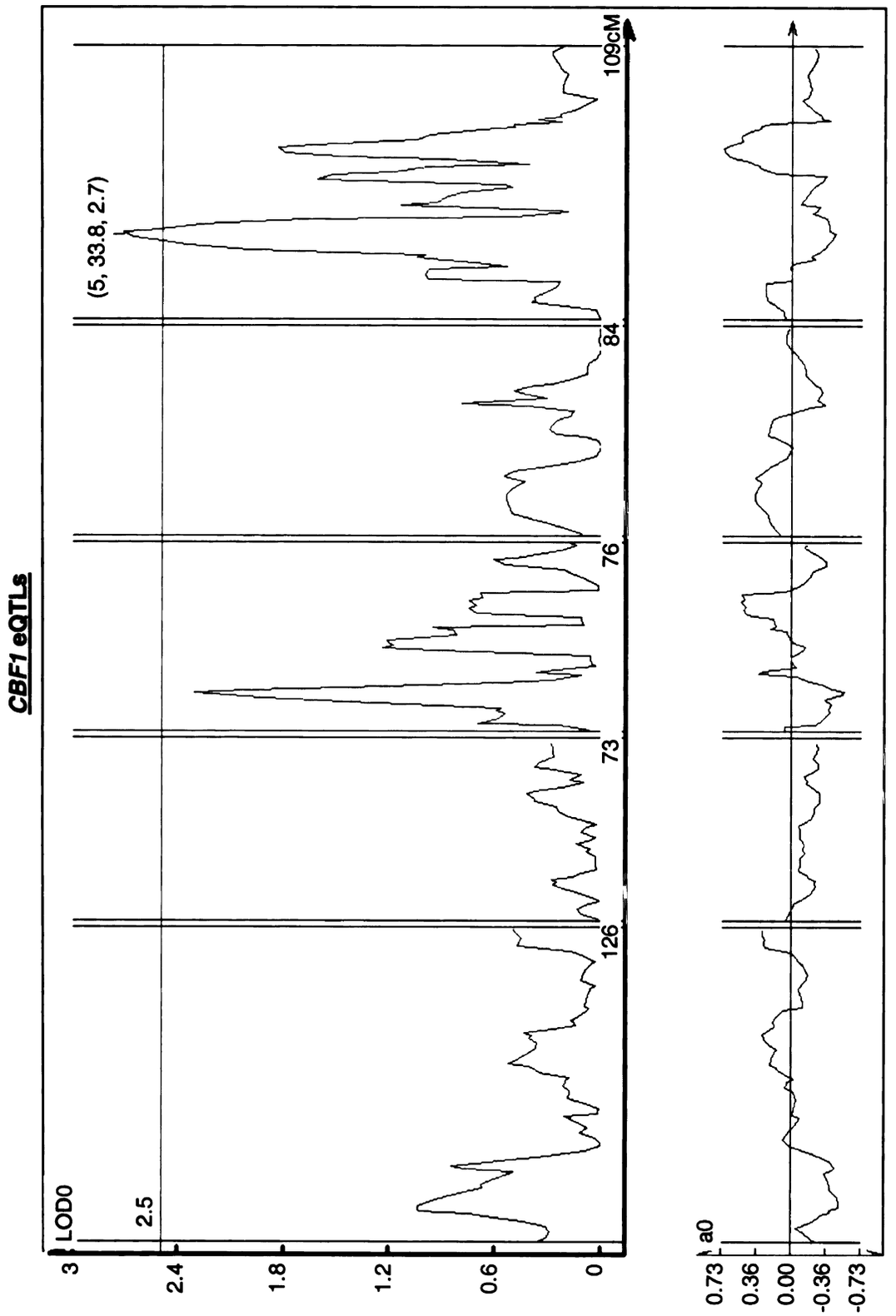


Figure 2.10. **eQTLs for *CBF1* expression.**

In addition to being regulated by eQTLs common to *CBF1* and *3* expression, the low temperature-induced expression of *CBF2* gene was found to be regulated by a unique small-effect eQTL on chromosome 2 (Figure 2.8). This eQTL was identified with a LOD score of 2.7 and the positive allele at this location was contributed by Cvi-0 (Figure 2.8).

Therefore, these analyses led to the identification of regulatory loci for *CBF1*, *2*, and *3* genes, revealing similarities and differences in their regulation based on the assumption that eQTLs overlapping in chromosomal location result from the same underlying gene (Table 2.1). Although *CBF1* expression was found to be regulated by *trans*-acting eQTLs, *CBF2* and *3* expression revealed a potential c*is*-acting eQTL in addition to the *trans*-acting eQTLs identified. Additionally, both parents, L*er* and Cvi-0, contributed alleles leading to higher accumulation of transcripts.

2.2.3.3 Uncovering Additional Small-effect eQTLs for *CBF* expression

Co-localization of the large effect eQTL on chromosome 4 with the *CBF* genes and its very strong statistical support suggested it to be a *cis*-acting eQTL. If this were true, eliminating sequence variation at the *CBF* locus by holding it constant in the population as L*er* or Cvi-0 would eliminate this eQTL. Moreover, this approach could potentially uncover additional small-effect eQTLs that were being statistically masked by this eQTL. To determine if this was the case, the RIL population was divided into two groups, one comprised of lines containing the *CBF* locus from Cvi-0 and the other from L*er* (see Materials and Methods for details).

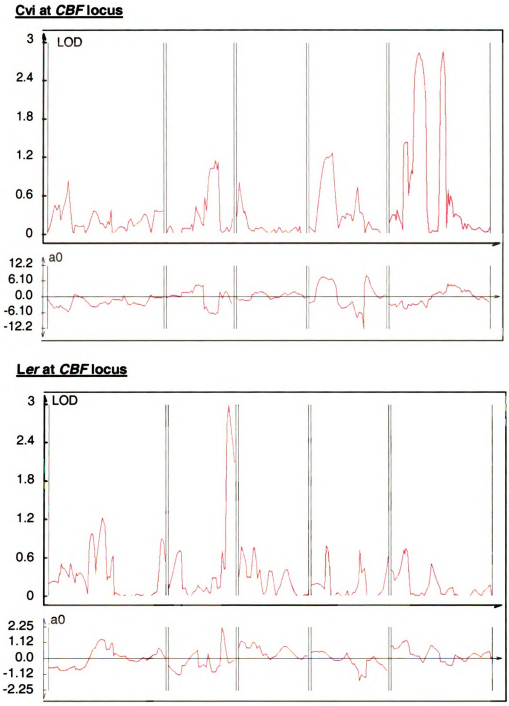


Figure 2.11. **eQTLs for *CBF2* expression after holding *CBF* locus constant.** CIM analysis of *CBF2* transcript levels in RILs containing the *CBF* locus from either Cvi-0 or L*er* as indicated.

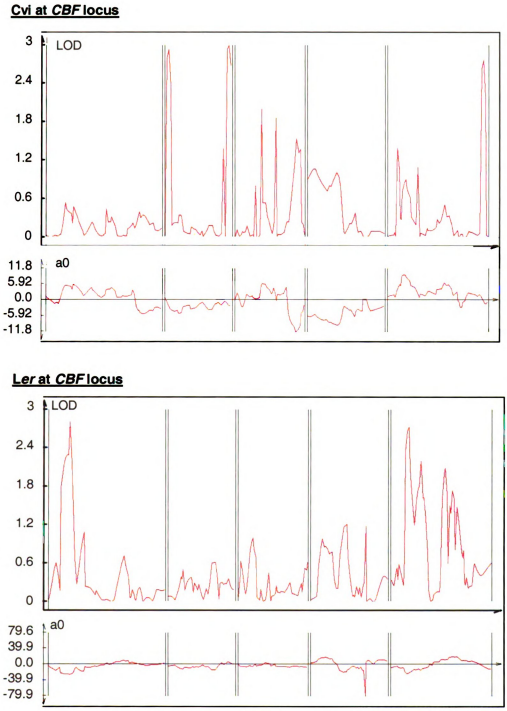


Figure 2.12. **eQTLs for *CBF3* expression after holding *CBF* locus constant.** CIM analysis of *CBF3* transcript levels in RILs containing the *CBF* locus from either Cvi-0 or L*er* as indicated.

The two subpopulations of RILs thus created were analyzed by CIM. The division of the RIL population into smaller subgroups increased the critical LOD score required to determine eQTLs. As would be expected, elimination of sequence variation around *CBF* locus led to disappearance of the eQTL on chromosome 4 from both subpopulations for *CBF2* and *3* expression (Figures 2.11 and 2.12). The graphs do not show a threshold line because it is higher than the maximum LOD value represented on the y-axis. The subdivision of the population resulted in identification of an additional *trans*-acting eQTL for the regulation of *CBF1* expression (Figure 2.13). This eQTL was located on on chromosome 3 and was supported by a 7.3 LOD (Figure 2.13). Cvi-0 contributed the positive allele for this eQTL. Furthermore, none of the small-effect eQTLs identified for the *CBF* genes using the entire population were detected using the subpopulations generated in this analysis (Figures 2.11, 2.12, and 2.13). Division of the RIL population into subpopulations for elimination of sequence variation at the *CBF* locus may have inadvertently abolished variation at additional genomic regions. This would occur as a result of the smaller number of RILs in each subpopulation, which would lead to reduction in overall genomic variation represented in each subpopulation. If these additional regions overlapped with the genomic locations of the small-effect eQTLs identified using the entire population, they would no longer be detected in the analysis of the subpopulations.

2.2.3.4 Use of Additional Normalization Control for Expression Analysis

The choice of internal reference gene to normalize loading differences between samples determines the accuracy of transcript quantification using

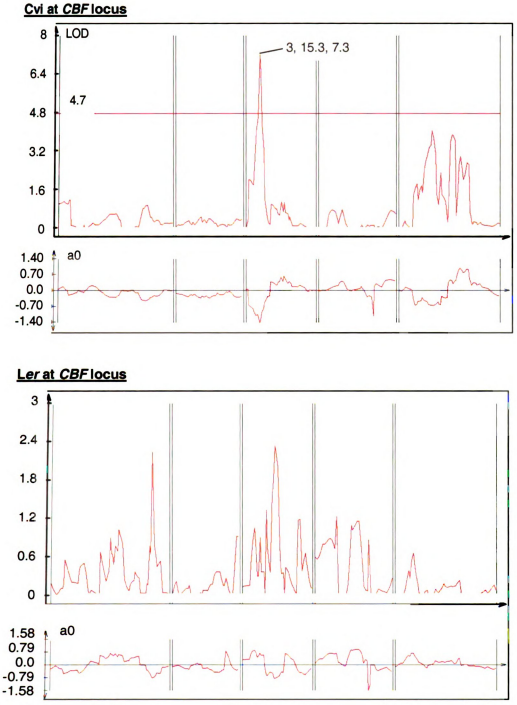


Figure 2.13. **eQTLs for *CBF1* expression after holding *CBF* locus constant.** CIM analysis of *CBF1* transcript levels in RILs containing the *CBF* locus from either Cvi-0 or L*er* as indicated.

real time RT-PCR. There is no single reference gene considered to be a universal standard for all systems. Thus, it is necessary to determine the gene that would serve as an ideal internal reference for the system being studied (De Boever et al., 2008; Lyng et al., 2008; Remans et al., 2008). The choice of reference genes is based on two main factors, the relative abundance of the reference gene and its unaltered expression under the experimental conditions being tested (De Ketelaere et al., 2006; Maccoux et al., 2007; Skovgaard et al., 2007; Hoogewijs et al., 2008). Although a reliable reference for normalization, 18S rRNA is not ideal for quantification of low abundance mRNAs. The relative abundance of this transcript in the RILs was, of course, significantly higher than *CBF* transcripts since cDNA samples had to be diluted 2000-fold to quantify 18S rRNA levels. Studies investigating changes in gene expression have also demonstrated that using different reference genes can uncover additional differences in regulation of transcription (Deutsch et al., 2005).

The actin-encoding gene, *Act1*, was selected for use as a second internal reference as transcript levels of *Act1* are more comparable to *CBF* transcript levels than 18S rRNA and remain unaltered at low temperatures (Figure 2.14). Furthermore, analysis of *Act1* expression in the RIL population did not reveal any eQTLs for *Act1* (Figure 2.15).

2.2.4 Identification of eQTLs Regulating of *CBF1, 2* and *3* Expression using *Act1* as Internal Reference

Using *Act1* normalized transcript levels, eQTLs were identified for *CBF1, 2,* and *3* genes (Figures 2.16, 2.17, and 2.18). The eQTL identified for

CBF

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CBF1

CBF2 and *3* expression overlying *CBF* locus on chromosome 4 using 18S rRNA normalized dataset was reproducibly detected in the *Act1* normalized data (Figures 2.17 and 2.18). L*er* contributed the positive allele for both *CBF2* and *3* and the LOD scores of the peaks were 7.8 and 6.8, respectively. Further in agreement with the 18S rRNA normalized dataset, this eQTL on chromosome 4 was not found to regulate *CBF1* expression (Figure 2.16). However, this analysis was not able to identify the small-effect eQTLs for *CBF1*, *2*, and *3* expression identified on chromosome 5 using 18S rRNA normalization.

Additional information was obtained for regulation of *CBF1* and *2* genes by using *Act1* as the internal reference. A common pattern of two adjoining eQTLs at overlapping genomic locations was identified for both *CBF1* and *2* genes on chromosome 1 (Figures 2.16 and 2.17).

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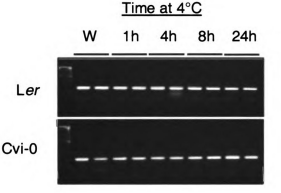

Figure 2.14. **Actin expression in response to low temperature.** RT-PCR showing Actin transcript levels in L*er* and Cvi-0 seedlings grown in warm (W) and treated for 1h, 4h, 8h, or 24h at 4°C.

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Figure 2.15. **eQTLs for *Act1* expression.** CIM analysis of 18S rRNA normalized *Act1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

Actin eQTLs

Figure

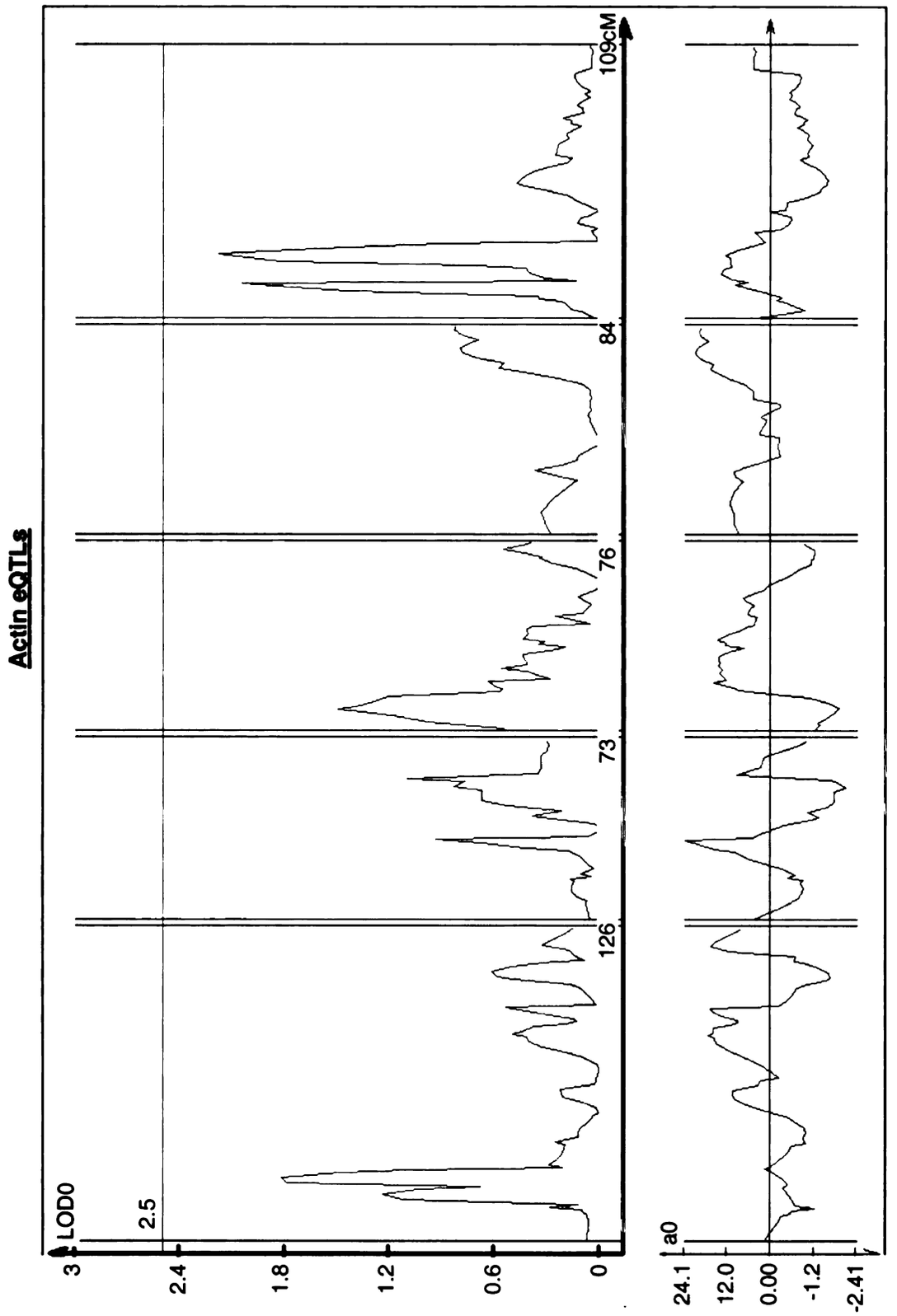

Figure 2.15. **eQTLs for *Act1* expression.**

Figure 2.16.

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Figure 2.16. **eQTLs for *CBF1* expression.** CIM analysis of *Act1* normalized *CBF1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

CBF1 eQTLs

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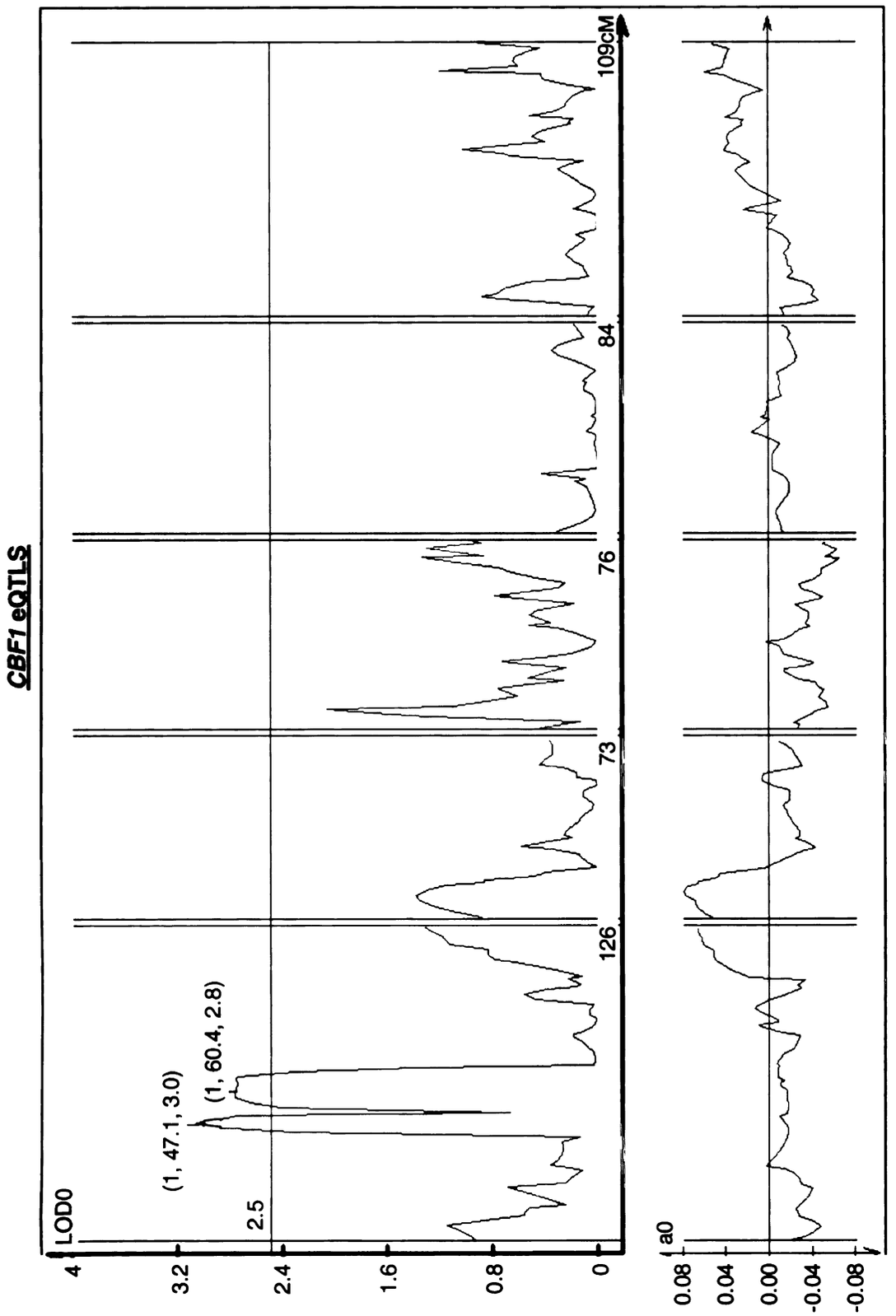

Figure 2.16. **eQTLs for *CBF1* expression.**

Figure 2.17

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Figure 2.17. **eQTLs for *CBF2* expression.** CIM analysis of *Act1* normalized *CBF2* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

CBF2 eQTLs

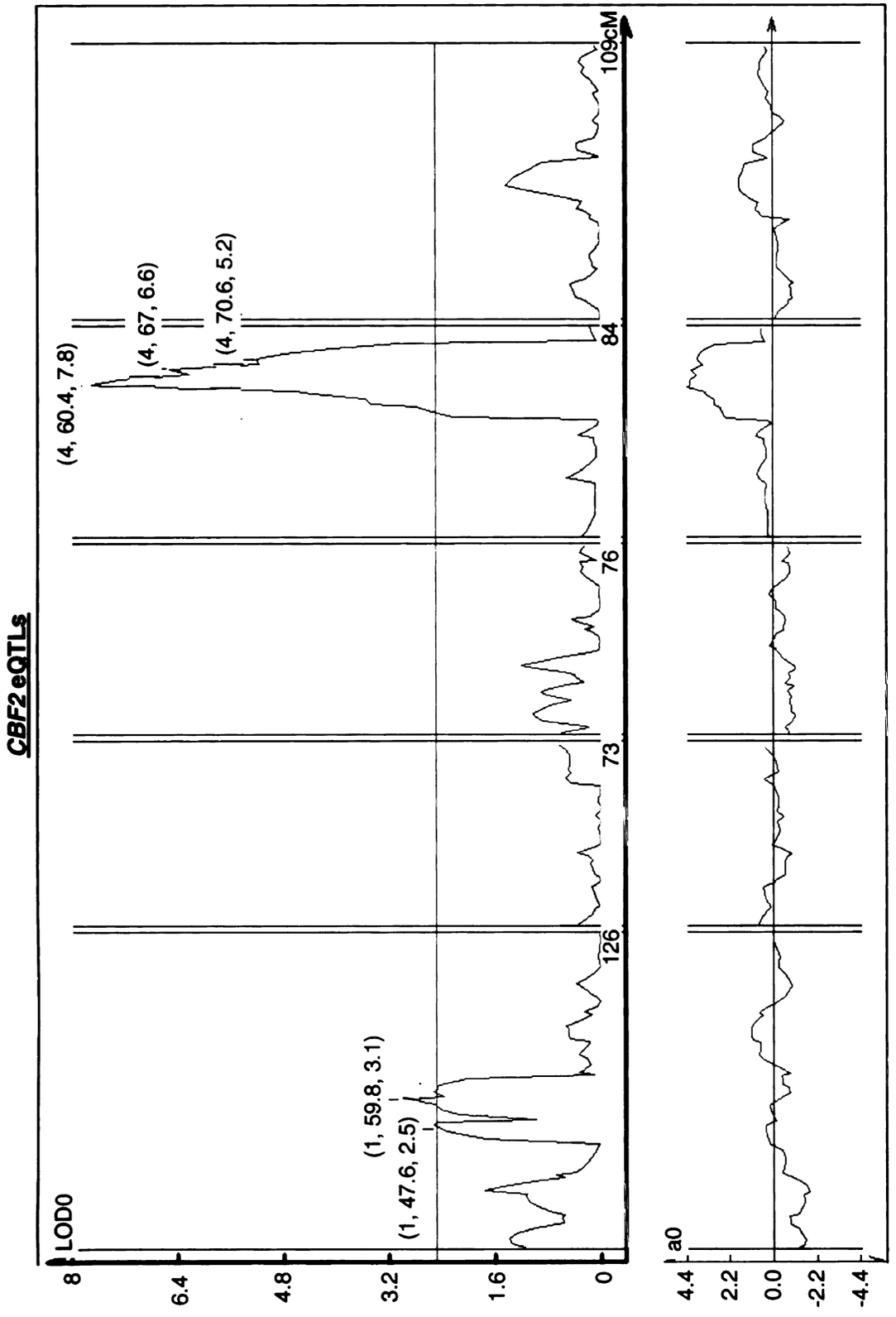

Figure 2.17. **eQTLs for *CBF2* expression.**

Figure 2.18

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Figure 2.18. **eQTLs for *CBF3* expression.** CIM analysis of *Act1* normalized *CBF3* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

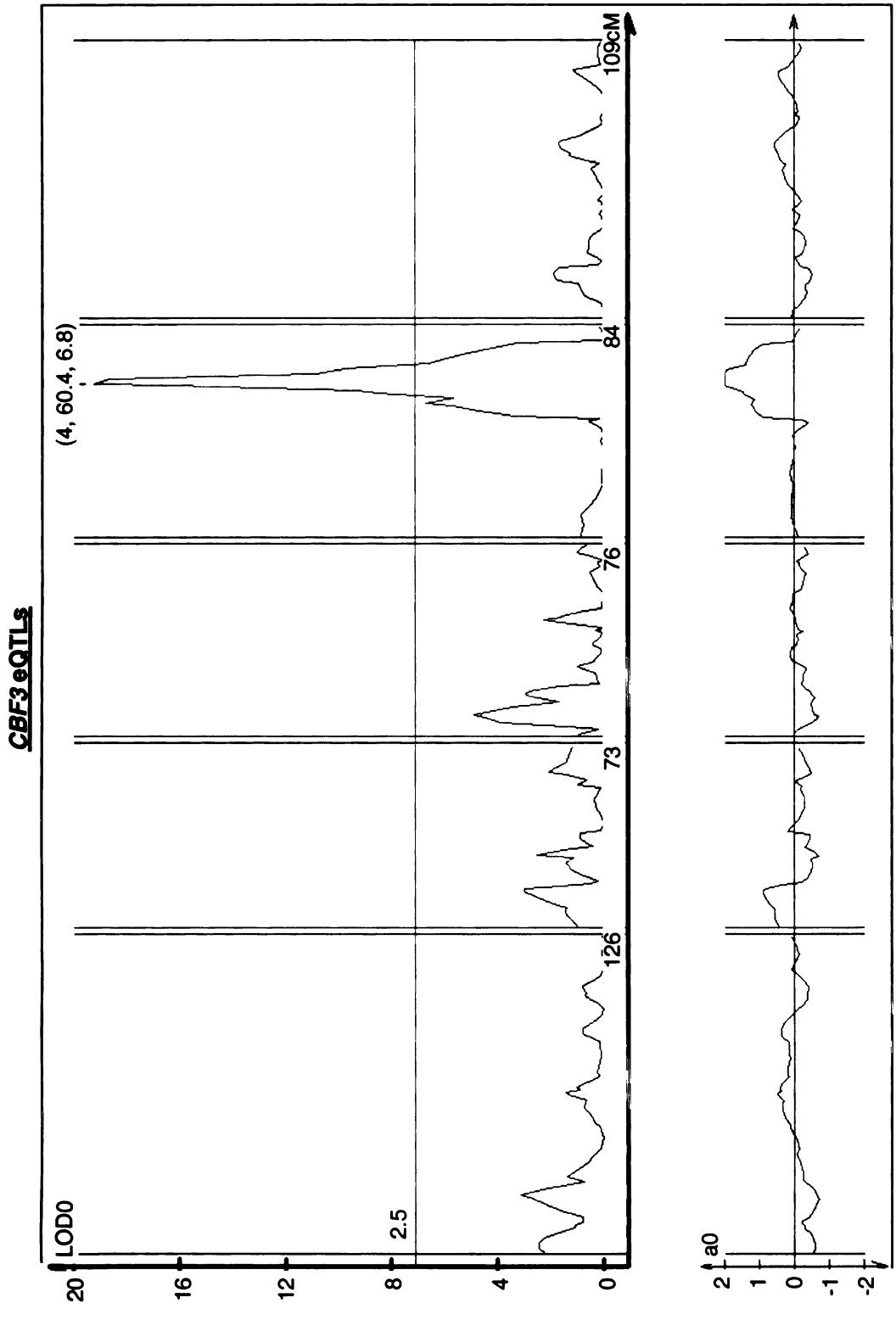

Figure 2.18. **eQTLs for *CBF3* expression.**

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2.3 Discussion

Comparison of different accessions of Arabidopsis *thaliana* indicated natural variation in low temperature responsive *CBF* expression among accessions collected from different environments (Figures 2.3, 2.4, and 2.5). Evaluation of an RIL population derived from a cross between Cvi-0 and L*er* (Alonso-Blanco et al., 1998), accessions from warm and cold environments, respectively, revealed transgressive segregation of *CBF* transcript accumulation (Figure 2.7). This suggested presence of allelic variation(s) in the parent accessions that were independently segregating to result in significantly higher levels of *CBF* transcripts observed in some RILs.

In past two years several studies have demonstrated that quantitative variation in gene expression is modulated by heritable factors that can be identified as eQTLs (Bing and Hoeschele, 2005; Carlborg et al., 2005; Kliebenstein et al., 2006). QTL mapping performed using *CBF1*, *2*, or *3* expression as the molecular phenotype in the L*er*/Cvi RIL population led to identification of eQTLs regulating low temperature responsive expression of these three genes.

2.3.1 Identification of a Major-effect eQTL Common for Regulation of *CBF2* and *3* Expression

A major-effect eQTL eQ C4, with the highest statistical support (upto LOD score ~20), was detected on chromosome 4 for *CBF2* and *3* expression (Figures 2.8, 2.9, 2.16, and 2.17). This eQTL overlapped the chromosomal location of *CBF* genes. The L*er* allele at this eQTL was found to contribute to

Gene	eQTL	
	18S rRNA	*Act1*
***CBF1* (At4g25490)**	**eQ C5-1**	**eQ C1-3** **eQ C1-4**
***CBF2* (At4g25470)**	**eQ C2-1** **eQ C4** **eQ C5-1** **eQ C5-2**	**eQ C1-3** **eQ C1-4** **eQ C4**
***CBF2* (At4g25480)**	**eQ C1-1** **eQ C4** **eQ C5-1** **eQ C5-2**	**eQ C4**

Table 2.1. List of eQTLs identified using 18S rRNA and *Act1* normalized gene expression data. The nomenclature is based on the location of the eQTL.

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higher expression of both *CBF2* and *3* genes. Using the same RIL population, Alonso-Blanco et al reported a freezing tolerance QTL at this location, which was suggested to arise from variation in the *CBF2* gene (Alonso-Blanco et al., 2005). Sequence comparison of this locus between Cvi-0 and L*er* demonstrated variations at the *CBF* locus, including a 1.6kb deletion of the *CBF*2 promoter in Cvi-0. Transformation of Cvi-0 with *CBF2* gene from L*er* resulted in higher levels of *CBF2* transcript and restoration of the freezing tolerance of transgenics to levels comparable to L*er*. It was reasonable to consider the possibility that the eQTL observed for *CBF2* expression on chromosome 4 was a *cis*-acting eQTL arising from the sequence variation existing in the promoter.

In several systems *cis*-acting eQTLs, which arise due to allelic variation in the gene itself, were consistently identified with higher linkage statistics than *trans*-acting eQTLs, which arise due to allelic differences in regulatory factors involved in the expression of the gene (Bystrykh et al., 2005). In mice, analysis of eQTLs contributing to variation in several thousand genes revealed about one-third of the eQTLs lying within 10 cM of the gene being used as the trait and were classified as *cis*-acting eQTLs (Doss et al., 2005). Further analysis was able to show that, out of these *cis*-acting eQTLs 64% were true positives (Doss et al., 2005). The remaining 36% genes that could not be confirmed as truly *cis*-acting may arise due to *trans*-regulatory factors located in physical proximity of the gene. Subsequent studies have addressed eQTLs lying within 5cM of the gene as local acting eQTLs, which could be *cis*- or *trans*-acting (West et al., 2007).

The eQTL identified for *CBF2* expression on chromosome 4 was supported by a high LOD score (Figures 2.8 and 2.15), which is regarded a characteristic of *cis*-eQTLs. An eQTL at the same location, with similar high LOD score, was also identified for *CBF3* expression (Figures 2.9 and 2.16). However, analysis of *CBF1* expression did not reveal an eQTL at this position (Figures 2.10 and 2.14). The *CBF* genes are located adjacent to each other on chromosome 4 in the order *CBF1*, *3* and *2*. These transcriptional activators display high sequence conservation (*CBF1* and *2*, 86% identity; *CBF1* and *3*, 86% identity; *CBF2* and *3*, 87% identity) (Liu et al., 1998) and a significant overlap in their target genes, suggesting functional redundancy (Gilmour et al., 2004).

When comparing the sequence of the *CBF* locus from Cvi-0 and L*er*, the *CBF1* promoter has been found to exhibit highest conservation with only seven SNPs in 1kb region upstream of the transcriptional start site, whereas *CBF2* and *3* promoters were found to be more divergent with a 1.6 kb deletion and 23 SNPs, respectively (Alonso-Blanco et al., 2005). A study in mice comparing the frequency and density of SNPs in the promoters of genes identifying cis-eQTLs and those not regulated by cis-eQTLs (total of 4752 genes) found that the cis-eQTL genes have a significantly higher frequency of cis-SNPs compared to non-cis-eQTL genes. Furthermore, these SNPs are present in the phylogenetically non-conserved regions. These SNPs were also found in regions predicted to affect predicted transcription factor binding sites, a likely consequence of the higher cis-SNPs density in these genes (GuhaThakurta et al., 2006). In studies using microarrays, sequence variation in coding regions can manifest falsely as *cis*-acting eQTLs since the probe is

usually based on one parent and has a considerable influence on the hybridization efficiency with the other (Alberts et al., 2007). However, this possibility can be ruled out in the present analysis as the coding regions of *CBF1-3* genes were determined and primers were designed based on conserved sequence.

It has also been reported that Cvi-0 shows reduced *CBF2* transcript accumulation and increased levels of *CBF1* and *3* transcripts (Alonso-Blanco et al., 2005). Furthermore, *CBF2* has been suggested to have a negative regulatory effect on *CBF3* (Alonso-Blanco et al., 2005). In a separate study, analyzing publicly available microarray data for low temperature response of wild type *Arabidopisis* and cold tolerance mutants, suggested *CBF2* and *3* to negatively regulate each other (Benedict et al., 2006).

The eQTL on chromosome 4 identified for *CBF2* could be attributed to the higher sequence divergence of its promoter, rendering it less active in Cvi-0. Therefore, supporting the possibility of this being a *cis*-acting eQTL for *CBF2* expression. If *CBF2* exerts a negative regulatory effect on *CBF3* expression, the eQTL identified for *CBF3* on chromosome 4 could arise due to altered levels of *CBF2* transcript, therefore making it a local-acting eQTL. *CBF2* has also been suggested to have a negative regulatory effect on *CBF1* expression; however, an eQTL overlying *CBF2* was not identified for *CBF1* expression. This suggests involvement of distinct regulatory mechanisms for low temperature responsive expression of the different members of *CBF* gene family. Analysis of RIL subpopulations with *CBF* locus held constant, as either L*er* or Cvi-0, resulted in disappearance of the eQTL on chromosome 4, thus suggesting it to be a local-acting eQTL.

2.3.2 Identification of Additional eQTLs Using Actin mRNA for Normalization

As discussed in Section 2.2.3.3, the selection of internal reference genes for normalization of expression depends on the system under study. Although 18S rRNA is routinely used as a reference, it is not ideal for normalization of transcripts that are much lower in abundance, such as the *CBF* mRNAs. It was therefore important to use another reference gene for this analysis and the *Act1* gene was selected as a second normalization control. The validity of *Act1* for use as the second reference gene was established in two ways. Firstly, it was demonstrated that the *Act1* gene did not show differential expression at low temperature (Figure 2.14). Secondly, eQTL analysis was performed to ascertain that this gene did not show heritable variation in expression in the RIL population (Figure 2.15).

Consistent with the 18S rRNA normalization, *CBF2* and *3* expression at low temperature identified common eQTLs on chromosome 4 overlapping with the genomic location of the *CBF* locus (Figures 2.8, 2.9, 2.17, and 2.18). Furthermore, *CBF1* expression analysis did not reveal any eQTL at chromosome 4 (Figure 2.16). Moreover, use of *Act1* as the internal reference to quantify *CBF* transcript levels in the segregating population further expanded the search for regulatory loci controlling *CBF1* and *2* expression. Two adjoining *trans*-acting eQTLs at overlapping genomic locations on chromosome 1 were identified for both *CBF1* and *2* genes (Figures 2.16 and 2.17).

Thus, natural genetic variation found in *CBF* expression at low temperatures was successfully used to identify regions in the Arabidopsis

genome (eQTLs), characterization of which will lead to identification of genes whose products are involved in regulating low temperature responsive transcription. The independent detection of freezing tolerance QTLs in chromosomal regions that were found to contain eQTLs for *CBF* expression substantiates the evidence for a regulatory role of these eQTLs during cold acclimation (Alonso-Blanco et al., 2005). In addition to the eQTL on chromosome 4, other freezing tolerance QTLs were found to overlap with eQTLs identified for regulation of *CBF* expression in the present study. Overlap was observed between a short-day photoperiod specific freezing tolerance QTL and eQTLs for *CBF1, 2,* and *3* expression found through this analysis. The finding that none of these eQTLs were detected for regulation of a gene non-responsive to low temperature, *Act1,* provides further support for the association of these eQTLs with cold acclimation.

Future fine mapping studies in the future would lead to identification of the sequence variations underlying these eQTLs and thus identify the genes responsible for *CBF* transcript regulation at low temperatures. Regulatory factors with pleiotropic effects on the low temperature response of plants may be uncovered in these regions. These may include potential thermosensor(s), signal transduction components, transcriptional activators or repressors, regulators of transcript stability, etc.

2.3.3. Genes Located in Regions Showing eQTLs

Genes previously reported to show co-regulation with cold tolerance (identified through mutational analysis) and genes analyzed in this study were mapped on the chromosomes to compare their location with the eQTLs

observed in the current study (Figure 2.19). The *ICE1* gene, located in the upper arm of chromosome 1, was found to be coincident with an eQTL identified for regulation of *CBF3* expression. This eQTL however, was not identified for *CBF1* or *2* expression, which is in agreement with the finding that *ice1* mutants are altered in expression of *CBF3*, but not in *CBF1* and *2* expression (Chinnusamy et al., 2003). This suggests that this eQTL regulating *CBF3* may result from variation in the *ICE1* gene.

Although the *HOS9* gene, shown to have an effect on freezing tolerance in Arabidopsis, is co-localized with the eQTL for *CBF2* expression on chromosome 2, it can be ruled out as a candidate since *hos9* mutants do not display altered *CBF* gene expression (Zhu et al., 2004).

There were no other genes, previously identified to play a role in low temperature stress tolerance, found to overlap in chromosomal location with the eQTLs identified in this study. Thus, the eQTLs will lead to novel genes regulating the low temperature responsive expression of *CBF1-3* genes.

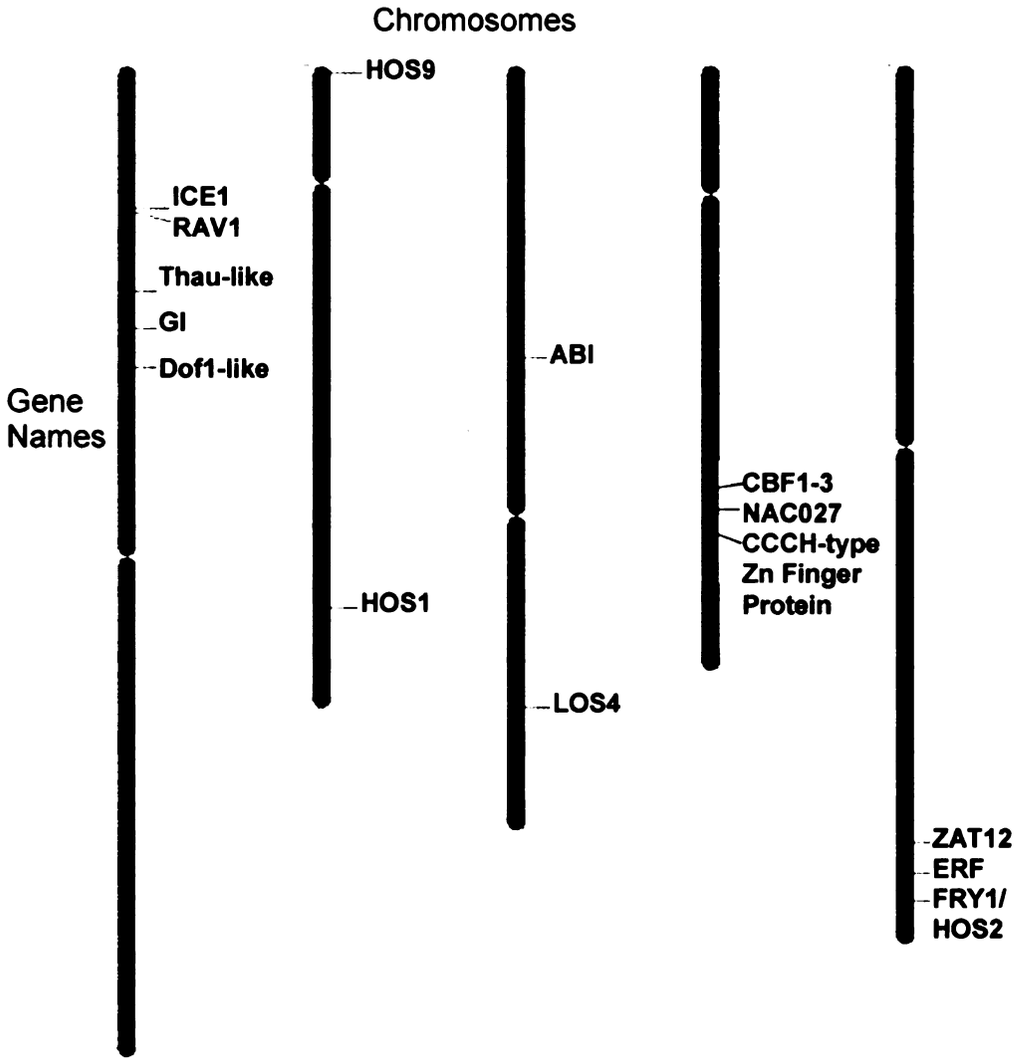

Figure 2.19. **Chromosomal locations of genes known to be linked with cold tolerance, identified either through mutational studies or transcriptional profiling.**

2.4 Material and Methods

2.4.1 Plant Growth and Treatments

Seeds were surface sterilized by incubating with 1 ml of 50% bleach + 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO) solution with shaking for 7 mins followed by four washes with sterile distilled water. They were re-suspended in sterile 0.1% agar and cold hardened at 4˚C for 3-4 days following which they were spread (density of approx 120-150 seeds/100 x 20 mm Petri plate) on solid Gamborg's B5 (Caisson Laboratories, Logan, UT) or MS nutrient medium (Sigma-Aldrich, St Louis, MO) with 0.8% (w/v) phyta agar (Invitrogen, Carlsbad, CA). Seeds on nutrient media were grown in controlled environment chambers (Conviron, Winnipeg, Canada) maintained at 22˚C illuminated with cool-white fluorescent lights at the intensity of approx 100-150 µmol m^{-2} s^{-1}. For the gradual temperature march down experiments, 10 to 12-day-old seedlings grown in continuous light were moved to controlled environment chambers maintained at 21˚C with cool-white fluorescent light. The temperature of the chamber was decreased by 2˚C, at a rate of 0.5˚C/15mins, after every 4h. Tissue was harvested in tubes maintained on a liquid nitrogen bath after 1h and 4h at 17°C, 15°C, 13°C, 11°C, 9°C, 7°C, 5°C, 3°C, and 1°C and was immediately transferred to -80˚C. For the cold treatments involving drop to specific temperatures, 100-150 seedlings of ecotype were grown as described above. At 10-12 days of age, the seedlings were moved to controlled environment chambers maintained at either 10°C, 8°C, 6°C, 4°C, 2°C or 0°C with cool-white fluorescent lights. After 2h incubation at the given temperature, tissue was harvested as described

above. For the analysis of kinetics of gene expression, time course treatment was performed at 4°C. Seedlings grown as described above were moved to controlled environment chambers maintained at 4°C, with cool-white fluorescent lights. After selected time intervals, a plate of seedlings for each ecotype was removed from the cold chamber and tissue was harvested in liquid nitrogen.

2.4.2 RNA Isolation

Total RNA was extracted from tissue using Qiagen RNeasy Mini RNA extraction kits (Qiagen Inc., Valencia, CA) by following a modified protocol of the manufacturer's instructions. Briefly, tissue was ground to a fine powder using liquid nitrogen and 900 μL to 1 mL of RLT extraction buffer (+10 μL β-mercaptoethanol/mL buffer) was added to it. 700 μL of this sample suspended in the RLT buffer was pipetted into the Qiashredder columns provided in the kit and centrifuged at 14,000 rpm for 2 minutes. The clear supernatant from the flow through was transferred to fresh tubes and 1/2 volume of 100% ethanol was added to the sample. Following a thorough mixing of ethanol with the sample, 700 μL was transferred to the Qiagen spin columns and centrifuged for 15-20 sec. The flow-through was discarded and the column was washed with 700 μL RW1 buffer by centrifuging for 15-20 sec. This was followed by two washes with 500 μL of RPE buffer. The RNA was finally eluted in 60μLs of RNase free H_2O.

2.4.3 Northern Blot Analysis

10 µg of total RNA was fractionated on a denaturing formaldehyde agarose gel and transferred to a positively charged nylon membrane (Hybond H^+, Amersham Pharmacia Biotech, CA) using the downward transfer method. Blots were prehybridized for 2 h with prehybridization buffer (5X SSC buffer, 10X Denhardt's reagent, 0.1% SDS (w/v), 0. 1M KPO_4 pH 6.8, 100 µg/ml denatured herring sperm DNA) and hybridized for 16 h with radioactive probe in hybridization buffer (5X SSC buffer, 10X Denhardt's reagent, 0.1M KPO_4 pH 6.8, 100 µg/ml denatured herring sperm DNA, 10% dextran sulfate (w/v), 30% (v/v) deionized formamide). 30-50 ngs of probe DNA was labeled with $dCT^{32}P$ using the Random Priming Kit (Invitrogen Inc., Carlsbad, CA) with manufacturer's instructions. Unincorporated nucleotides were removed by passing the labeling mix through a sephadex G-50 column. Hybridization of membranes with the labeled probe was performed in hybridization chambers maintained at 49°C. Membranes were washed three times with 1XSSC and 0.1% SDS (w/v) solution at 55°C. Phospho-screens were exposed overnight to the radioactively labeled membranes and the signal was developed using the Phospho Imager.

2.4.4 RT-PCR

2 µg of total RNA was reverse transcribed using random hexamers with the Superscript II Reverse Transcriptase Kit (Invitrogen Inc., Carlsbad, CA) following manufacturer's instructions. The cDNA was diluted to a total volume of 200 µls with sterile distilled water. Equilibration was performed using 18S rRNA transcript levels, as this gene does not respond to low temperature.

Primers were designed for the RT-PCR of all genes using the Primer 3 available at http://www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi. For 18S rRNA equilibration, forward (5' GAC GAA CAA CTG CGA AAG CAT 3') and reverse (5'CCC CCA ACT TTC GTT CTT GA3') primers were used to obtain similar amplicon levels for all samples after 18 cycles. 2 µls of the equilibrated cDNA was then used for PCR using Arabidopsis *Act1* forward (5' GGT CGT ACT ACC GGT ATT GTG CT 3') and reverse (5' TGA CAA TTT CAC GCT CTG CT 3') primers using 28 cycles. For PCR, each sample was tested in technical triplicates.

2.4.5 Taqman Real-Time RT-PCR for Quantification of Molecular Phenotype

Gene expression levels for *CBF1*, *2*, and *3* genes were measured in the RILs following exposure of 10-day old seedlings to 4°C for 2h. Expression values were obtained for two independent biological experiments. For each experiment, the RNA used for measurement of transcript levels was obtained from a pool of 70-100 seedlings of individual RIL. After removing contaminating genomic DNA using Turbo DNA free kit from Ambion (ABI, CA) treatment, 10 µg of total RNA was used for total cDNA synthesis (Archive cDNA Synthesis Kit). Random primers were used for cDNA synthesis to allow detection of 18S rRNA, which was used as the normalization control. The cDNA synthesis was performed for RNA samples from both biological replicates using reaction components from a common master mix to perform the reactions in parallel. This helped minimize differences in the efficiency of the cDNA synthesis reaction in different samples. Similarly, components of

the qPCR step were also prepared as a common master mix to quantify samples from both biological replicates in parallel. The table below shows the primer and probe sequences that were designed using the Primer Express® (ABI) program and were subsequently used for quantification of the respective genes.

Gene	Forward Primer Sequence	Probe Sequence	Reverse Primer Sequence
18S rRNA (At2g16590)	5' GAC GAA CAA CTG CGA AAG CAT 3'	5' FAM-TGC CAA GGA TGT TTT C 3'	5' CCC CCA ACT TTC GTT CTT GA 3'
Rav1 (At1g13260)	5' GGC GGA GGC ACT GTT TGA 3'	5' FAM-AAA GCG GTA ACG CCA AG 3'	5' AAC GGT TTA GCT TCC CAA CGT 3'
CBF2 (At4g25470)	5' GGA TGC TCA TGG TCT TGA CAT G 3'	5' FAM-AGA CCT TGG TGG AGG C 3'	5' TCT TCA TCC ATA TAA AAC GCA TCT TG 3'
Zat12 (At5g59820)	5' CGC ATC CTT GTC CCA TAT GTG 3'	5' FAM-TGG AGT TTC CGA TGG GA 3'	5' TCA TGT GTC CTC CCA AAG CTT 3'

Primer and probe mixes for the remaining genes were ordered from ABI (Applied Biosystems Inc., CA). Due to company policy only the probe sequences for these genes were provided, which are shown in the table below.

Gene	Probe Sequence
ACT1	5' FAM-TGG TCG TAC TAC CGG TAT TGT GCT C 3'
Thaumatin Family Protein (At1g20030)	5' FAM-CCA ACA CCA GCC TAA AAT CAG CTG A 3'
Dof-1 Domain Protein (At1g26790)	5' FAM-GAC CAC ACA TGA TGA ATA ACC AAT C 3'
CBF3 (At4g25480)	5' FAM-TGA AGT CGA CGG CGA TGA TGA CGA C 3'
CBF1 (At4g25490)	5' FAM-CGC CGC CGT CTG TTC AAT GGA ATC A 3'
CCCH-type Protein (At4g29190)	5' FAM-AAT TAT GGA TTT TGG GGC CTG ATT T 3'
ERF Subfamily B3 (At5g61600)	5' FAM-ACG TTT TCA CTT GCG TCA CTC TCA T 3'

2.4.6 MADs calculation

Median Absolute Deviation (MAD), an alternate method of determining the variability of data, is defined as

$$MAD = Constant * Median (\left| Y_i - \tilde{Y} \right|)$$

where $\tilde{Y}$ is the median of the data. The value of constant is 1.4826. The MAD value was calculated for each expression data value. The median of these values was then multiplied with ($\left| Y_i - \tilde{Y} \right|$), which was calculated for the expression value of each RIL. RILs showing MAD values >+3 and <-3 were considered as outliers and were removed from the dataset. These RILs were represented as missing values in the phenotype data used for Composite Interval Analysis (CIM) using the QTL Cartographer package.

2.4.7 eQTL Mapping

Information for genotype of each RIL was downloaded from www.arabidopsis.org and was prepared for QTL cartographer by manually eliminating the redundant marker information along the length of the chromosomes. MAD values were calculated for the expression levels of genes for 155 RILs and those showing MAD values >+4 and <-4 were considered as outliers. These outliers were removed from the dataset to reduce noise and it was re-analyzed using QTL cartographer. In the CIM analysis, the walk speed, which is a parameter that determines the interval along the genetic map at which the QTL calculations are performed, was set at 2cM. Permutation analysis using 1000 permutations was performed to determine the critical LOD threshold. A confidence level of 95% was

determined for a LOD score of 2.4 and thus the critical threshold levels for determining the eQTLs was set at the LOD score of 2.5.

2.4.8 Sub-grouping RILs Based on Composition of *CBF* Locus

The composition of the *CBF* locus in the lines was determined first by examining the marker data available for these lines on the TAIR website. Two chromosomal markers, g4539 and GB.490 C, flanking the *CBF* locus were selected and each line was scored for the presence of Cvi-0 or Ler marker on these two positions. The genetic positions of g4539 and GB.490 C were 44 cM and 73 cM respectively. If these flanking markers were Cvi-0, the *CBF* locus was considered as Cvi-0 and if both the markers were L*er*, it was considered as L*er*. Using this method, 114 lines were determined to belong to one group or the other, given the caveat that error in sorting the individuals can arise if two recombination events occurred within the region being examined such that the *CBF* locus was not from the same parent as the flanking markers. For 48 lines that displayed the presence of different parental markers in the flanking positions, PCR was performed to determine the composition of the locus. Two primer pairs were used for the differential PCR, both based in the promoter region of *CBF2*. The first primer pair (sequence generously provided by J Salinas) amplified a ~2 kb fragment from the promoter of L*er* and ~200 bp fragment from the Cvi-0 promoter. The second primer pair, also based in the promoter region of *CBF2*, amplified a ~500 bp region from L*er* and did not amplify anything from the Cvi-0 promoter. Thus, lines showing a 2 kb fragment and a 500 bp fragment were genotyped as L*er*

at the *CBF* locus and those containing a 200 bp fragment were genotyped as Cvi-0.

Chapter 3

eQTLs Regulating Expression of Genes Induced Early During Cold Acclimation

3.1 Introduction

In addition to the *CBF1-3* genes, hundreds of other genes are induced in response to low temperature (Fowler and Thomashow, 2002). Many of the early expressed genes encode transcription factors, suggesting the existence of multiple regulatory networks at low temperatures (Fowler and Thomashow, 2002). However, apart from the CBF regulon, which is activated by the *CBF1-3* genes, the regulatory pathways active during cold acclimation are poorly understood.

Multiple functionally related genes are often co-regulated through a common regulatory gene. For instance, the *COR* genes, which are induced with similar kinetics at low temperature, are collectively activated by the *CBF* genes. In this instance, mutations affecting the *CBF* genes will affect expression of multiple *COR* genes. Analogously, allelic differences in a common regulator will affect the expression of multiple genes, leading to co-localization of their eQTLs to this regulator. Thus analysis of multiple low temperature-regulated genes for eQTLs will provide the opportunity to identify common regulatory pathways.

Many early expressed genes at low temperature display temporal expression profiles similar to the *CBF1-3* genes. Furthermore, some of these genes, including *CBF1-3*, are also induced in response to mechanical

agitation and cycloheximide treatment, suggesting shared regulatory pathways (Vogel et al., 2005). It was sought to determine if, using eQTL analysis, common *trans*-acting eQTLs could be identified for this group of low temperature-induced genes. Genes showing common eQTLs could possibly constitute branches of the same regulatory pathway. Finally, to broaden the scope of the study and include different regulatory pathways, genes suspected not to be co-regulated were also included for identification of eQTLs.

3.2 Results

In order to identify additional genetic loci containing regulators of low temperature responsive transcription, six candidates were chosen for eQTL mapping. The L*er*Cvi RIL population was evaluated for transcript accumulation of *Rav1* (At1g13260), *Zat12* (At5g59820), Ethylene Response Factor (ERF; At5g61600), CCCH-type Zn-Finger transcription factor (At4g29190), Dof-1 domain containing transcription factor genes (At1g26790), and Thaumatin-like protein encoding gene (At1g20030).

The expression profiles of these genes at low temperature were determined through investigation of publicly available microarray data (compiled from several research groups) accessible through the Genevestigator database (http://www.genevestigator.ethz.ch/at). Examination of AtGenExpress data for low temperature stress treatments of Arabidopsis plants showed ERF, *Rav1*, and *Zat12* to be induced early in response to low temperature, with peak levels of expression at 1h (ERF) or 3h (*Rav1* and *Zat12*) and subsequent decline in expression by 24h (Figure 3.1). These profiles were similar to the *CBF1-3* genes, which also peaked after 3h of exposure to low temperature (Figure 3.2). In addition, all of the above genes were induced by cycloheximide treatment (Table 3.1) and mechanical agitation (Vogel et al., 2005) suggesting shared regulatory pathways. Identification of common eQTLs for regulation of these genes would provide additional evidence to support existence of shared pathways.

Analysis of the Genevestigator data also revealed a group of low temperature-induced genes that were not induced by cycloheximide

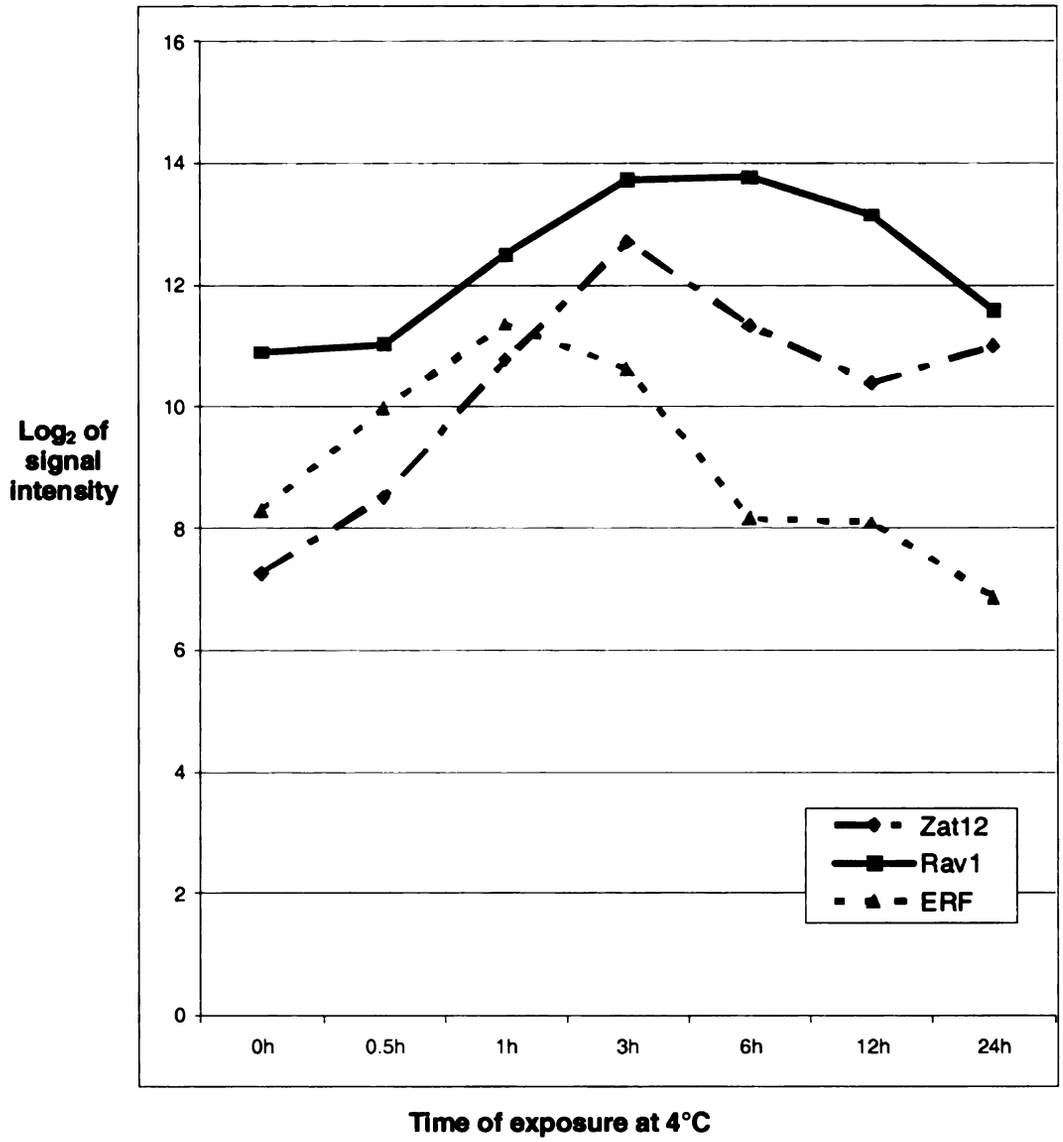

Figure 3.1. **Expression profiles of *Zat12*, *Rav1*, and ERF genes at low temperature.** Digital Northern from Genevestigator database showing expression levels of ERF, *Rav1*, and *Zat12* genes after 0.5, 1h, 3h, 6h, 12h, and 24h at 4°C in aerial parts of seedlings of Col-0, measured using microarrays. The y-axis displays the log2 of signal intensities obtained for the gene.

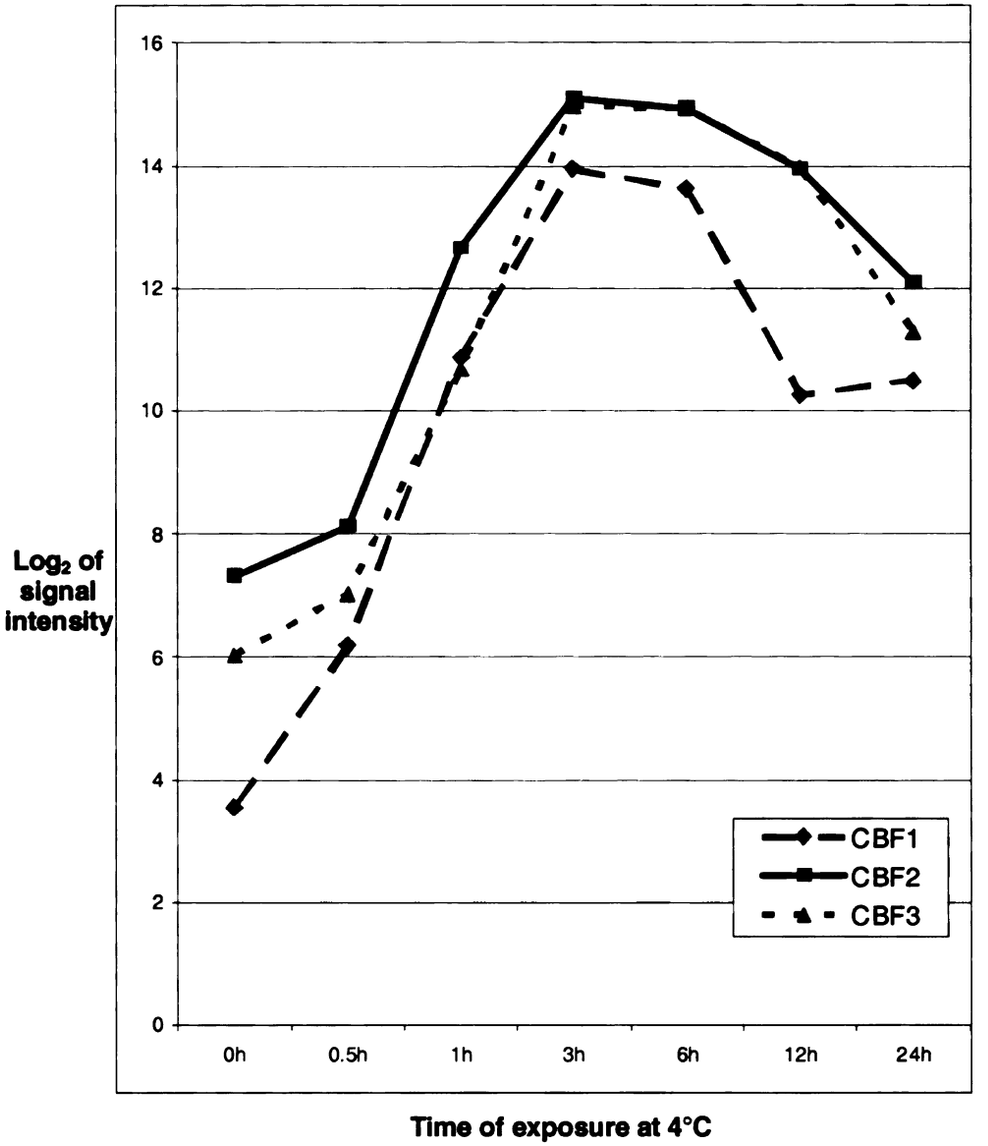

Figure 3.2. **Expression profiles of *CBF1*, *CBF2*, and *CBF3* genes at low temperature.** Digital Northern from Genevestigator database showing expression levels of ERF, *Rav1*, and *Zat12* genes after 0.5, 1h, 3h, 6h, 12h, and 24h at 4°C in aerial parts of seedlings of Col-0, measured using microarrays. The y-axis displays the log2 of signal intensities obtained for the gene.

AGI Numbers	Gene Name	Cold Treatment (log2 of signal)	Cycloheximide Treatment (log2 of signal)
At5g59820	Zat12	10.913	91.445
At5g61600	ERF	2.054	78.742
At1g26790	Dof1-domain containing	5.853	-0.003
At4g29190	CCCH-type Zn Finger	7.115	-0.611
At1g13260	Rav1	6.578	6.872
At1g20030	Thaumatin-like	14.382	-0.804
At4g25470	CBF2	144.658	7.997
At4g25490	CBF1	142.391	8.870
At4g25480	CBF3	78.764	1.721

Table 3.1. **Expression of selected genes in response to cycloheximide treatment and cold.** Heat map from Genvestigator database showing response of *Rav1* (At1g13260), *Zat12* (At5g59820), Ethylene Response Factor (At5g61600), CCCH-type Zn Finger transcription factor (At4g29190), Dof-1 domain containing transcription factor genes (At1g26790), and Thaumatin-like protein encoding gene (At1g20030) upon cycloheximide and cold treatment.

treatment, suggesting distinct regulatory pathways for these genes. This group included Thaumatin-like protein, CCCH-type Zn-Finger transcription factor, and Dof-1 domain containing transcription factor genes (Table 3.1).

The gene encoding a Dof-1 domain containing protein was an interesting candidate for analysis because of its potential to be involved in conveying the low temperature signature to proteins or DNA. Dof (DNA-binding with one finger) domain proteins are plant-specific transcription factors with a highly conserved DNA-binding domain containing a single C_2-C_2 zinc finger. This Dof domain has been shown to possess DNA-binding as well as protein-protein interaction abilities (Yanagisawa, 2004). Their ability to interact with protein as well as DNA gives them the ability to act at the intermediate step of conveying the signal from a protein to DNA. Furthermore, of all the abiotic stress conditions for which data was available at the Genevestigator site (http://www.genevestigator.ethz.ch/at), the Dof-1 domain-containing gene was induced only by low temperature and hydrogen peroxide treatment (Genevestigator), suggesting specific functional role at low temperature and oxidative stress. The other candidate, the Thaumatin like protein, also provides an interesting low temperature-regulated gene as it is predicted to be a GPI-anchored protein based on sequence homology data (TAIR). Both these genes are of further interest as they do not encode usual transcription factors like the other candidates included in this analysis, thus furthering the objective of broadening the investigation of low temperature-induced regulatory pathways. The CCCH-type Zn-Finger transcription factor-encoding gene could be involved in regulation of a subset of genes that are differentially expressed later in response to low temperature.

If they were indeed regulated through distinct pathways, eQTL mapping for these genes would potentially expand the search for loci involved in regulation of transcription at low temperature. Using the Genevestigator database, expression profiles of these genes were found to be similar to *Rav1*, *Zat12*, and ERF genes, showing early induction in response to low temperature treatment (Figure 3.3). Thus the candidates represented groups of genes that were possibly regulated through common and distinct pathways acting at low temperatures.

3.2.1 eQTL mapping

Using the Taqman® real-time RT-PCR method, transcript levels for *Rav1*, *Zat12*, ERF, Thaumatin-like protein, CCCH-type Zn-Finger transcription factor, and Dof-1 domain containing transcription factor genes were estimated in 155 RILs that were treated at 4°C for 2h. The amplification profiles generated during qPCR for different transcripts are shown in Figure 3.4. $2^{-\Delta Ct}$ values were determined using 18S rRNA and *Act1* as the internal reference genes (see Materials and Methods).

Transcript accumulation for the six selected low temperature responsive genes, measured through independent normalizations with 18S rRNA and *Act1*, was analyzed using CIM to identify eQTLs. Analysis of the 18S rRNA normalized expression data led to identification of eQTLs for two genes that are not induced by cycloheximide treatment, CCCH-type Zn-Finger transcription factor and the Thaumatin-like gene (Figures 3.5 and 3.6, respectively). However, using this normalization, no eQTLs were detected for

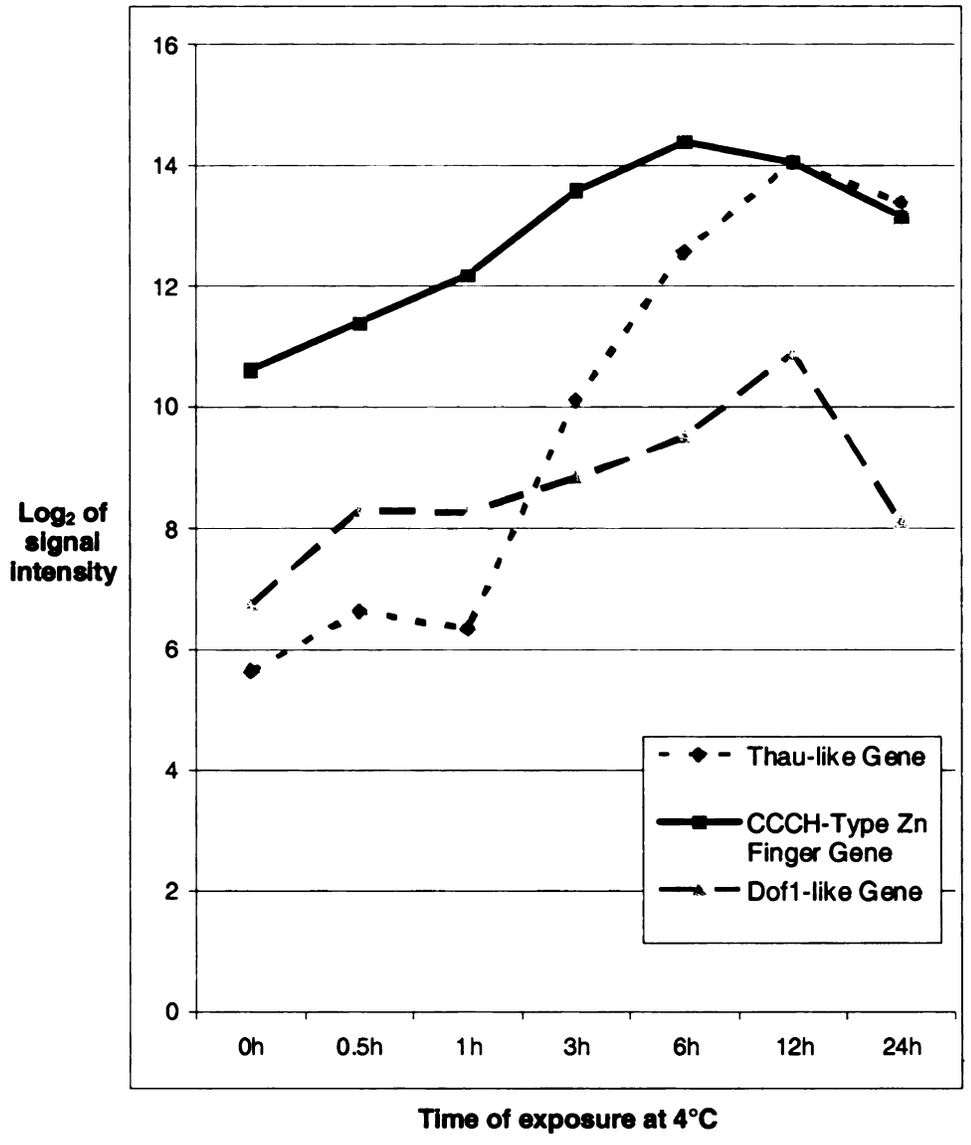

Figure 3.3. **Expression profiles of Thau-like, CCCH-type Zn Finger containing transcription factor, and the Dof1-domain containing genes at low temperature.** Digital Northern from Genevestigator database showing expression levels of Thau-like, CCCH-type Zn Finger containing transcription factor, and the Dof1-domain containing genes after 0.5, 1h, 3h, 6h, 12h, and 24h at 4°C in aerial parts of seedlings of Col-0, measured using microarrays. The y-axis displays the log2 of signal intensities obtained for the gene.

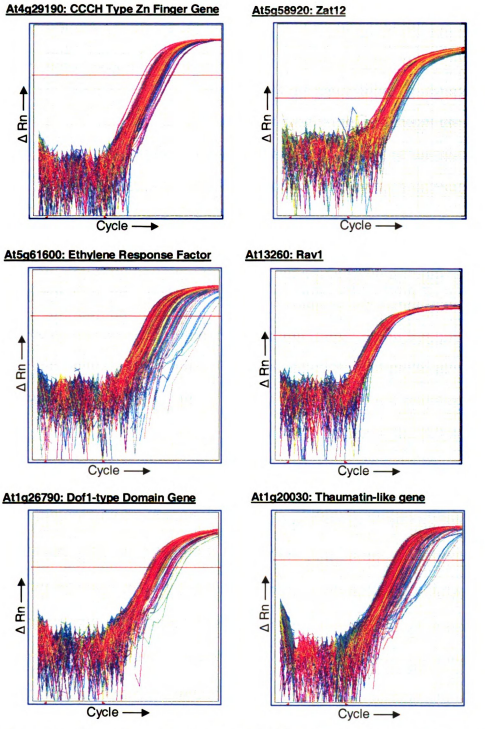

Figure 3.4. **Taqman real-time RT-PCR amplification profiles for transcripts of indicated genes.** The y-axis represents fluorescence emission and the x-axis represents cycle numbers. The horizontal red line depicts the threshold at which the C_t value for each amplification curve was determined.

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Figure 3.5. **eQTLs for regulation of CCCH-type Zn Finger containing transcription factor expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

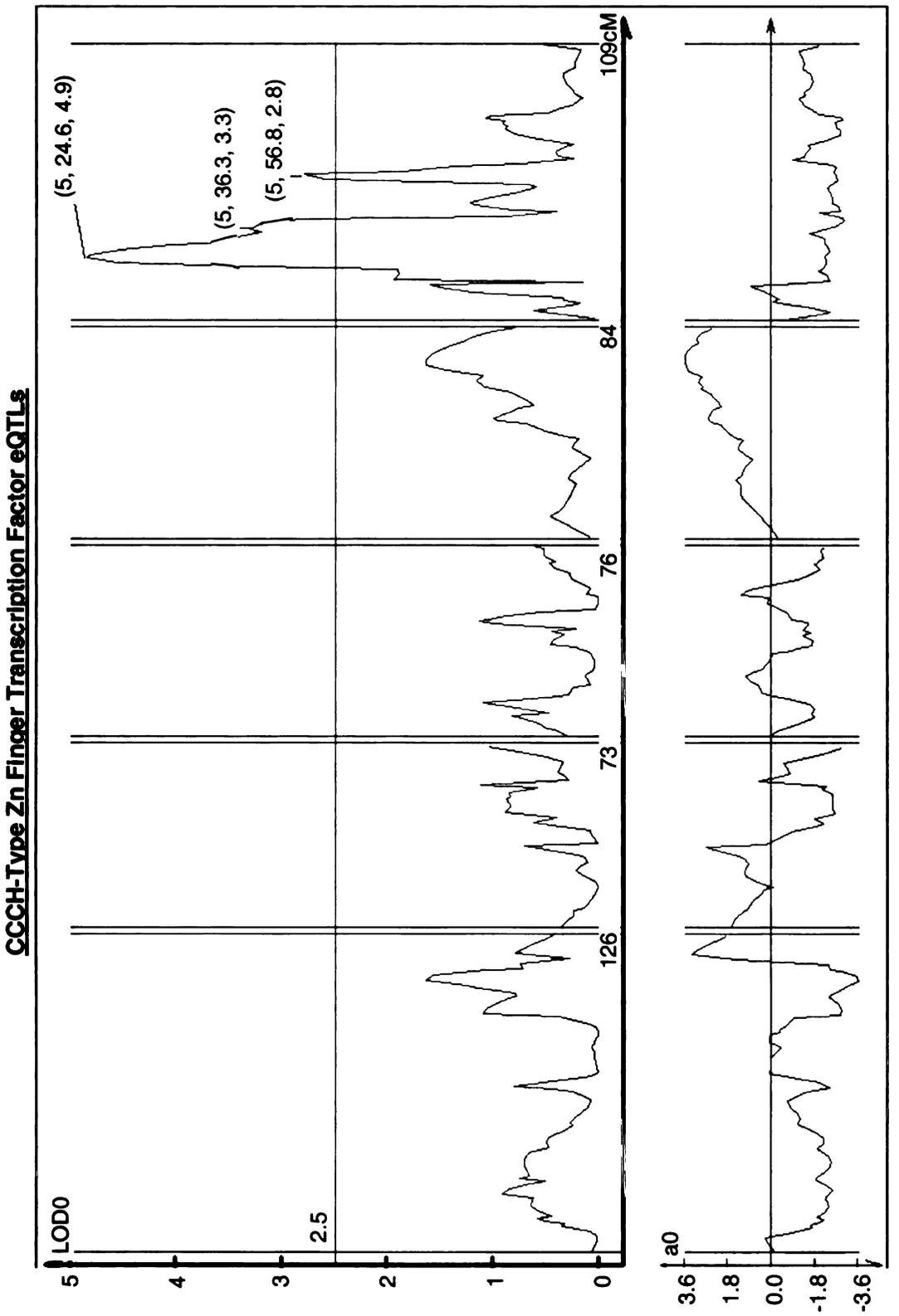

Figure 3.5. **eQTLs for regulation of CCCH-type Zn Finger containing transcription factor expression.**

Figure 3.6. **eQTLs for regulation of Thaumatin-like gene expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

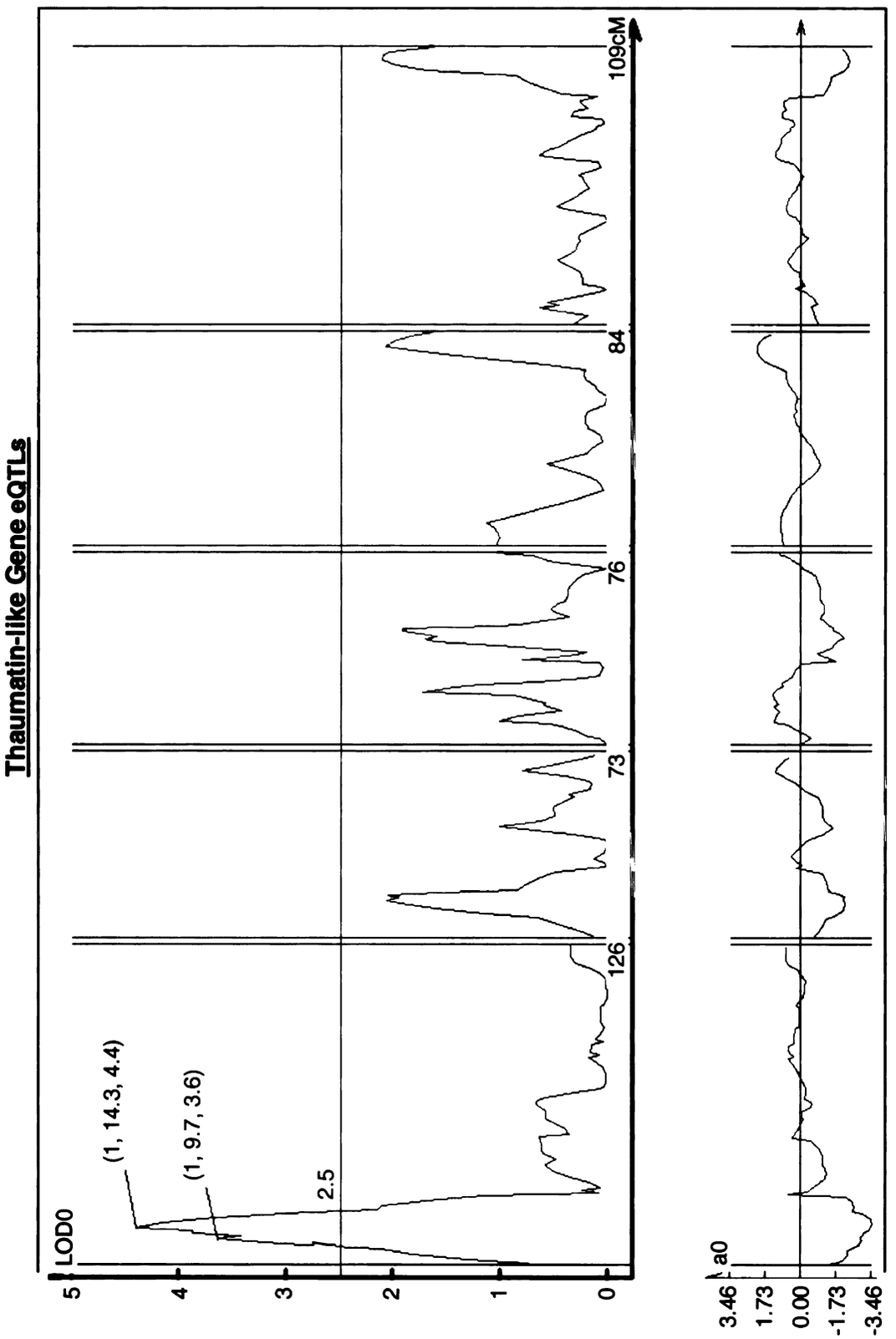

Figure 3.6. **eQTLs for regulation of Thaumatin-like gene expression.**

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Figure 3.7. **eQTLs for Dof1-domain containing gene expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

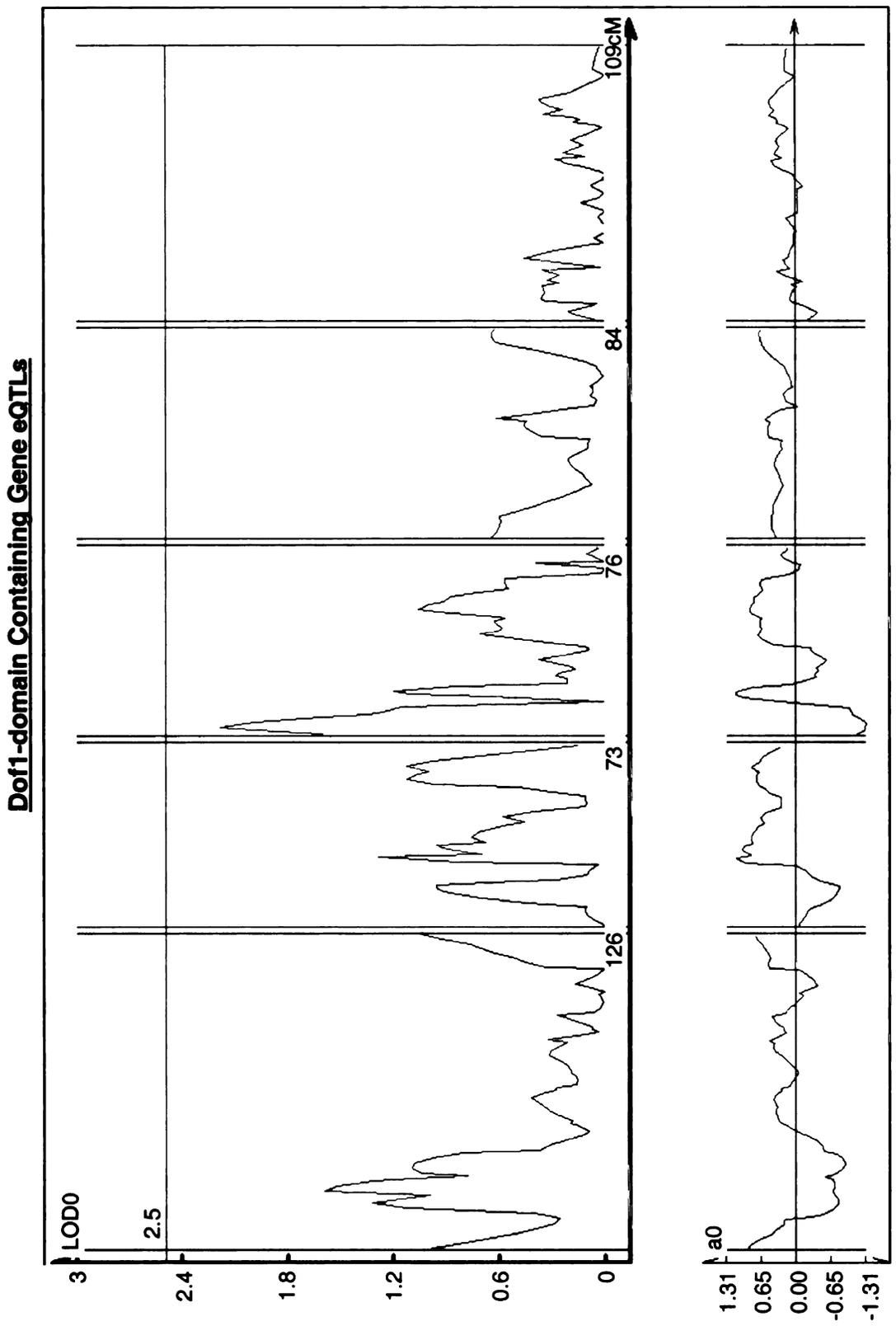

Figure 3.7. **eQTLs for Dof1-domain containing gene expression.**

the Dof1-like domain containing transcription factor, *Zat12*, and ERF genes (Figures 3.7, 3.9, and 3.10, respectively).

3.2.2. Identification of eQTLs using 18S rRNA as the Internal Reference

The eQTLs identified for CCCH-type Zn-Finger containing transcription factor and Thaumatin-like gene co-localized with eQTLs identified for regulation of *CBF* genes. Differential expression of the CCCH-type Zn-Finger containing transcription factor gene at low temperature was found to be regulated by eQTLs on chromosome 5 that were overlapping in position with eQTLs identified for *CBF1*, *2*, and *3* expression (Figure 3.5). The first eQTL on chromosome 5 identified for CCCH-type Zn-Finger containing transcription factor gene, with a strong statistical support of 4.9 LODs, was common with the eQTL detected for all three *CBF*s at the same location (see Chapter 2 section). The second eQTL identified with a LOD score of 2.8 was shared with *CBF2* and *3* genes. The location of eQTL identified for Thaumatin-like gene on chromosome 1 overlapped with an eQTL identified for *CBF3* expression (Figure 3.6). Cvi-0 contributed the positive allele for this eQTL, which was supported with 4.4 LODs.

3.2.3. Identification of eQTLs using *Act1* as Internal Reference

Expression data obtained for the six genes using *Act1* levels as internal reference identified eQTLs for additional genes. This normalization uncovered eQTLs for the *Rav1*, *Zat12*, ERF, and Dof1-like domain containing transcription factor genes (Figures 3.11, 3.12, 3.13, and 3.14 respectively),

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Figure 3.8. **eQTLs for *Rav1* expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

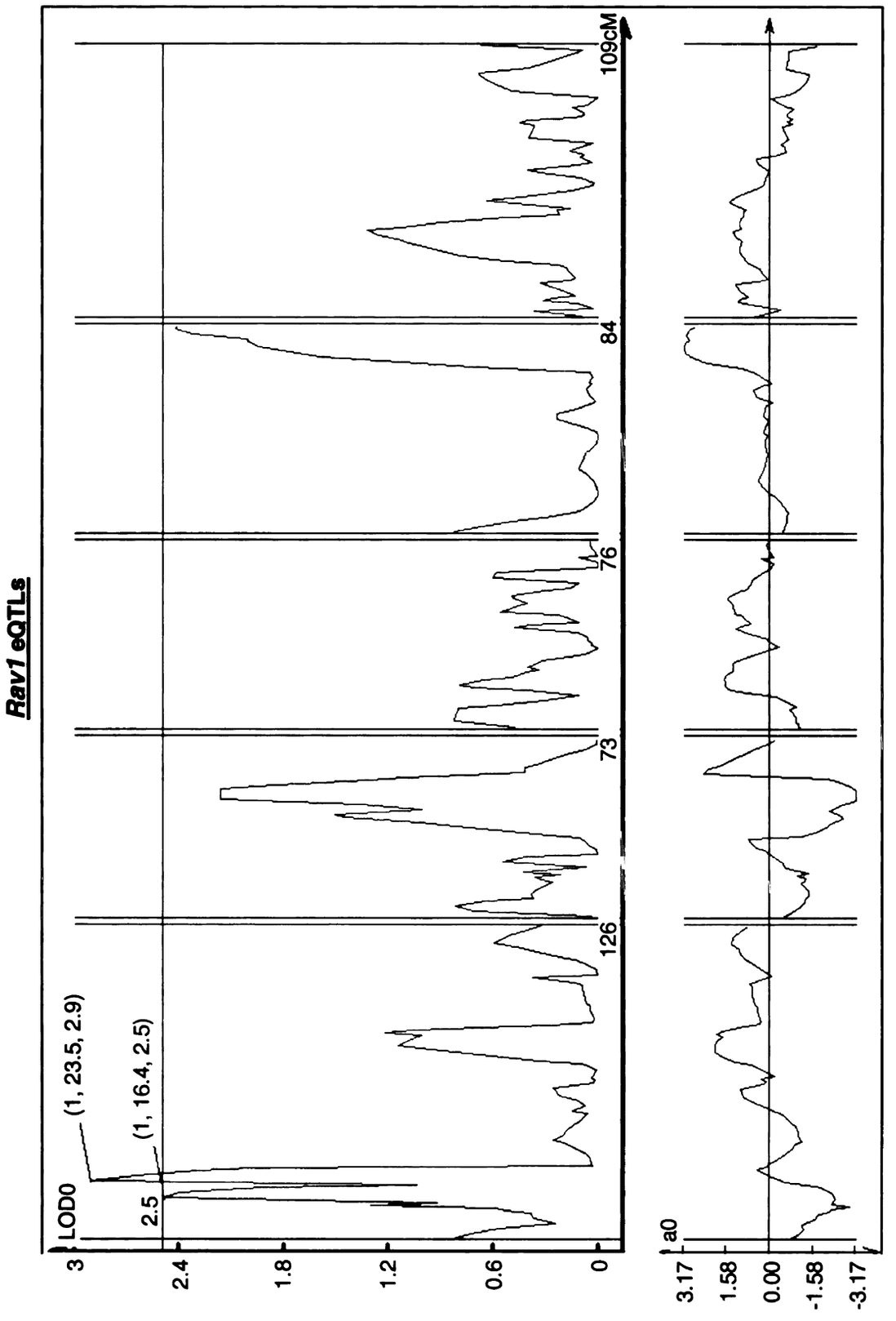

Figure 3.8. **eQTLs for *Rav1* expression.**

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Figure 3.9. **eQTLs for *Zat12* expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

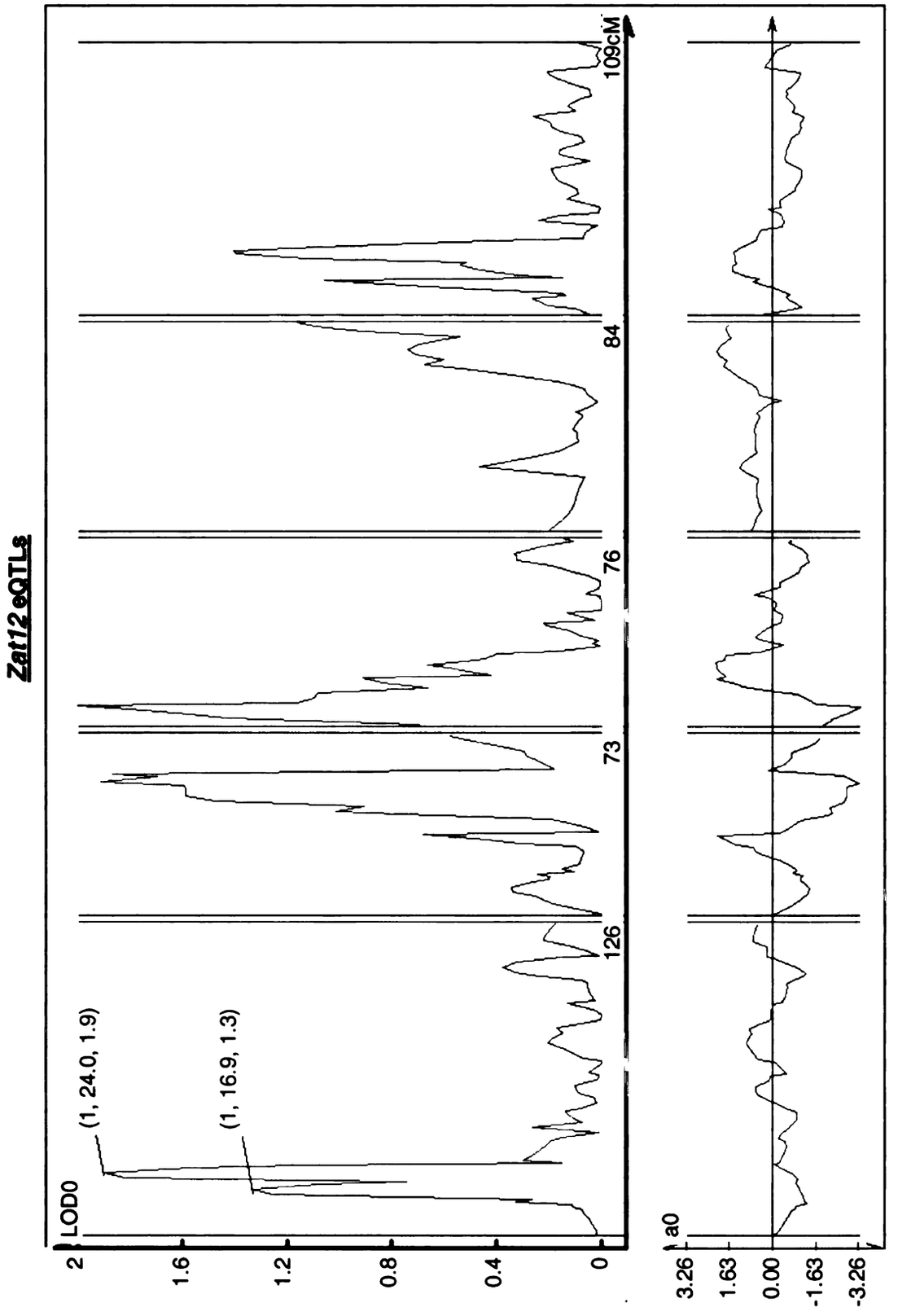

Figure 3.9. **eQTLs for *Zat12* expression.**

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Figure 3.10. **eQTLs for Ethylene Response Factor gene expression.** CIM analysis of 18S rRNA normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

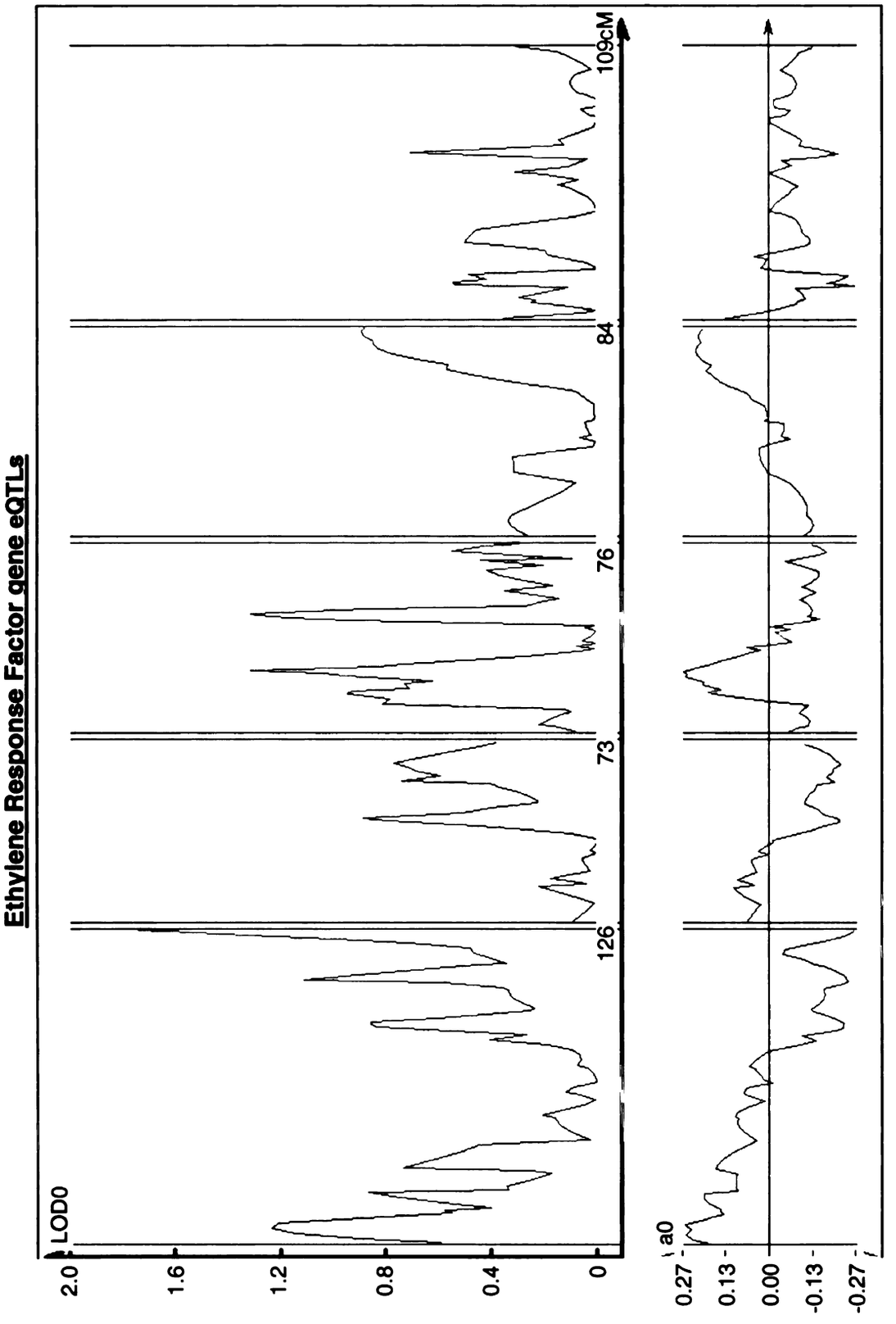

Figure 3.10. **eQTLs for Ethylene Response Factor gene expression.**

which were not identified using 18S rRNA for normalization of expression data. Additional eQTLs were also identified for CCCH-type Zn-Finger transcription factor gene (Figure 3.15). The expression of Thaumatin-like gene, however, did not identify any eQTLs through this analysis (Figure 3.16).

Act1 normalized data revealed common eQTLs with similar patterns for *Rav1*, *Zat12*, Dof1-like domain containing transcription factor, and CCCH-type Zn-Finger transcription factor genes (Figures 3.11, 3.12, 3.14, and 3.15 respectively) on chromosome 1. Furthermore, eQTLs at the same location were also identified for the regulation of *CBF1* and *2* expression using *Act1* as the internal reference gene (see Chapter 2, Section 2.2.3.3).

An additional eQTL for CCCH-type Zn-Finger containing transcription factor was identified on chromosome 1, followed by eQTLs regulating ERF and the Dof1-like domain containing transcription factor (Figures 3.13, 3.14 and 3.15).

Figure 3.11. **eQTLs for *Rav1* expression.** CIM analysis of *Act1* normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

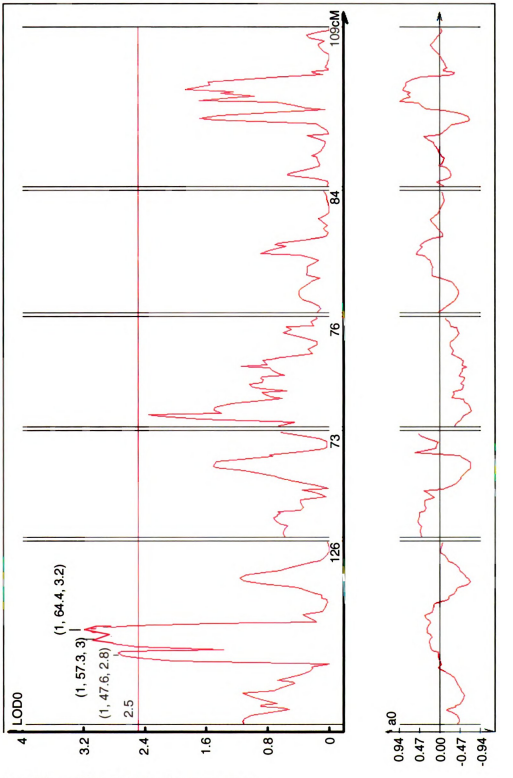

Figure 3.11. **eQTLs for *Rav1* expression.**

Figure 3.12. **eQTLs for *Zat12* expression.** CIM analysis of *Act1* normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

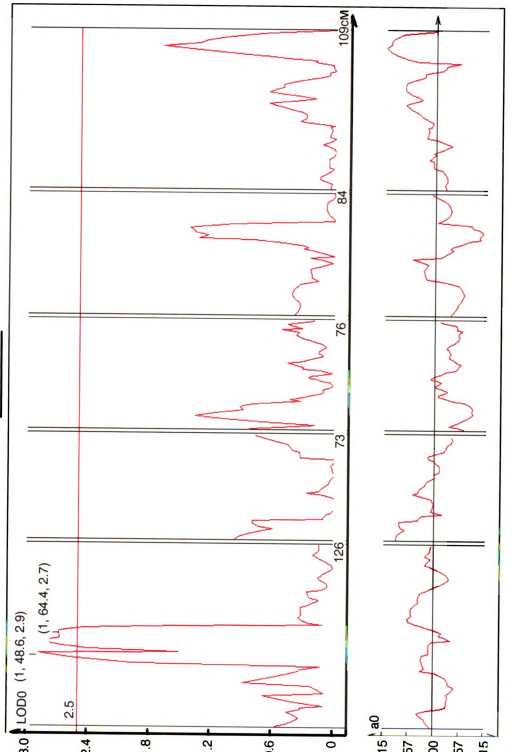

Figure 3.12. **eQTLs for *Zat12* expression.**

Figure 3.13. **eQTLs for Ethylene Response Factor gene expression.** CIM analysis of *Act1* normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

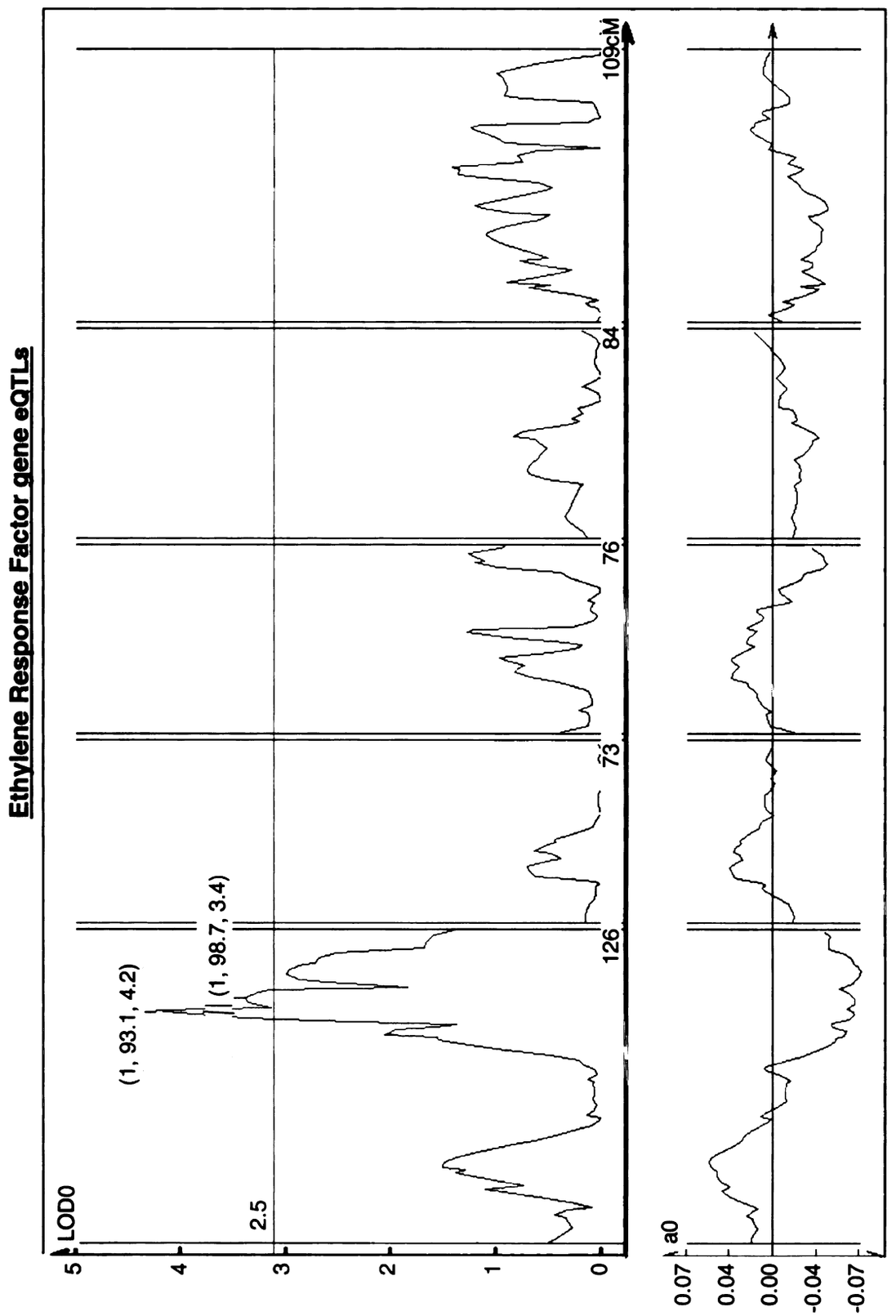

Figure 3.13. **eQTLs for Ethylene Response Factor gene expression.**

Figure 3.14. **eQTLs for CCCH-Type Zn Finger Transcription Factor gene expression.** CIM analysis of *Act1* normalized *CBF1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

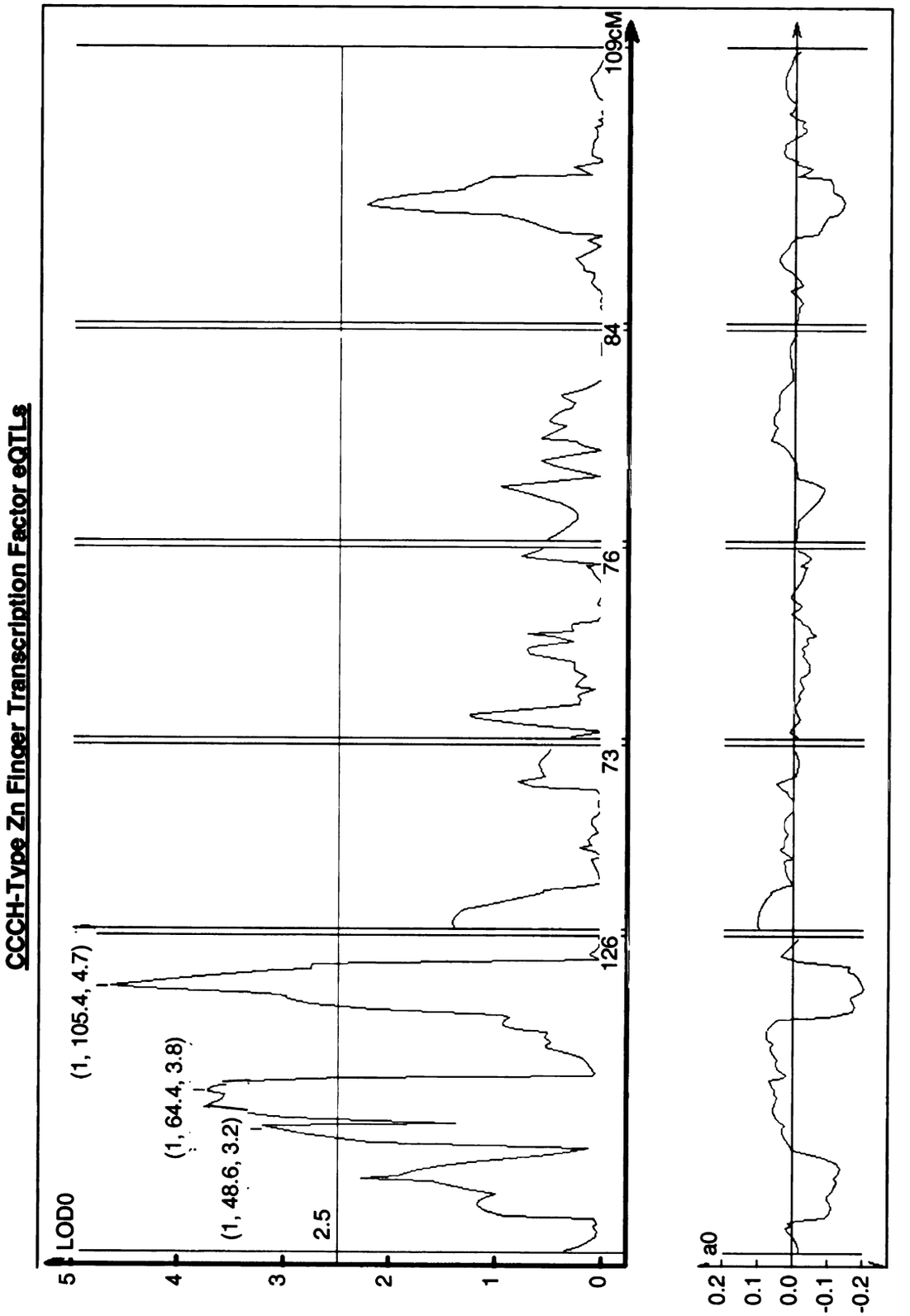

Figure 3.14. **eQTLs for CCCH-Type Zn Finger Transcription Factor gene expression.**

Figure 3.15. **eQTLs for Dof1-domain containing gene expression.** CIM analysis of *Act1* normalized transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

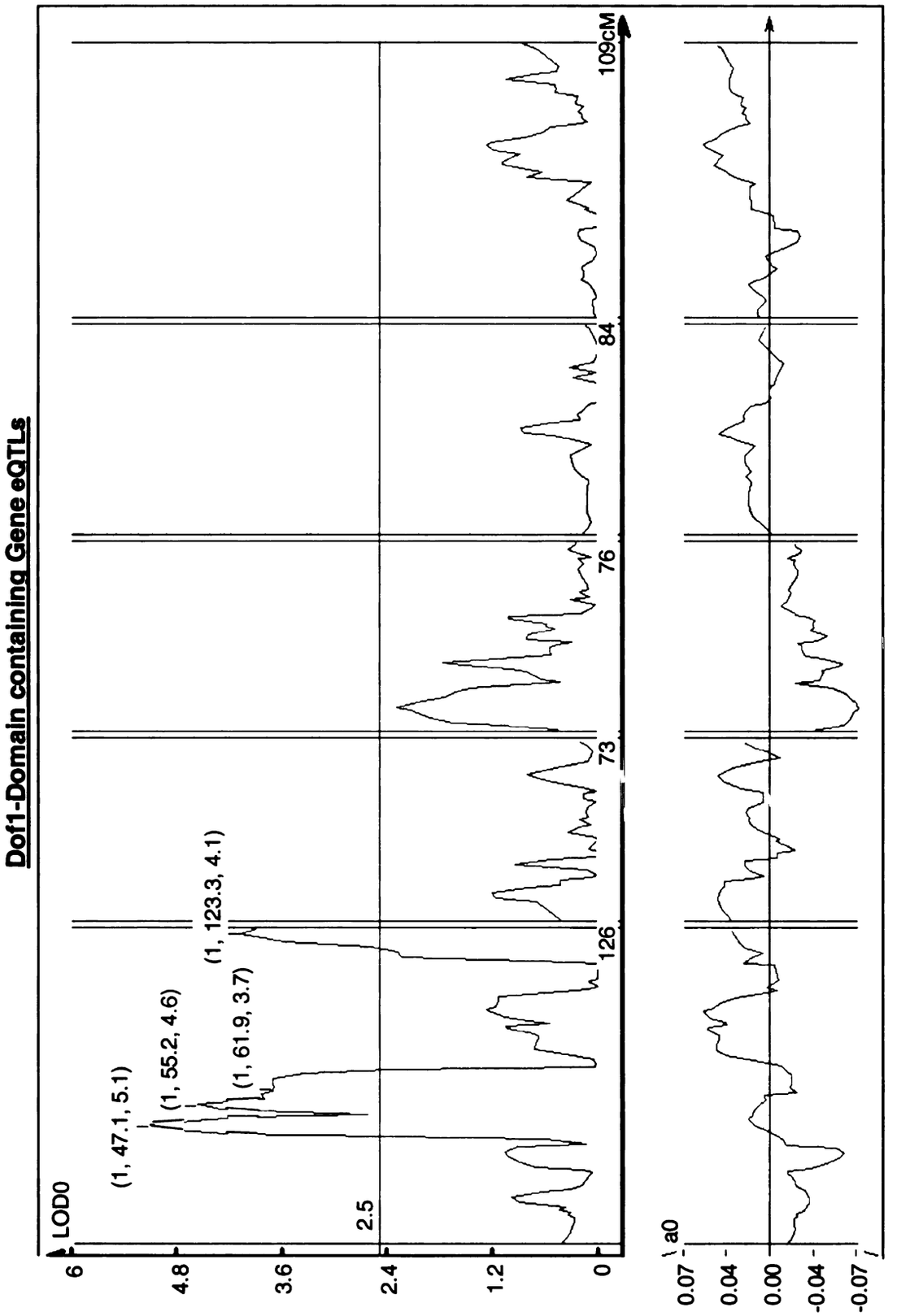

Figure 3.15. **eQTLs for Dof1-domain containing gene expression.**

Figure 3.16. **eQTLs for Thaumatin-family gene expression.** CIM analysis of *Act1* normalized *CBF1* transcript levels in RILs treated at 4°C for 2h, using QTL Cartographer. In the top panel, the y-axis represents the LOD scores and the x-axis represents the five chromosomes of Arabidopsis. The area between each set of vertical double lines represents the length in cMs of the indicated chromosome. The horizontal red line represents the threshold LOD score for 95% confidence level. The numbered values above the peaks represent the chromosome number, the position in cMs, and the LOD score, respectively. The lower panel shows a plot of estimated additive effects where a positive value indicates that the allele from the high expressing parent is associated with higher levels of transcript, which is L*er*.

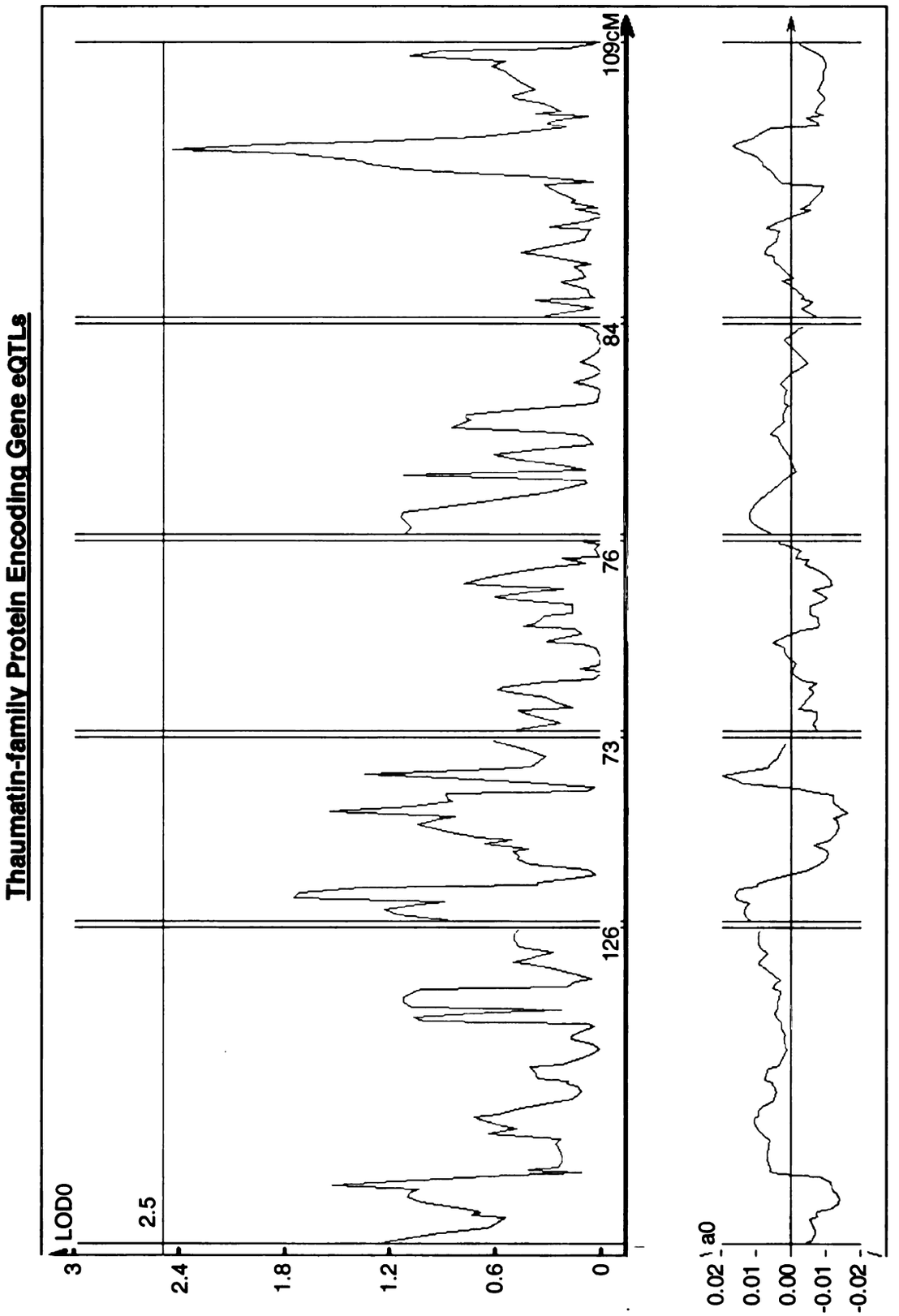

Figure 3.16. **eQTLs for Thaumatin-family gene expression.**

3.3 Discussion

Cold acclimation involves a network of regulatory pathways that ultimately result in developing tolerance to low temperatures. Genes upregulated upon exposure of Arabidopsis to 4°C include several transcription factors that reach peak expression levels between 1-4h and subsequently decrease to lower levels (Vogel et al., 2005). A subset of these genes is also induced by mechanical agitation and cycloheximide treatment, suggesting shared regulatory pathways. This subset includes *CBF1-3*, *Rav1*, *Zat12*, and ERF genes (Table 3.1). A different subset of genes, including Thaumatin-like protein, CCCH-type Zn-Finger transcription factor, and Dof-1 domain-containing transcription factor, are only induced in response to low temperature but not to mechanical agitation or cycloheximide treatment (Figure 3.3). The expression of the Dof-1 domain containing gene was found to be unaffected by cycloheximide treatment, whereas Thaumatin-like and CCCH-type Zn-Finger transcription factor genes were downregulated by the treatment. These differences in expression present two interesting groups of genes, the analysis of which may lead to identification of different pathways responsive to low temperature.

Altogether, using 18S rRNA and *Act1* normalization for quantification of phenotypic data, eQTLs were detected for all genes analyzed in this study (Table 3.2). The eQTLs identified were all *trans*-acting and were supported by statistically significant LOD scores.

Gene	eQTL	
	18S rRNA	*Act1*
Rav1 (At1g13260)		eQ C1-3 eQ C1-4
Zat12 (At5g59820)		eQ C1-3 eQ C1-4
CCCH-Type Zn Finger TF gene (At4g29190)	eQ C5-1 eQ C5-2	eQ C1-3 eQ C1-4 eQ C1-6
Dof1-domain containing gene (At1g26790)		eQ C1-3 eQ C1-4 eQ C1-7
Thau-like gene (At1g20030)	eQ C1-1	
ERF gene (At5g61600)		eQ C1-5

Table 3.2. List of eQTLs identified using 18S rRNA and *Act1* normalized gene expression data. The nomenclature is based on the location of the eQTL.

3.3.1. Common eQTLs Regulating Multiple Low Temperature-Induced Genes.

Several eQTLs were found to commonly regulate more than one gene. Regulation of functionally related genes through a common gene is a widely employed strategy (Keurentjes et al., 2007). Genes suggesting co-regulation, based on similar induction responses to low temperature, mechanical agitation, and cycloheximide treatment indeed identified common eQTLs on chromosome 1. These genes included *CBF1*, *CBF2*, *Rav1*, and *Zat12*. Identification of a common eQTL regulating the expression of these genes strengthened the evidence for a shared regulatory pathway.

In addition, through identification of common eQTLs at this position, the CCCH-type Zn-Finger transcription factor and Dof-1 domain-containing transcription factor genes were also found to share regulatory pathways with the above group. Therefore, this analysis helps bin *CBF1*, *CBF2*, *Rav1*, *Zat12*, CCCH-type Zn-Finger transcription factor and Dof-1 domain-containing transcription factor genes into a common regulatory pathway. Co-localization of eQTLs controlling more than one functionally related gene would indicate coregulation by a common gene underlying the eQTL. It is possible that the *trans*-regulatory factor underlying the common eQTL identified for the above genes functions upstream of a branch point in the regulatory pathway, which is manifested as the difference in response to mechanical agitation and cycloheximide treatment. Therefore, the CCCH-type Zn-Finger transcription factor and Dof-1 domain-containing transcription factor genes may be regulated through an arm downstream of the branch-point that is not responsive to mechanical agitation and cycloheximide treatment.

The grouping of the CCCH-type Zn-Finger containing transcription factor gene with this group is further supported by common eQTLs shared with *CBF1*, *2*, and *3* genes on chromosome 5.

3.3.2. Unique eQTLs Identified for Regulation of Specific Low Temperature-Responsive Genes.

In addition to common eQTLs, the low temperature responsive genes analyzed also identified distinct eQTLs. The ERF, CCCH-type Zn-Finger containing transcription factor, Dof1-like domain containing transcription factor and Thaumatin-like genes identified eQTLs that were not shared with any other gene, indicating regulation by different pathways or different arms of a branched pathway. The expression of the ERF, Dof1 domain-containing transcription factor, and the CCCH-type Zn-Finger containing transcription factor genes revealed distinct eQTLs on chromosome 1 (Figures 3.13, 3.14 and 3.15).

Chapter 4

Optimization of Taqman® Real Time RT-PCR for Molecular Trait Measurement

4.1 Introduction

QTL mapping experiments estimate the effect of genotype on phenotypic variance observed in a population. Low environmental variance improves such correlation of genotype with phenotypic data. Using an RIL population allows reduction in environmental variances by allowing biological replicates. In addition, technical variance may contribute to increased noise that needs to be minimized to reveal the genetic variation in a population.

In order to improve the power of eQTL detection, accuracy and reproducibility of RNA quantification is essential. This has been facilitated by the development of highly sensitive and reproducible methods of RNA quantification. Microarrays are employed when simultaneous analysis of several hundred genes is desired and have been used extensively to identify eQTLs for multiple genes (Carlborg et al., 2005; de Koning et al., 2005; Rosa et al., 2006; Alberts et al., 2007; Shi et al., 2007). Real-time RT-PCR is a quantitative method that provides an accurate means to determine the levels of specific DNA and RNA templates for a smaller set of genes (Reist et al., 2003; Huggett et al., 2005; Luu-The et al., 2005; Stahlberg et al., 2005; Wacker and Godard, 2005; Botteldoorn et al., 2006; Wells, 2007). The latter method provides a broad dynamic range—at least five orders of magnitude—for detecting specific DNA or RNA templates. This technique can produce

data for high throughput samples with a short turn around time. The high sensitivity of real-time RT-PCR allows detection of subtle differences in transcript levels between samples, which may have arisen due to variation in genetic regulators. However, the inherent sensitivity of this technique also leads to the detection of even minute amounts of contaminating RNA (fewer than 5 copies) (Valasek and Repa, 2005). Optimization of this technique is therefore necessary to ensure reproducibility.

4.2 Results

4.2.1 Primer and Probe Optimization for Taqman® Real-time RT-PCR

Coding regions of *CBF2*, *Rav1*, and *Zat12* were PCR amplified from Cvi-0 and L*er* and were cloned to detect sequence variation, if any, between the two genotypes (see Materials and Methods). Pairwise alignment of the consensus sequence obtained for coding regions of these genes from Cvi-0 and L*er* displayed high sequence identity. This indicated that common primers could be used to detect transcripts arising from both genotypes. Candidate primer and probe pairs for *CBF2*, *Rav1* and *Zat12* were obtained using Primer Express® (ABI). Using additional criteria, optimal primer and probe sequences were selected.

CBF1, *2*, and *3* genes show a high degree of sequence identity often resulting in cross-reactivity of primers (Figure 4.1). The 3'UTRs of genes often show higher sequence variation among different members of a family and were therefore chosen as the template for primer design. PCR was performed using selected primers and plasmids containing coding regions of *CBF1*, *2*, and *3* genes (Figure 4.2). The primers were found to cause non-specific amplification of *CBF1* and *3* genes. Optimization experiments revealed the requirement of higher annealing temperature to achieve specificity for *CBF2* amplification (Figure 4.3). Additionally, these primers amplified a fragment of expected size using coding region of *CBF2* gene from Cvi-0 (Figure 4.1). Since the *CBF* genes are intron-less it was not possible to design primers that would distinguish between genomic and cDNA template and they detected an

Figure 4.1. **Sequence alignment of *CBF1-3* genes.**

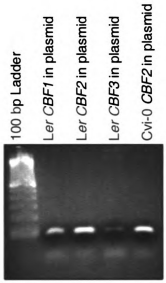

Figure 4.2. **Cross-reactivity of *CBF2* primers with *CBF1* and *3*.** PCR showing amplification of pGEM T-easy plasmid containing *CBF1*, *2*, and *3* coding regions from L*er* and *CBF2* coding regions from Cvi-0 using with primers designed to be specific for *CBF2* coding region. The PCR conditions used were as follows: denaturation at 94°C for 5min, 28 cycles of 94°C for 30 sec, annealing at 58°C for 1min, extension at 72°C for 1min, and a final extension at 72°C for 5 mins.

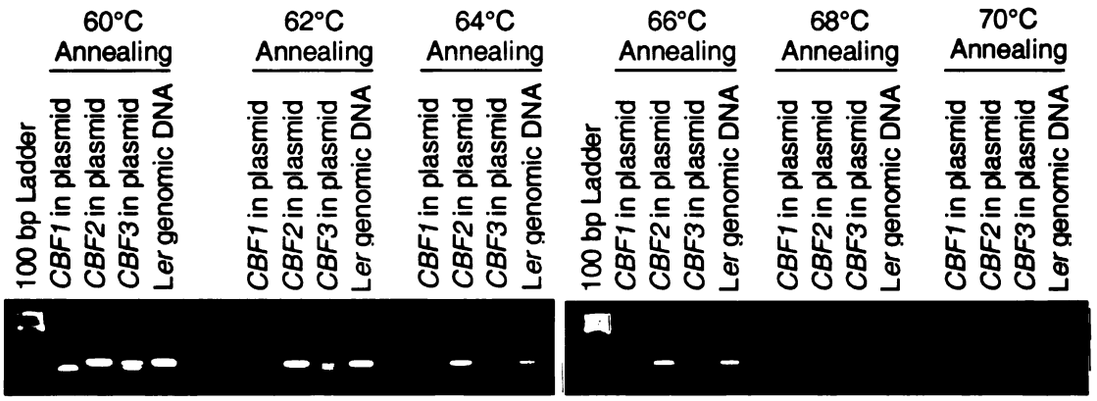

Figure 4.3. **Gradient PCR to determine temperature for specific amplification of *CBF2*.** Gradient PCR performed with indicated annealing temperatures to check primers for amplification of *CBF1*, *2* and *3* coding regions and genomic DNA from Ler, respectively. The PCR conditions used were as follows: denaturation at 94°C for 5 min, 28 cycles of 94°C for 30 sec, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

amplicon of expected size using genomic DNA from Cvi-0 and L*er* (Figure 4.4).

Primers for the 18S rRNA gene (*ATRRN18*; At2g16590), which was selected as the normalization control, were also designed using the Primer Express® program. Primers and probes were further tested in the ABI Prism 7900 HT with serial dilution of plasmid DNA containing the target gene. The real-time amplification plot of technical triplicates from a 1 in 10 dilution series for gene 18S rRNA gene is shown in Figure 4.5. The amplification curve of each sample is approximately 3 cycles apart as would be expected for the dilution series.

4.2.2 Comparison of One- and Two-Step Method Towards Optimization of Real-Time RT-PCR

cDNA synthesis and quantitative PCR (qPCR) in real time can be performed in a single step or in two steps, where cDNA synthesis and qPCR are performed separately (Wang et al., 2004; Psallida and Argyropoulos, 2005; Wacker and Godard, 2005). Both methods were tested in technical triplicates to assess and compare reproducibility. Estimates of standard error with multiple runs of the one-step method revealed huge variation between different samples analyzed in the same light cycler run (Figure 4. 6). Two-step RT-PCR, on the other hand, showed uniformity in the standard error for different samples, indicating lower variation within a single light cycler run (Figure 4.7). The variation between samples measured in the same light cycler run has been previously reported to be lower than that observed for

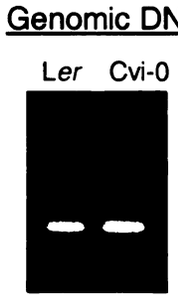

Figure 4.4. **PCR using genomic DNA of L*er* and Cvi-0 as template.** The PCR conditions used were as follows: denaturation at 94°C for 5min, 28 cycles of 94°C for 30 sec, annealing at 58°C for 1min, extension at 72°C for 1min, and a final extension at 72°C for 5 mins.

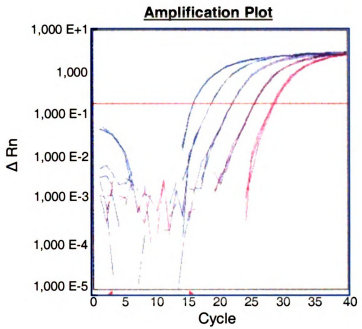

Figure 4.5. **Taqman real-time RT-PCR amplification profiles.** Profiles obtained for a 1 in 10 dilution series of the 18S rRNA gene cloned in a plasmid. The x-axis represents fluorescence emission and the y-axis represents cycle numbers. Each amplification curve represents a single well, and every dilution is measured in triplicate wells. The horizontal red line depicts the threshold at which the C_t value for each amplification curve was determined.

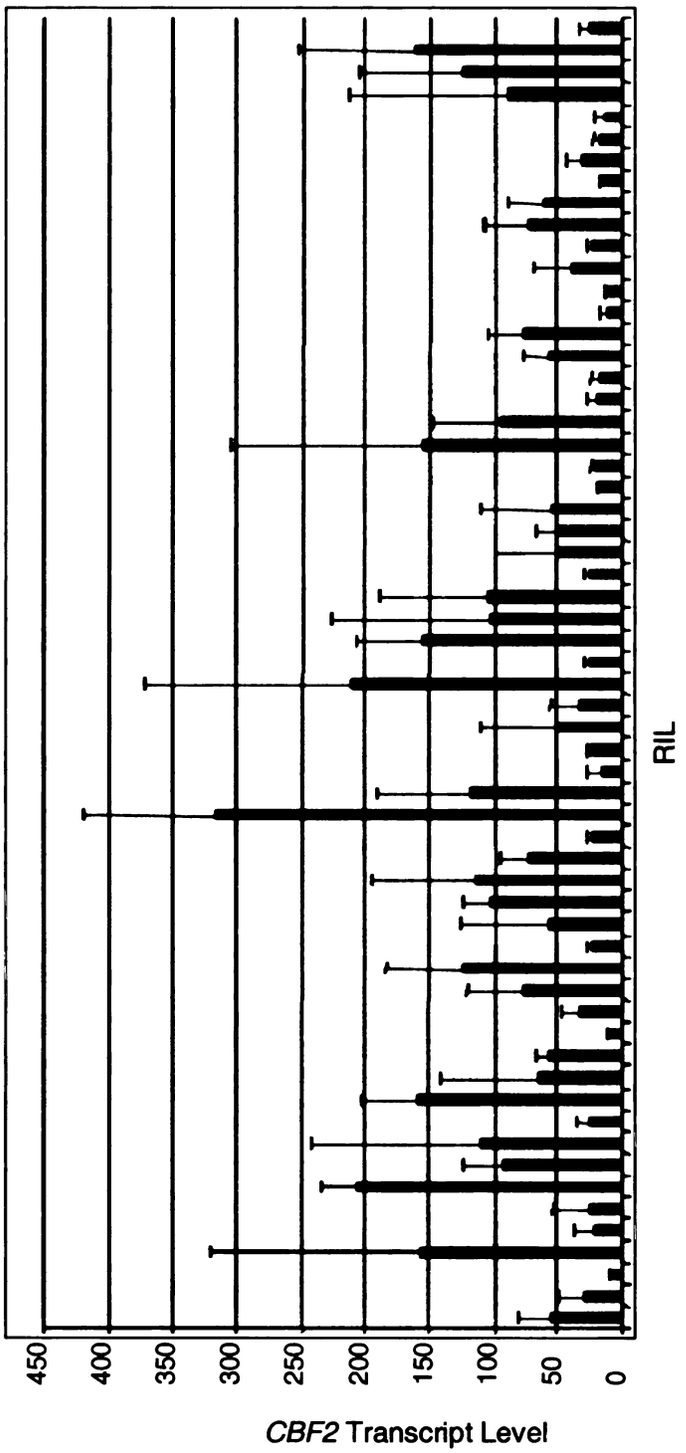

Figure 4.6. **Technical variation for one-step real time RT-PCR.** One-step real time RT-PCR estimates of *CBF2* transcript levels with standard error obtained from three technical replicates.

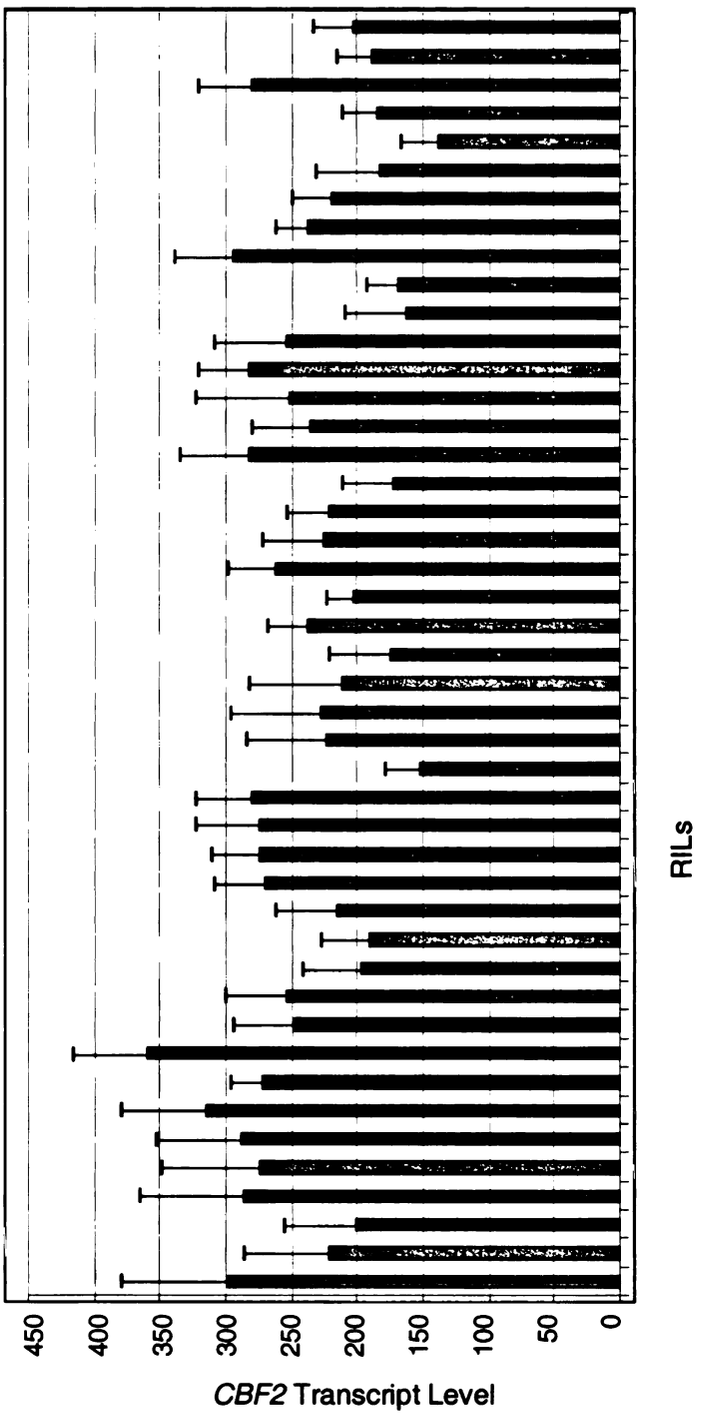

Figure 4.7. **Technical variation for two-step real time RT-PCR.** Two-step real time RT-PCR estimates of *CBF2* transcript levels with standard error obtained from three technical replicates. The RILs analyzed are different from those depicted in Figure 4.6

different light cycler runs. Therefore, the two-step method revealed higher accuracy and reproducibility. There were several other factors that needed careful attention in order to optimize detection and reproducibility of template RNA. Use of random primers for cDNA synthesis allows the use of 18S rRNA and is more helpful for detection of mRNAs with significant secondary structure. Variable enzymatic efficiencies between different samples during the cDNA synthesis step were avoided by using a common reaction mix for all the samples of two biological replicates.

4.2.3 Optimization to Remove DNA Contamination

Genomic DNA contamination poses a significant challenge during analysis of transcripts arising from intron-less genes. The primers designed for real-time RT-PCR were shown to not differentiate between genomic DNA and cDNA templates and therefore could lead to inaccurate estimation of gene expression. Genomic DNA contamination in the extracts leads to inaccurate trait measurements.

PCR of total RNA from RILs using primers designed for real-time qPCR showed significant amplification without reverse transcription of these samples, revealing genomic DNA contamination. Comparison of real-time qPCR amplification profiles pre- and post-DNase I treatment for *CBF2* and 18S rRNA revealed the effect of contamination on overestimation of transcript levels (Figure 4.8a. and b., respectively). A prominent effect of genomic DNA contamination was observed for 18S rRNA quantification, which amplified the overall inaccuracy of target mRNA estimation due to the comparative method

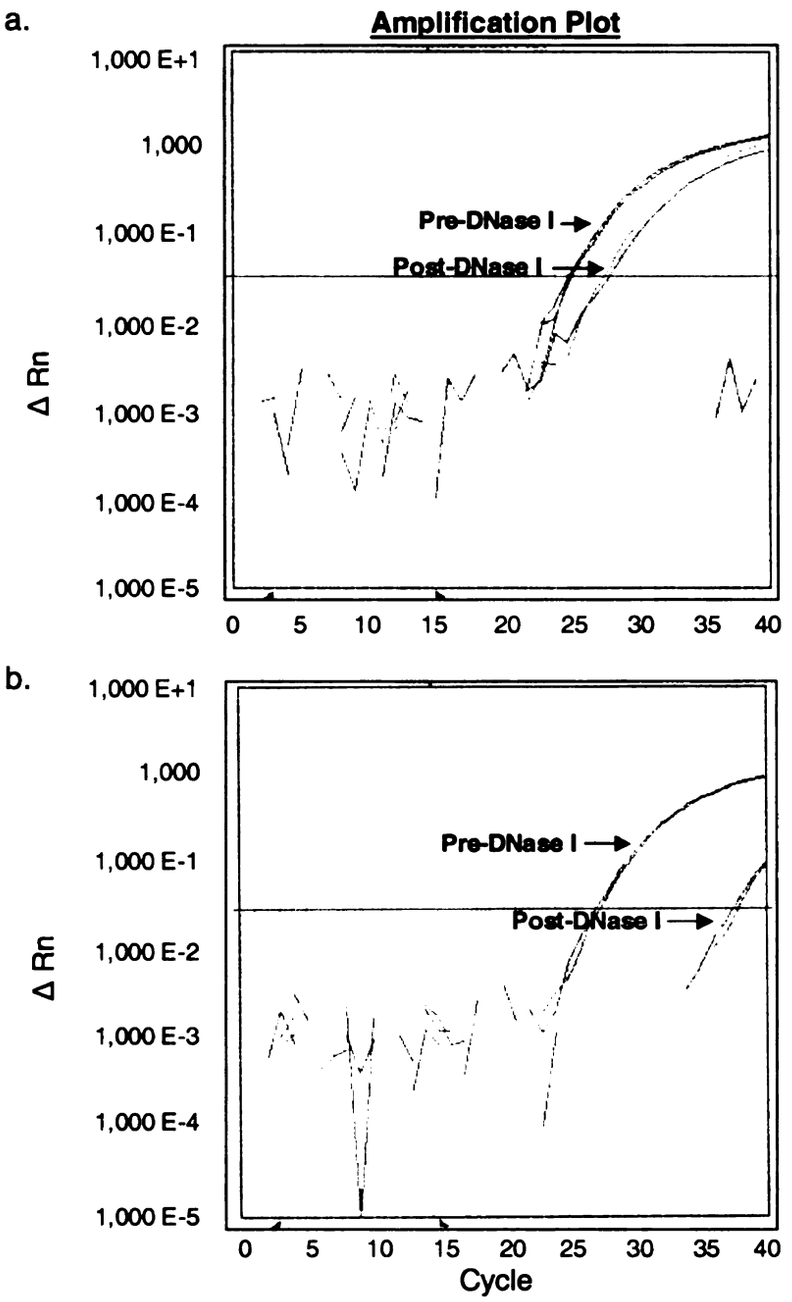

Figure 4.8. **Effect of genomic DNA contamination on RNA estimation.** Taqman real-time RT-PCR amplification before profiles pre- and post-DNAse I treatment of samples for a. *CBF2* and b. 18S rRNA, in technical duplicates. The y-axis represents fluorescence emission and the x--axis represents cycle numbers. Each amplification curve represents a single well, and every RIL sample is measured in triplicate wells. The horizontal red line depicts the threshold at which the C_t value for each amplification curve was determined.

of RNA quantification. Different methods of DNase I treatment, including the On-column® DNase I treatment kit from Qiagen, DNase I (Roche) treatment followed by heat inactivation of enzyme at 65°C, and Turbo DNA free kit from Ambion®, were compared to remove genomic DNA contamination without compromising the RNA integrity and quality of sample for subsequent real-time RT-PCR analysis. The Turbo DNA free kit from Ambion® (ABI) was found to be most effective in removing genomic DNA contamination, following which no amplification was observed for *CBF2* and 18S rRNA in samples before reverse transcription (Figure 4.9a. and b., respectively).

4.2.4 Identification of Outliers and Their Effect on eQTL Mapping

To quantify template levels following real-time qPCR, the cycle number at which the reporter dye emission intensities rise above background noise, called the threshold cycle (C_t), is determined for the exponential phase of the amplification plot. The amount of transcript present in each sample is calculated using the mean C_t values of technical triplicates of each cDNA to account for technical error. ΔC_t values are then determined as the mean of the triplicate C_t value of target transcript minus the mean of the triplicate C_t value for the internal reference transcript (18S rRNA or *Act1* mRNA) and $2^{-\Delta Ct}$ is determined to represent normalized transcript levels in each sample.

Initial analysis of $2^{-\Delta Ct}$ values of RILs, performed using QTL Cartographer, produced peculiar outputs where the program was unable to separate the eQTL signal from the noise (Figure 4.10a). In order to reduce noise in the expression data, it was important to determine the nature of the noise being generated. A normal plot of gene expression values of the entire

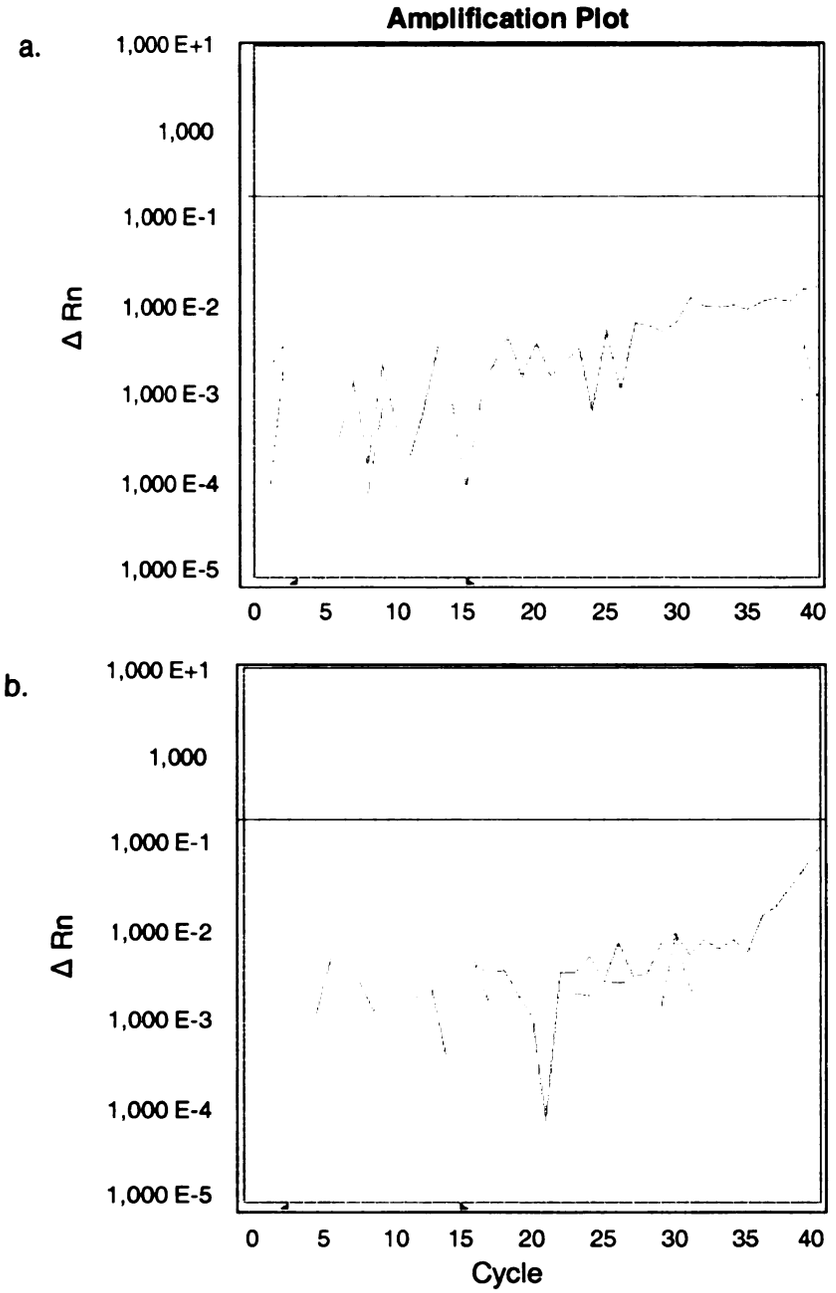

Figure 4.9. **No-RT (reverse transcriptase) samples after DNase I treatment.** Taqman real-time RT-PCR amplification profiles for a. *CBF2* and b. 18S rRNA transcripts in total RNA samples without reverse transcription in samples treated with Turbo DNA free kit (Ambion). The x-axis represents fluorescence emission and the y-axis represents cycle numbers. The horizontal red line depicts the threshold at which the C_t value for each amplification curve was determined.

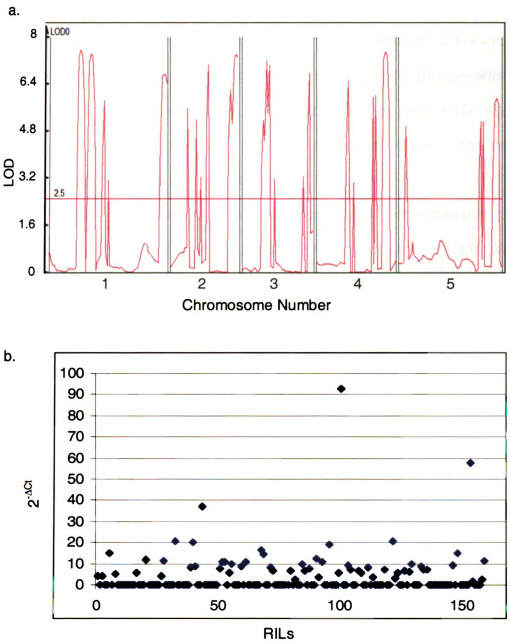

Figure 4.10. **QTL Cartographer output with background noise in data.**

a. eQTL profile generated by QTL Cartographer v2.0 using the complete expression dataset.

b. Scatter plot of expression values for different RILs. The x-axis depicts the RILs and the y-axis shows $2^{-\Delta Ct}$ values (measure of transcript levels)

population, depicting the variability of data, revealed a long tail suggesting the presence of outliers, which was also evident from a scatter plot of the values (Figure 4.10b.). Data points showing very high expression levels were omitted and subsequent analysis resulted in elimination of the false eQTL signal (Figure 4.11a. and b.). This confirmed presence of outliers that were introducing noise in the phenotype data.

In order to remove this noise, statistical analysis of expression data was performed to identify the deviants. Standard deviation is the most commonly used measure for assessing the variability of a dataset. It is defined as the square root of variance, which is roughly the arithmetic average of the squared distance from the mean. As expected, squaring the distance from the mean would result in higher impact of values that are further from the mean. Therefore, the standard deviation can be greatly affected by outliers. To avoid the effect of outliers on the estimate of deviation, Median Absolute Deviation (MAD), an alternate method of determining the variability of data was used to detect outliers in the expression data. MAD is the variation of the average absolute variation, which is based on the median value and therefore is not affected by outliers.

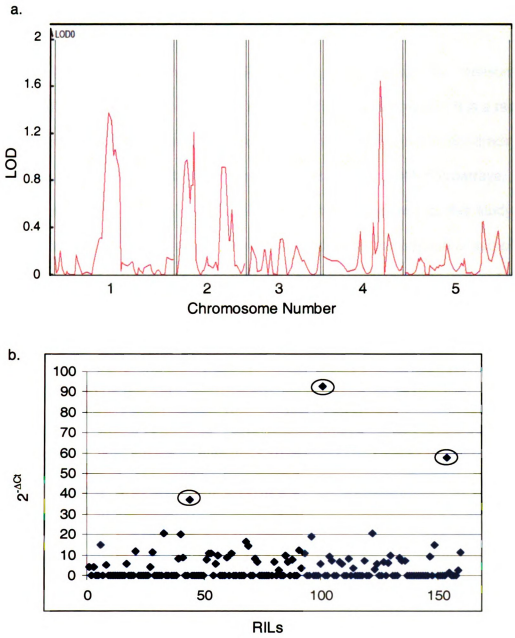

Figure 4.11. **Removal of outliers reduced noise in the dataset.**

a. eQTL profile generated by QTL Cartographer v2.0 using the expression data lacking information form three RILs.

b. Scatter plot of expression values for different RILs depicting, in circles, the values that have been ommitted. The x-axis depicts the RILs and the y-axis shows $2^{-\Delta Ct}$ values (measure of transcript levels).

4.3 Discussion

Taqman® real-time RT-PCR was an ideal method for measuring molecular phenotype with the accuracy desired for eQTL analysis. It is a rapid and high-throughput method that allows simultaneous analysis of hundreds of samples at a cost lower than the alternate method using DNA microarrays. An advantage of real time RT-PCR assay particularly significant for this study is the wide dynamic range of this method, which permits comparison of samples containing hugely different levels of RNA. Furthermore, the sensitivity of this technique allows detection of poorly transcribed mRNA and subtle differences in RNA levels amongst samples.

Several steps were taken to deal with the challenges that can arise while using this method and to reduce variability of the experimental procedure. Sequence comparison of *CBF2*, *Rav1*, and *Zat12* genes from the parental accessions, Cvi-0 and L*er*, was performed to ensure that common primers and probes could be used to detect cDNAs arising from both genotypes. This comparison revealed high sequence conservation of the coding regions of these genes between the two accessions, allowing design of primers and probes demonstrating equal efficiency of amplification and detection of transcript arising from both genotypes. The specificity and optimal conditions for the primers and probes were established using regular PCR as well as real-time PCR in the ABI Prism 7900 light cycler.

The high sensitivity of this technique resulted in its susceptibility to minute amounts of contaminating RNA that could be introduced through aerosol contamination of the surrounding work area, amongst others. Another

source of contamination that is intrinsic to this technique is the carryover contaminating DNA in the recombinant *Taq* polymerase commercial enzyme preparations. Inclusion of no-template controls along with each run was important to preclude contamination of samples. No-template controls using 18S rRNA specific primers and probes revealed amplification signals that were significantly above the background noise. Furthermore, detection of such signals varied with the batch of enzyme used. This contamination was found to be specifically problematic for 18S rRNA estimations. It was critical to resolve this problem due to the impact of the internal reference on the overall accuracy of transcript measures for the genes of interest. The contamination problem evident in no-template controls was overcome by the use of two-step method for real time RT-PCR.

The most critical consideration for accuracy of transcript measurement is the quality of RNA sample. This relates to the integrity of the RNA (free of degradation) and purity of sample. Inhibitors that tend to co-purify during RNA extraction and are carried over from reverse transcription of the sample can have a significant impact on the method. To obtain high quality RNA, extractions were performed using a commercially available silica column-based method (RNeasy RNA extraction kit from Qiagen, Inc.). However, this resulted in purification of contaminating DNA in total RNA extracts of RILs. This method has been previously reported to contain contaminating DNA, with RNA constituting only 50-80% of total nucleic acid. The genomic DNA contamination detected in the samples was interfering with the accuracy of transcript measurement, necessitating use of DNase I treatment before real-time RT-PCR.

Consistency in the reagents used for different samples and transcripts was essential to make data comparable across the biological replicates and different genes analyzed in this study. To achieve consistency, cDNA synthesis for RILs from the two independent biological experiments were performed in parallel, using a common reagent mix. Furthermore, for qPCR of each gene, common reagent mix was used for both biological replicates and each cDNA sample was measured in triplicates within each light cycler run.

Although real-time RT-PCR provides several benefits for use in analysis involving large sample sizes, it is critical to optimize the method at several levels. The need for high technical reproducibility of transcript measurement in this study required careful consideration of factors mentioned above. Ultimately, through these optimization studies, this method was able to achieve high reproducibility for identification of eQTLs for the different genes included in this study.

Chapter 5

Discussion and Implications for the Future

Cold acclimation has been the subject of intense research for several years. Despite considerable success in understanding various biochemical, physiological and gene expression changes occurring during cold acclimation, it is not yet clear how plants perceive the low temperature signal and regulate early changes in gene expression that ultimately lead to freezing tolerance. Whole genome transcriptional analysis has led to the identification of genes that are differentially expressed in response to low temperature. Clustering of these genes based on common expression patterns suggests existence of several gene regulatory pathways at low temperatures.

In this study, natural variation observed in low temperature responsive gene expression was successfully used to identify genetic loci that regulate the differential expression of early responsive genes. Analysis of nine low temperature responsive genes identified ten regulatory eQTLs involved in both common and unique regulation of different genes. Collectively, the data allowed construction of a pathway depicting the regulatory positions of the identified eQTLs (Figure 5.1).

As mentioned previously, extensive "cross-talk" is known to occur between different abiotic stresses such as cold, drought and salinity. Due to significant overlap in the gene regulatory networks, many regulatory components may be shared for drought and cold. Although these genes would lack a selective pressure with regard to low temperature, they would experience selective pressure due to other stresses such as drought and

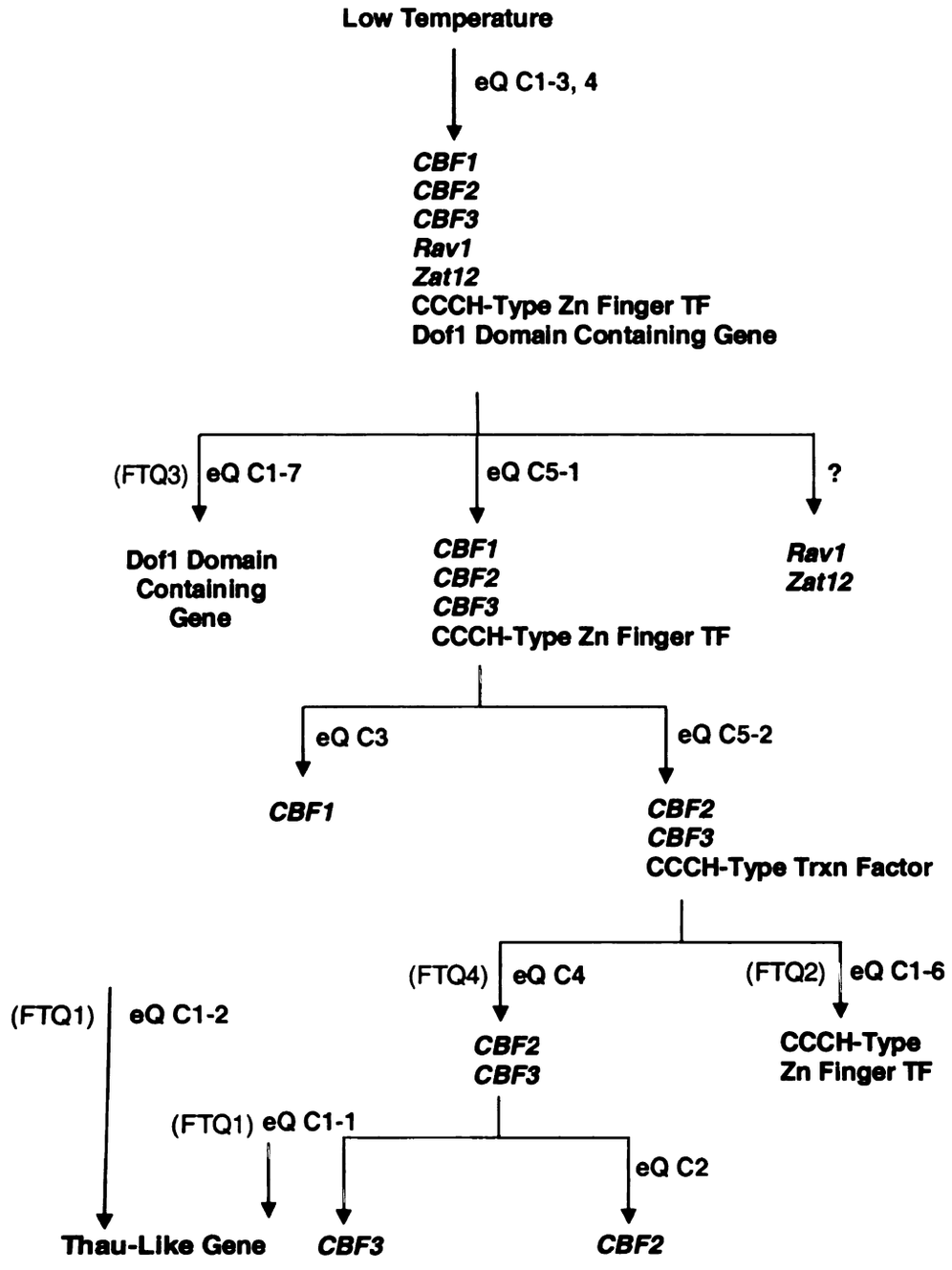

Figure 5.1. **Model of gene regulatory pathway**. The regulation of nine low temperature responsive genes has been proposed to be to regulated by the eQTLs (denoted in blue text) identified in the present study. Overlapping freezing tolerance QTLs, identified by Alonso-Blanco et al. (2005), have been indicated within brackets next to the corresponding eQTL.

salinity. Consequently, regulatory genes shared between cold and drought stress would not be identified using this population. Therefore, eQTLs identified in this study are likely to contain genes whose products are specifically involved in regulation of the low temperature response in Arabidopsis. This is supported by the observation that a limited number of eQTLs were identified in this study, which were commonly regulating several low temperature responsive gene. The independent identification of these eQTLs for more than one low temperature responsive genes and not for the housekeeping gene *Act1* raised the confidence in their regulatory role at low temperatures. Additional confidence in the eQTLs comes from the observation that some of them coincide with freezing tolerance QTLs reported by Alonso-Blanco et al (Alonso-Blanco et al., 2005). The QTL contributing to freezing tolerance on chromosome 4, FTQ4, was found to overlap with eQ C4 identified for *CBF2* and *3* regulation (Figure 5.2, 2.8 and 2.9, respectively). Two photoperiod-dependent freezing tolerance QTLs on chromosome 1, FTQ2 and FTQ3, were coincident with eQTLs identified for ERF and Dof1-like domain containing genes (Figure 5.2, 3.13, and 3.14). The short-day photoperiod specific FTQ2 was co-incident with eQ C1-5 identified for ERF, whereas the long-day photoperiod specific FTQ3 was co-incident with eQ C1-7 identified for Dof1-like domain containing transcription factor gene. Finally, the freezing tolerance QTL FTQ5 on chromosome 5, specific for short-day photoperiod, was found to overlap with eQ C5-1 identified for *CBF1, 2, 3* and CCCH-type Zn finger containing transcription factor gene expression. In addition to identification of eQTLs in regions overlapping with freezing tolerance QTLs, this study

uncovered eQTLs for regulation of low temperature responsive gene expression, which were not detected in the study by Alonso-Blanco et al.

The above-mentioned study on freezing tolerance QTLs demonstrated that higher freezing tolerance was attributable to the L*er* allele at the QTLs, whereas mostly Cvi-0 alleles were found to lead to higher expression of low temperature responsive genes in the current study. This could be explained, firstly, by the physical proximity of the genes giving rise to freezing QTLs or eQTLs for low temperature responsive gene expression, where one may be contributed by L*er* parent and the other by Cvi-0. In fact, several low temperature regulatory genes may be located in clusters, causing certain regions of the genome to be hotspots for genes involved in different aspects of cold acclimation. Secondly, it is possible that QTLs identified through analysis of freezing tolerance and low temperature responsive gene expression are due to the same gene.

Although induction of specific genes is a common strategy employed to combat stressful environmental conditions, negative regulation of genes plays an equally important role in the adaptation process. The transcripts analyzed in this study are undetectable under normal growth conditions, quickly increasing in levels in response to low temperature followed by a subsequent decrease. The adjustment in expression levels of these genes may involve the role of transcriptional repressors, which may be underlying the eQTLs and freezing tolerance QTLs. Negative regulators that play a role in ensuring specific transcript levels of target genes during cold acclimation would lack the selective pressure to maintain highly effective alleles in Cvi-0 (because of warm temperatures in its natural habitat). Weaker alleles of these genes could

result in increased expression of their targets and therefore result in Cvi-0 contributing a positive allele for gene expression. However, the ultimate effect of an inability to decrease expression of some genes could have a negative effect on cold tolerance. Thus, the same allele could be identified as having a positive effect on gene expression, while having a negative effect on cold tolerance.

Previous studies indicate that plants appear to sense the temperature shifts in their environment and fine-tune gene expression levels through the different stages of cold acclimation (Zarka et al., 2003). Zarka et al demonstrated the elegance of the *CBF* transcriptional response to changes in environmental temperature. The study showed that repeated transfer of plants between warm and cold environments de-sensitized *CBF* genes such that the levels of *CBF* transcripts obtained in subsequent cold shock were significantly diminished. Cold-acclimated seedlings did not show a detectable increase in *CBF* transcript levels after a short (<8h) shift to warm temperatures and return to cold, however, longer (>8h) exposure to warm temperatures caused induction of *CBF* genes. These observations indicate tight regulation of *CBF* expression through a refined cold-sensing mechanism that must use transcriptional activators as well as repressors. Other low temperature responsive genes may also experience such tight transcriptional regulation through roles of positive and negative regulators as suggested by the finding that certain Cvi-0 alleles result in higher expression of genes.

Identification of eQTLs in this study was achieved by using two different internal references for the normalization of expression data. Analysis of expression data normalized with 18S rRNA and *Act1* mRNA levels resulted

in identification of common and distinct eQTLs. For instance, the eQ C4 found to regulate *CBF2* and *3* expression was detected using both normalization genes. However, eQ C1-3 and C1-4 were identified for *CBF1*, *2*, *Rav1*, *Zat12* etc only in the *Act1* normalized dataset. Use of more than one reference genes is ideal to obtain maximum information from a gene expression study. There is still debate in the scientific community about whether the data from different normalization controls should be combined and how best to do so. It has been suggested that geometric mean of multiple normalization controls be used for accurate RT-PCR expression analysis (Vandesompele et al., 2002).

For the purpose of this study, the expression data normalized with the two reference genes was analyzed separately and the results presented independently. Some differences observed in the eQTL profiles generated using 18S rRNA and *Act1* mRNA normalized data can be explained in two ways. Firstly, the vast diference in transcript abundance for 18S rRNA and the genes being analyzed in this study would limit the accuracy of normalization. Secondly, the rRNA genes, which are present as arrays in the nucleolus organizer regions (NOR) in the genome, show variable cytosine methylation in different accessions of Arabidopsis *thaliana* (Riddle and Richards, 2002). This variation in cytosine methylation of NOR has been attributed to five QTLs using the RIL population from Cvi-0 X L*er* (Riddle and Richards, 2002). Cytosine methylation is used to achieve heritable transcriptional silencing of genes and variable levels of it in the segregating population would imply variable 18S rRNA levels (Riddle and Richards, 2002). Therefore, it was considered likely that some information on variation in low temperature

responsive gene expression was not recovered using 18S rRNA as a normalization control. To reduce the error rate in identification of eQTLs using the normalized gene expression data, it was necessary to select a normalization gene that was not differentially expressed in the segregating population. Thus, for the second reference gene, *Act1*, expression data from the RIL population was analyzed using CIM to ensure there were no eQTLs leading to variable expression of this gene in the population.

A common approach used to identify genes contributing to the eQTLs involves analysis of select candidate genes located in the regions containing the eQTLs. The genomic regions underlying each eQTL contained hundreds of genes in the support interval of 2 LODs. Based on functional predictions, a list of putative candidate genes encoding regulators of transcription at low temperature was generated. This list included several signaling proteins such as GTPases, mitogen-activated protein kinases, receptor-like kinases, tyrosine protein kinases, protein phosphatases, phosphatidylinositol-4-phosphate 5-kinases, two-component response regulators, receptor kinases, Ca^{2+} binding proteins, Calcineurin B-like proteins and Ca^{2+}-dependent kinases. Additionally, these regions also contain transcription factors, chromatin-remodeling factors, histone deacetylases, miRNAs, ankyrin-repeat containing proteins, and ubiquitin ligases.

Low temperature stress is known to elicit spatial and temporal Ca^{+2} signaling in plants and Ca^{+2} has been shown to act as a second messenger mediating low temperature response (Monroy and Dhindsa, 1995; Monroy et al., 1998), a Ca^{+2}-dependent protein kinase (CDPK) has been shown to upregulate a low temperature responsive promoter in barley (Sheen, 1996).

The Ca^{2+} signal in plants is relayed by several known classes of Ca^{2+}-binding proteins, including calmodulins, Ca^{2+}-dependent protein kinases (CDPKs), and calcineurin B-like proteins. CDPKs comprise a class of Ca^{2+} sensors, possessing both protein kinase and Ca^{2+}-binding domains in a single polypeptide, allowing them to detect and relay the Ca^{2+} signal (Roberts and Harmon, 1992). The Arabidopsis genome is predicted to contain 34 CDPK genes, some of which are induced in response to abiotic stresses, such as the *AtCDPK1* and *AtCDPK2* genes, which are induced in response to drought and salt stress (Urao et al., 1994). Furthermore, *AtCPK10* and *30* have been demonstrated to activate a stress-inducible promoter in protoplasts (Sheen, 1996). In rice, *OsCDPK13* responds to low temperature stress and its overexpression results in enhanced cold tolerance of the transgenics (Abbasi et al., 2004). The genomic regions encompassing the eQTLs identified in the present study contain *CDPK2*, *6* and *9*, which may play a role in low temperature signaling mediated by a Ca^{+2} signal.

In addition to the CDPKs, the list also contains Ca^{2+} sensing proteins such as calmodulins (CaM) and Calcineurin B-like (CBL) proteins. CaM is a ubiquitous calcium-binding protein found to regulate diverse cellular functions by modulating the activity of a variety of proteins (Kim et al., 2007). CBL proteins are similar to the regulatory B subunit of calcineurin protein of animals (Kudla et al., 1999), that can bind Ca^{2+} and the CBL interacting protein kinases (CIPKs) to relay the signal in cell (Shi et al., 1999). In rice, calreticulin CRT1, is suggested to play a role in low temperature stress (Li et al., 2003). The region encompassing the eQTLs contain *CAM1*, *4*, and *6*,

CBL3, *7*, and *8*, *CRT2* and *3*, *AtCIPK6*, *8*, and *25* and several Ca^{2+} binding proteins.

A Ca^{2+} sensing GTPase in Arabidopsis was found to play a role in stress-related Ca^{2+} signal transduction. Its activity was regulated by changes in Ca^{2+} concentration, and the gene was found to be induced by ABA and salt stress and knock-out mutants were found highly sensitive to ABA and salt treatments (Jayasekaran et al., 2006) GTPase mediated signal transduction in Arabidopsis *thaliana* has been reported to be involved in diverse developmental processes and hormone signaling and the genome is predicted to encode 93 small GTPases (Li et al., 2001; Yang, 2002).

Mitogen-activated protein (MAP) kinase cascades have been shown to regulate several signaling pathways in diverse organisms (Teige et al., 2004). The MAP Kinase Kinase 2 (MKK2) is specifically activated by low temperature in Arabidopsis (Teige et al., 2004). Furthermore, plants overexpressing MKK2 exhibit increased freezing tolerance and *mkk2* null mutants show hypersensitivity to cold stress. In relevance to this study, the MKK2 overexpressors revealed upregulation of the low temperature responsive genes *CBF2*, *CBF3 Rav1*, *ZAT12*, and several ERF genes (Teige et al., 2004). The eQTL regions contain several components of the MAPK pathway, including ATMKK3 and 10, ATMPK1 and 13, MAPKKK7, 13 and 18, and MEK1.

Members of another family of signaling proteins, protein phosphatases, were detected in the regions of interest. PP2A is suggested to function in low temperature signaling in alfalfa (Monroy et al., 1993; Monroy and Dhindsa, 1995) and *AtPP2CA* gene is proposed to negatively regulate cold acclimation

in Arabidopsis since antisense inhibition of the protein caused inhibition of cold acclimation in transgenic plants (Tahtiharju and Palva, 2001).

Additional signaling genes that may be involved in the low temperature responsive gene expression and were found to be present in the genomic regions identified using eQTL analysis encode receptor protein kinases such as ARK1 and 2, histidine two-component response regulators such as ARR4 and 24, and inositol polyphosphate 5 phosphatase such as IP5P1 and other members of this family.

The transcription factor encoding genes present in the chromosomal regions underlying the eQTLs include members of the MYB, NAC, and PHD family of proteins. The nascent polypeptide-associated complex (NAC) domain-containing proteins constitute one of the largest families of transcription factors in Arabidopsis with more than a hundred members. These proteins are characterized by a conserved N-terminal NAC domain that can bind DNA as well as proteins. Five Arabidopsis NAC proteins are predicted to contain two NAC domains in tandem, a transmembrane region and nuclear import and/or export signals (At1g60280, At1g60300, At1g60340, At1g60350 and At1g60380). These proteins were found to exist as membrane-bound precursors that are proteolytically cleaved following a stimulus, allowing nuclear localization of the transcription factor (Olsen et al., 2005).

miRNAs (microRNAs) comprise a class of noncoding RNAs with regulatory roles in gene expression (Vella and Slack, 2005; Gottwein et al., 2007; Guarnieri and Dileone, 2008; Mattes et al., 2008; Negrini and Calin, 2008; Schultz et al., 2008; Tang et al., 2008). Several miRNAs are located in the regions encompassing the eQTLs. MIR319 has been reported to be

upregulated in response to abiotic stress (Sunkar and Zhu, 2004). MIR319, along with MIR159, is known to target several members of the *MYB* transcription factor genes and five *TCP* gene (Palatnik et al., 2007). However, its role at low temperatures is not yet established. Sequence homology of the miRNAs with low temperature regulated genes would suggest direct regulation of the homologous targets.

In conclusion, using natural genetic variation, this study narrowed down the genomic regions of Arabidopsis containing regulatory genes with a role in low temperature responsive gene expression. These regions, except eQ C1-1 that coincides with location of *ICE1*, contain novel regulatory genes since none of the known low temperature associated genes were located in these regions. These previously known low temperature associated genes have mostly been identified using mutational approaches, which are unable to detect subtle changes in genome that may not have profound effects on gene expression despite their occurrence in the regulatory genes. Using the eQTL approach it was possible to identify genomic regions responsible for the subtle differences in low temperature responsive gene expression. Several putative candidates are present the in the regions underlying the eQTLs. Ultimately, the genes underlying the eQTLs would have to be identified to determine the genetic pathways functioning at low temperature. Fine mapping of the eQTLs and confirmation of their regulatory effect through generation of NILs (near isogenic lines) will lead to identification of the underlying genes. Efforts are ongoing to develop a resource for a tilling array of NILs along the entire length of Arabidopsis chromosomes. Using NILs containing regions identified in this

study, the process of identifying the underlying sequence variations will be greatly hastened.

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