Effects of Chronic Cold Treatment on Root Elongation and Gene Expression in *Arabidopsis thaliana*

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Summary

Low temperature is a major limitation of plant growth. Cold adaptation is important for the survival and distribution of plant species at high elevations and high latitudes. Much is known about the molecular basis for cold acclimation and freezing tolerance, which are triggered by acute cold treatment. The causes of growth limitation at low, non-freezing temperatures are largely unexplored. To better understand the mechanisms limiting plant growth in cold environments, I studied the elongation-growth of roots and patterns of gene expression in *Arabidopsis* accessions from diverse habitats. *Arabidopsis thaliana* (L.) Heyhn is a small, annual weed that is widely distributed in different growth environments and is well-suited for molecular genetic studies. My initial study of 23 accessions failed to detect ecotypic differentiation for root elongation rates at low, non-freezing temperatures (10 °C); however, evidence was obtained implicating the cell-cycle gene *CYCB1;1* as part of a compensatory mechanism for maintaining proliferation under these conditions.

I used microarray technology to obtain a global picture of cold-responsive gene expression in the temperate Col-0 accession and the high-altitude (3400 m) Sha accession, which is expected to be adapted for a cold environment. I compared the effects of acute-cold treatment (4 h at 10 °C) and chronic-cold treatment (6 weeks at 10 °C) using plants grown at 21 °C as a control. Cold-treatment had major effects on gene expression at the mRNA level: 11% of the 24,000 genes represented on the Affymetrix ATH1 GeneChip responded by at least 2-fold to either or both cold treatments. A substantial fraction of cold-responsive genes, 35%, responded specifically to chronic cold treatment. This suggests there are fundamental differences in the response of plants to acute-cold treatment and growth at low, nonfreezing temperatures. Datasets of annotated genes were screened for significant, non-redundant enrichment for Gene Ontology (GO) terms to identify functional groups and processes. GO-term enrichment provided a rough picture of major trends in gene expression associated with cold-responses, which were then verified by examining the expression patterns of individual genes. Flavonoid biosynthesis, particularly the activation of anthocyanin biosynthesis, was the only major function induced by both acute- and chronic-cold treatment. In contrast, genes concerned with electron transport and light-reactions in photosynthesis were repressed by both cold treatments. This is consistent with the well-documented, general reduction of these functions associated with growth at low temperatures. Thus,

regulation at the mRNA level appears to be an important mechanism for down-regulating energy metabolism in cold environments.

Acute-cold treatment induced numerous genes concerned with responses to pathogen infection, cold, drought, salt stress, and UV damage. The breadth of these stress responses emphasizes that brief exposure to cold, even at temperatures as high as 10 °C, is perceived by plants as a form of stress. Unexpectedly, global induction of stress-related genes was restricted primarily to the acute-cold response. This strongly suggests that in contrast to “cold shock,” growth at low, non-freezing temperatures is not recognized by *Arabidopsis* plants as a stress per se. Therefore, mechanisms exist for suppressing prolonged stress responses in the cold. This implies that general stress responses are not essential for growth of *Arabidopsis* at low temperatures.

Several other processes and pathways responded primarily to chronic- cold treatment and are likely to be relevant to growth at low temperatures. Sha-specific, chronic-cold induction of genes encoding ion transporters; genes concerned with compensation for Pi deprivation; and, genes required for formation of root hairs, comprised the only major functional group showing ecotypic differentiation. Induction of genes encoding primary wall constituents and enzymes concerned with cell enlargement and pectin metabolism were induced specifically by chronic-cold treatment, while those genes important for secondary wall formation such as those encoding cellulose synthase and laccase required for lignification were repressed. These findings and the cold-repression of genes concerned with fiber and vascular tissue formation suggest as a working hypothesis that chronic cold treatment increases the flexibility of roots and cell wall extensibility as a compensatory response to the reduced root growth in the cold. In summary, the present study identified several functional groups of genes showing novel regulation by chronic cold treatment. These findings provide the starting point for future studies using informative mutants and biochemical profiling to establish causal relationships between gene expression and adaptations for growth in cold environments.

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1.0 Introduction

Low temperature is a major limitation of plant growth. Adaptation to low temperature is important for the survival and distribution of high elevation or latitude plant species. Although cold-adapted plants are commonly not damaged by temperatures between 0 °C and 10 °C, tissue formation is inhibited at these temperatures, while photosynthesis continues at relatively high rates (Körner 2006). Much is known about acclimation and regulation in response to freezing temperatures, but the causes of growth limitation at low positive temperatures remained unexplored.

Although many studies have been carried out to understand how very low, but still positive temperatures affect plant growth, much less is known about the actual mechanisms involved that facilitate meristematic activity at low temperatures in some taxa or genotypes and not in others. This is an issue of fundamental significance for cold-climate agriculture, but also for the understanding of plant life in arctic and alpine life conditions. To better understand the mechanisms of low temperature limitation of plant growth in cold environments, I studied the elongation-growth of roots and patterns of gene expression in natural *Arabidopsis thaliana* accessions from diverse habitats. This chapter provides an introduction and summary of the present knowledge of biochemical, physiological, and molecular changes of plants in response to low temperatures. I also provide the background and rational for using *Arabidopsis* accessions as model system for studying these responses.

1.1 Plants and cold environments

More than 70% of the earth exists as cold ecosystems that have stable temperature below or close to the freezing point of water (Strahler 1984). Plants have adapted to living in cold climate environments, enabling them to colonize high mountains and sub-arctic regions. Numerous investigations have shown that ambient temperatures recorded from a nearby meteorological station is not relevant to the temperature of plant tissues (Körner & Larcher 1988). Plant structure can exaggerate large altitudinal differences in air temperature. For example, temperature measured during day time at the leaf of a highland tree can be lower than the ambient temperature, while a leaf of prostrate plants, may be warmer at high altitude than at low altitude. Direct sunlight radiation in arctic and alpine regions can even raise the temperature of compact, low

rosette plants above their heat tolerance (Körner 2003). In the greenhouse under controlled conditions, daytime plant temperatures are generally higher than the air temperatures, while nighttime plant temperatures are generally lower than the air temperature. For example, in greenhouse-grown *Vinca* the nighttime temperature of the shoot tip was 0.5 - 5.0°C below air temperature, whereas the daytime temperature of the shoot tip was up to 4 °C higher than the air temperature (Faust & Heins 1998).

Low temperature, especially below-ground low temperature could limit growth of plants at high latitudes or altitudes (Körner 1998; Körner 2003; Körner & Paulsen 2004). By using a data-logger buried below-ground in the high altitude treeline regions, the global minimum ground temperatures allowing growth of root of tree species has been estimated to be between 6 °C to 8 °C (Körner & Paulsen 2004). By maintaining the shoot growth temperature of tree species at 22 °C to allow optimum rates of photosysthesis and variable root temperatures ranging from 2 °C to 16 °C, critical temperature for significant root growth of tree species frequently found in both lowland and alpine treeline region is approximately 6 °C (Alvarez-Uria & Körner 2007). These experimental results indicated that these threshold temperatures are critical for plant metabolism and permits synthesis of new tissue that allows growth at such temperatures.

Depending on the cold tolerance of a species, freezing (sub-zero) or chilling (above zero) temperatures can cause injury and always interrupt growth (Hughes & Dunn 1990; Schneider, Nielsen & Somerville 1995; Pearce 1999). Most tropical and subtropical plant species lack the ability to adapt to freezing temperature and are typically injured by chilling temperature. On the other hand, most temperate plants exhibit cold acclimation, i.e., increased tolerance to freezing after exposure to low, non-freezing temperatures (Thomashow 1999). Cold acclimation in temperate plants is initiated when the ambient temperature progressively decreasing in late autumn or early winter. Depending on plant species, it may take a few days to several weeks to fully acclimated and reach maximum levels of freezing tolerance ranging from -10 °C to -30 °C (Gilmour, Hajela & Thomashow 1988; Webb, Uemura & Steponkus 1994; Antikainen & Griffith 1997b; Thomashow 1999).

The cell membrane is believed to be a primary site of freezing injury in plants. Formation of ice inside of plant cells directly and irreversibly damages plasma membranes resulting in the loss of selective permeability. For non-freezing tolerant plants, formation of ice in intercellular spaces can also lead to membrane damage as a result of cell dehydration due to the movement of liquid water from cells to the extracellular compartment. As a consequence, extracellular freezing leads to cellular dehydration (Steponkus 1984). Several forms of membrane damage resulted from this freeze-induced dehydration have been observed, including expansion-induced-lysis,

lamellar-to-hexagonal-II phase transition, and fracture jump lesions (Gordon-Kamm & Steponkus 1984; Steponkus 1984; Webb *et al.* 1994).

Freezing-tolerant plants have evolved mechanisms to protect against freezing injury. For example, changes in lipid composition during cold acclimation have been shown to stabilize membranes to prevent expansion-induced-lysis and the formation of hexagonal II phase lipids (Gordon-Kamm & Steponkus 1984; Steponkus 1984). During cold acclimation, winter rye leaves produce intrinsic ice nucleators that initiate the formation of extracellular ice during freezing. The formation of extracellular ice is essential for the survival of freezing-tolerance winter rye that have the ability to restrict ice formation to extracellular areas and prevent physical damage to the cells (Brush, Griffith & Mlynarz 1994). In some plants species, cold acclimation results in the accumulation of metabolites that promote supercooling and, hence, delay freezing of water in tissues (Ishikawa 1984; Lindstrom & Carter 1985; Körner 2003).

1.2 Cold sensing and the molecular basis of cold acclimation

The adaptation of plants to cold depends on perception of low temperatures, transduction of cold-induced signals, and finally the regulation of cold-responsive genes that encode transcription factors or effector proteins to cope with the diverse stresses resulting from the exposure to sub-zero temperatures. Many features of the response to cold stress are common to responses to other stresses such as drought and salinity. This implies the existence of mechanisms for integrating the diverse responses and linking the responses to growth and development. (Chinnusamy, Schumaker & Zhu 2004; Yamaguchi-Shinozaki & Shinozaki 2006).

1.2.1 Cold signaling pathways

The plasma membrane is both a primary site of freezing-induced injury and important for perception of cold signals. Increasing the rigidity of plasma membranes by treatment with dimethylsulfoxide and destabilizing actin microfilament by cytochalasin D treatment at 25 °C have been shown to increase calcium influx, expression of cold acclimatization-specific genes, and freezing tolerance of alfalfa protoplasts. Conversely, these effects, which are associated with cold acclimation, could be prevented by treating protoplasts at 4 °C with benzyl alcohol to increase membrane fluidity and by treating the protoplasts with jasplakinolide to stabilize actin microfilaments (Örvar *et al.* 2000). It is believed that a plasma membrane-associated two-component regulator transduces the signal generated by cold-reduced membrane fluidity to regulate down-stream genes (Los & Murata 2004). In the prokaryote *synechocystis,* the first component regulator in cold-sensing is the histidine kinases Hik33 and Hik19 and the second component is *Response regulator1* (*Rer1*) (Suzuki *et al.* 2000; Los & Murata 2004). It has been proposed that reduced membrane fluidity triggers the autophosphorylation of Hik33, and the subsequent transfer a phosphate group to Hik19 and then to Rer1 (Suzuki *et al.* 2000). Although no homologues of Hik33 and Hik19 have been identified in seed plants, there is evidence that the *Arabidopsis* histidine kinase AtHK1 is a sensor of abiotic stress (Urao *et al.* 1999). The expression of the AtHK1 is increased in response to changes in osmolarity resulting from to cold, salinity and dehydration stress. Moreover, *AtHK1* can complement yeast double mutants lacking the osmosensor SLN1 and SHO1 suggesting that AtHK1 histidine kinase has an evolutionarily conserved function in transducing signals induced by osmotic stresses to the nucleus through a phosphorylation cascade (Urao *et al.* 1999). To date, however, the actual sensor or sensors of low temperature in plants has not been identified.

It has been proposed that changes in membrane fluidity and the actin cytoskeleton are key, early steps in cold sensing. According to this view, cold decreases the fluidity of membranes, leading to rearrangement of the actin cytoskeleton. This then activates Ca^{2+} channels, and increases cytosolic Ca^{2+} levels that then trigger expression of *COR* genes in the cold-responsive pathway (Örvar *et al.* 2000; Sangwan *et al.* 2001). Previous studies have shown that cytosolic free calcium concentration controlling the cold-responsive gene expression (Knight, Trewavas & Knight 1996; Tähtiharju *et al.* 1997) and acquisition of freezing tolerance (Monroy, Sarhan & Dhindsa 1993; Monroy & Dhindsa 1995). The rapid influx of Ca^{2+} ions into plant cells under cold stress or release of cold-induced vacuolar Ca^{2+} ions suggest that Ca^{2+} channels or some non-specific ion

channels might function as cold sensors in plants (Knight & Knight 2000; Örvar *et al.* 2000; Sangwan *et al.* 2001).

The microtubules of higher plants are affected by cold treatment (Bokros *et al.* 1996; Nick 2000). In addition, microtubules depolymerize rapidly, become shorter, and less abundance after exposure to low, non-freezing temperatures (Bartolo & Carter 1991; Bokros *et al.* 1996; Olinevich & Khokhlova 2003) and this effect is correlated with decreased in plant cell growth that likely affected the microtubule-guided activity in morphogenesis, chromosome movement, and organelle transport (Pihakaski-Maunsbach & Puhakainen 1995). Such microtubule depolymerization also triggers the changes in cytosolic calcium and calmodulin calcium signaling that results in general plant reactions to cold (Durso & Cyr 1994; Mazars *et al.* 1997). Interestingly, microtubules of cold-resistant fish (Modig *et al.* 1999; Detrich *et al.* 2000), psychrophilic algae (Willem *et al.* 1999), and yeast (Gupta Jr *et al.* 2001) are cold resistant and have been attributed to specific substitutions of amino acids in the sequence of α-tubulin.

Endogenous ABA concentrations are transiently increased in response to low temperature in plants capable of cold-acclimation, but not in plants incapable of cold-acclimation (Chen, Li & Brenner 1983; Lång *et al.* 1994). Treatment with ABA at warm temperatures has been shown to enhance freezing tolerance in *Arabidopsis* and other plant species, which is associated with patterns of gene expression similar to those resulting from cold treatment. Cold acclimation obtained by ABA treatment is not, however, as effective as that induced by cold treatment (Lång, Heino & Palva 1989; Mantyla, Lång & Palva 1995; Shinozaki & Yamaguchi-Shinozaki 2000; Xiong, Schumaker & Zhu 2002). The current view is that ABA is a secondary signal acting between the primary cold signal and regulation of gene expression. ABA-responsive elements are present in the 5'-upstream sequences of many genes up-regulated at the level of transcription during cold acclimation or drought stress (Yamaguchi-Shinozaki & Shinozaki 1993; Yamaguchi-Shinozaki & Shinozaki 1994; Yamaguchi-Shinozaki & Shinozaki 2005; Yamaguchi-Shinozaki & Shinozaki 2006). The Arabidopsis *aba1* mutant deficient in ABA biosynthesis shows reduced cold acclimatization and freezing tolerance than the wild-type that can be rescued by exogenous application of ABA (Heino *et al.* 1990; Gilmour & Thomashow 1991). In contrast, ABA insensitive (*abi*) mutants show cold-induced freezing tolerance comparable to wild-type *Arabidopsis* plants. Although transcripts accumulation of several ABA-inducible genes is abolished in the warm-grown *abi1* mutant plants in warm condition, accumulation is cold-inducible comparable to wild type (Gilmour & Thomashow 1991). This suggests that both ABA-dependent and ABA-independent pathways contribute to cold acclimation and freezing tolerance (Gilmour & Thomashow 1991; Nordin, Heino & Palva 1991).

1.2.2 CBF-dependent pathway

The activation of upstream cold signaling pathways ultimately affects expression of cold-responsive genes. The most important milestone in understanding cold-regulated gene expression was the discovery of the *Arabidopsis* CBF (CRT binding factor)/DREB1 (DRE-binding factor 1) genes. The major features of the CBF-dependent cold-responsive pathway are summarized in Figure 1.1. Three members of the CBF/DREB1 family genes, CBF1/DREB1b, CBF2/DREB1c, and CBF3/DREB1a are present in a tandem array on chromosome 4 (Stockinger, Gilmour & Thomashow 1997; Gilmour *et al.* 1998; Liu *et al.* 1998; Shinwari *et al.* 1998; Kasuga *et al.* 1999; Thomashow 1999; Shinozaki & Yamaguchi-Shinozaki 2000; Thomashow 2001). They are members of the AP2/EREBP family of DNA-binding proteins (Riechmann & Meyerowitz 1998). These transcription activators bind to one or more copies of a cis-acting element with the core sequence CCGAC designated as the CRT (C-repeat)/DRE (dehydration response element) present in the promoters of *Cold-Regulated* (*COR*) and many other cold-responsive genes. Binding of CBFs to these elements stimulates transcription, and, in the case of the *COR* genes, has been shown to increase freezing tolerance (Baker, Wilhelm & Thomashow 1994; Yamaguchi-Shinozaki & Shinozaki 1994; Stockinger *et al.* 1997).

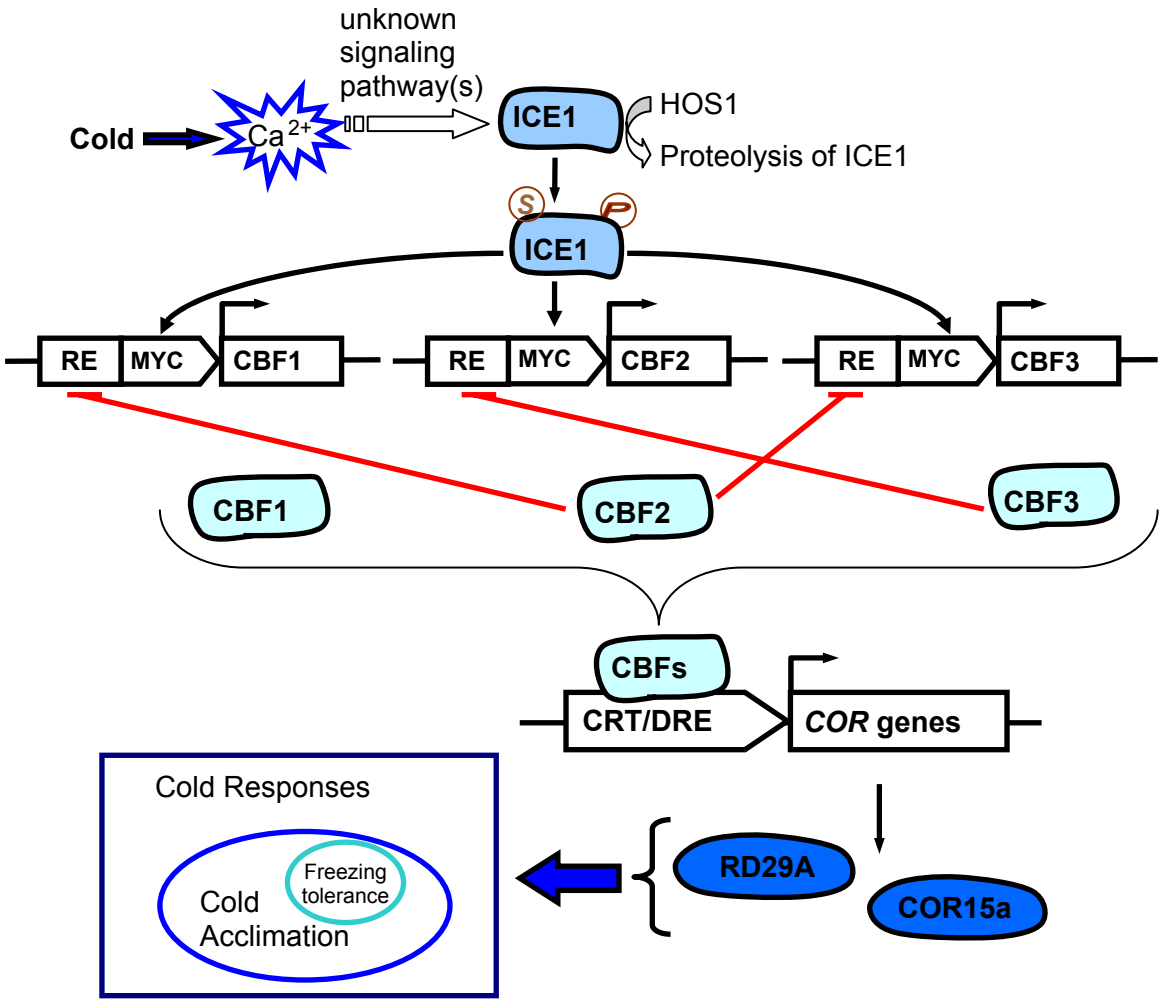


Figure 1.1. A simplified scheme of the CBF regulatory pathway in *Arabidopsis*.

CBF genes are conserved in various plant species including wheat (*Triticum aestivum* L. cv Norstar) and rye (*Secale cereale* L. cv Puma) that cold acclimate, in tomato (*Lycopersicon esculentum* var. Bonny Best, Castle Mart, Micro-Tom, and D Huang) and rice, freezing-sensitive plants that do not cold acclimate (Jaglo *et al.* 2001; Dubouzet *et al.* 2003; Badawi *et al.* 2007) as well as in *Populus spp.*, that are woody plants (Benedict *et al.* 2006). Considerable evidence suggests that *CBF* genes are the "master switches" that control the expression of a group of genes, the CBF regulon, which has roles in freezing tolerance. Expression of CBF/DREB1 under the control of the 35S promoter or stress inducible *RD29A* promoters in *Arabidopsis* results in the induction of a series of *COR* genes and accumulation of compatible solutes. The action of the CBF regulon proteins and accumulation of compatible solutes result in an increase in freezing tolerance in both nonacclimated and cold acclimated plants. This implies that

they are sufficient to trigger the cold acclimation response (Jaglo-Ottosen *et al.* 1998; Liu *et al.* 1998; Kasuga *et al.* 1999; Gilmour *et al.* 2000; Gilmour, Fowler & Thomashow 2004).

CBF genes are rapidly induced by exposure to cold within 15 minutes and reach peak expression after approximately 2 hours (Stockinger *et al.* 1997; Gilmour *et al.* 1998; Liu *et al.* 1998). Several components acting upsteam of the *CBF* genes have been identified. *Inducer of CBF Expression 1* (*ICE1*) encodes a transcriptional activator of the *CBF* genes (Chinnusamy *et al.* 2003). The dominant *ice1* mutation are impaired in cold acclimation and defective in cold regulated expression of *CBF3* and its target *COR* genes. ICE1 is a MYC-like bHLH transcriptional activator that is constitutively expressed in warm temperature. ICE1 binds specifically to the MYC cis-element in the *CBF3* promoter and activates the expression of *CBF3* in response to cold. Transgenic lines constitutively overexpressing *ICE1* do not express *CBF3* at warm temperatures but do show a higher level of *CBF3* but not *CBF1* and *CBF2* expression, and enhanced expression of the target genes *RD29A* and *COR15A* at cold temperatures. This suggests that cold-induced modification of ICE1 protein is needed (Chinnusamy *et al.* 2003). It has been shown that phosphorylation (Zhu, Dong & Zhu 2007) and sumoylation (Miura *et al.* 2007) are necessary for the modification of ICE1 protein to act as a transcriptional activator of CBF3 *in planta,* and hence to activate downstream CBFs and cold-responsive genes. Promoter sequence comparison of *CBF* genes indicated that two defined regions designated as ICEr1 and ICEr2 (Induction of CBF expression region 1 or 2) are *cis*-acting elements that also contribute to the cold responsiveness of *CBF* promoters (Zarka *et al.* 2003).

The *Arabidopsis* loss-of-function mutant *hos1* (*high expression* of *osmotically responsive gene 1*) shows enhanced cold induction of *CBF* genes and their downstream targets (Ishitani *et al.* 1998; Lee *et al.* 2001). *HOS1* encodes a 915-amino-acid-long polypeptide with ubiquitin E3 ligase activity and a RING-finger domain (Lee *et al.* 2001). HOS1 interacts physically with ICE1 and mediates its polyubiquitination. Cold-induced degradation of ICE1 protein is blocked in the *hos1* mutant. This indicates that HOS1 is a negative regulator of the CBF-pathway required for degradation of ICE1 and attenuation of cold responses (Dong *et al.* 2006).

There is evidence for regulatory interactions among the closely related CBF family of transcription factors (Fig. 2.1). Relative to wild-type *Arabidopsis*, the null mutant *cbf2* exhibits increased expression of *CBF1* and *CBF3* and enhanced tolerance to freezing, salt and dehydration. It has been proposed that the CBF2 protein down-regulates the expression of CBF1 and CBF3 during cold acclimation (Novillo *et al.* 2004).

This view is also consistent with the finding that enhanced expression *CBF2* in the *ice1* mutant is correlated with decreased *CBF3* expression (Chinnusamy *et al.* 2003).

1.2.3 CBF-independent pathway

CBF-independent cold-responsive pathways (Fig. 1.2) have also been identified (Xin & Browse 1998; Zhu *et al.* 2004; Zhu *et al.* 2005). For example, the *esk1* (e*skimo1*) mutant shows constitutive freezing tolerance without cold acclimation. The *esk1* mutation does not appear to affect the expression of *COR* genes under warm conditions but greatly enhances the induction of these genes in response to low temperature (Xin and Browse, 1998). ESK1 encodes a protein with a DUF231 domain of unknown biological function. RNA profiling shows that there is a poor overlap of sets of genes regulated by ESK1 and by CBF3 and ICE1, which are components of the CBF-dependent pathway. This suggests that ESK1 is a novel negative regulator of cold acclimation (Xin *et al.* 2007). Forward genetic screens in *Arabidopsis* have identified *HOS9* and *HOS10* genes as negative regulators of cold acclimation. HOS9 and HOS10 are homeodomain and AtMYB8 transcription factors, respectively, that are constitutively expressed and not induced by cold treatment (Zhu *et al.* 2004; Zhu *et al.* 2005). Loss of function mutants are hypersensitive to freezing, but show enhanced- or earlier cold-induction of *RD29A* and *COR15a* not correlated with changes in CBF-gene expression. This suggests that HOS9 and HOS10 are negative regulators of cold acclimation that do not involve the CBF pathway (Zhu *et al.* 2004; Zhu *et al.* 2005).

A T-DNA insertion mutation in the transcriptional adaptor protein ADA2b have been described that show enhanced freezing tolerance without cold acclimation. Induction of *CBF* genes was not affected in the mutants under cold acclimatizing conditions, but subsequent transcript abundance of cold-regulated genes was reduced in both mutants. This suggests that ADA2b may directly or indirectly repress a freezing tolerance mechanism that does not require the expression of *CBF* or *COR* genes (Vlachonasios, Thomashow & Triezenberg 2003). *SFR2* (*Sensitive to Freezing 2*) encodes a novel type of β-glycosidase and is essential for freezing tolerance in *Arabidopsis* (Thorlby, Fourrier & Warren 2004). Mutants deficent in the CBF pathway and *COR* gene expression, show a high level of electrolyte leakage in response to freezing, a characteristic of freezing damage of plasma membranes. In contrast, the deficient *sfr2* mutant exhibits irreversible effects on growth at low temperatures and a low level of electrolyte leakage in response to freezing. It is believed that SFR2 functions in a

CBF-independent alternative pathway for cold tolerance that might be involved in polysaccharide turnover in the cell wall, or protection of the cell membrane from cold stress damage (Thorlby *et al.* 2004).

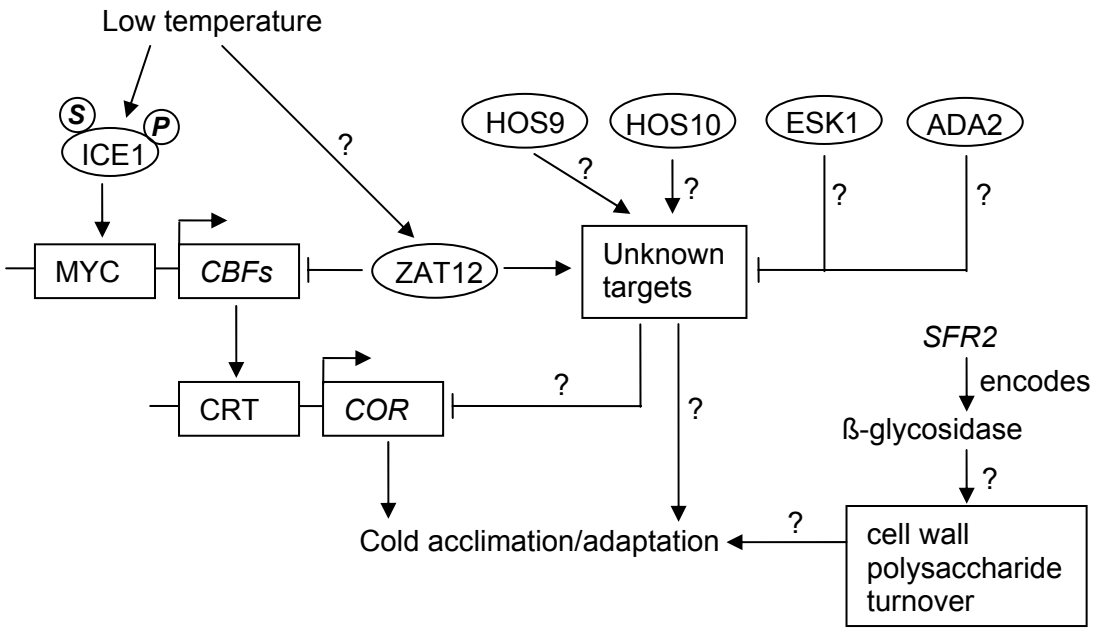


Figure 1.2. A simplified scheme of the CBF-independent regulatory pathways in *Arabidopsis*. Question marks indicate unknown effector(s)/repressor(s) that regulate the targets or involve in the regulatory network of gene expression in response to low temperature.

Arabidopsis ZAT12 is a C2H2-type zinc-finger protein of *Arabidopsis* (Meissner & Michael 1997) predicted to be a transcriptional repressor (Hiratsu *et al.* 2002) that has been shown to be involved in abiotic stress responses including to high light (Iida *et al.* 2000), wounding (Chen *et al.* 2002; Cheong *et al.* 2002; Rizhsky *et al.* 2004), low oxygen (Klok *et al.* 2002), heat and treatment with paraquat (Rizhsky *et al.* 2004); and, cold (Fowler & Thomashow 2002). Over expression of ZAT12 results in the repression of 15 genes down regulated in response to cold and induction of 9 genes upregulated in response to cold. Some of these genes are induced by both ZAT12 and CBF2. Constitutive expression of *ZAT12* at warm temperatures also led to a small, but reproducible, increase in freezing tolerance. In addition, ZAT12 down-regulated the expression of CBF1/2/3 indicating that ZAT12 as a negative regulator of CBF cold

responsive pathway. These findings have led to the hypothesis that ZAT12 is part of a pathway that functions in parallel with the known CBF cold-responsive pathways (Vogel *et al.* 2005). To summarize, the available evidence suggests that cold acclimation is associated with the activation of multiple low temperature regulatory pathways (Seki *et al.* 2001; Fowler & Thomashow 2002; Kreps *et al.* 2002; Vogel *et al.* 2005).

1.2.4 Role of cold-responsive genes in freezing tolerance

A large number of genes have been shown to be induced during cold acclimation. Some are homologs of genes encoding LEA (Late-Embryogenesis Abundant) proteins. LEA proteins are synthesized late in embryogenesis just prior to seed dessication and in seedlings in response to dehydration stress (Thomashow 1999). These proteins are unusually hydrophilic and composed largely of repeated amino acid sequence motifs. Many are predicted to contain regions capable of forming amphipathic α-helices that may have roles in stabilizing membranes against freezing damage (Thomashow 1999). Several *Arabidopsis* genes induced by both cold and drought are arranged in tandem in the genome, such as *LTI78/COR78/RD29A* and *LTI65/RD29B*, *KIN1* and *KIN2/COR6.6*, *COR15a* and *COR15b*, *LTI29/LTI45* and *COR47*, *RCI1A/RCI1* and *RCI1B/RCI2*, and *RCI2A* and *RCI2B*. These structurally related and closely linked genes pairs have been shown to exhibit differential responses to cold and dehydration stress and have been proposed to have functions specialized for different stress conditions (Hughes & Dunn 1996).

Some of these genes encode proteins with proven or potential contribution to freezing tolerance. For example, the *Arabidopsis FAD8* gene, encodes a fatty acid desaturase that might contribute to freezing tolerance by altering lipid composition (Gibson *et al.* 1994). The spinach *hsp70* gene (Anderson *et al.* 1994) and the *Brassica napus hsp90* gene (Krishna *et al.* 1995) encode molecular chaperones that might stabilize proteins against freeze-induced denaturation. The *Arabidopsis COR15a* gene encodes a 15-KDa polypeptide that is processed to a matured form, called COR15am, and targeted to the chloroplast. Constitutive expression of COR15a in non-acclimated transgenic *Arabidopsis* plants increase the freezing tolerance of chloroplasts frozen both *in situ* and in isolated leaf protoplasts (Artus *et al.* 1996). Overexpression of COR15a decreases the formation of the freeze-induced lamellar-to-hexagonal II phase found in the chloroplast membrane envelope (Steponkus *et al.* 1998). COR15am forms oligomers

localized to the stroma of chloroplast. Recombinant COR15am can associate *in vitro* with L-lactate dehydrogenase and protect it from freeze damage (Nakayama *et al.* 2007).

Anti-freezing proteins (AFP) have been isolated from plants that decrease the temperature at which ice is formed by binding to the surface of ice nuclei and inhibiting ice crystal growth (Antikainen & Griffith 1997a). Six polypeptides that accumulate in the apoplast of cold-acclimated winter rye leaves have been shown to exhibit potent antifreezing activity. These polypeptides are similar to members of three classes of pathogenesis-related (PR) proteins, namely, endochitinases, endo-β-1,3-glucanases, and thaumatin-like proteins when compared to the protein sequence database and characterized by enzyme activity assays. While purified endochitinases from non-acclimated rye or freezing-sensitive tobacco plants have no anti-freezing activity, tobacco class I endo-β-1,3-glucanase but not class I chitinase has been reported shows cryoprotective properties (Hincha, Meins Jr & Schmitt 1997).This suggest that either rye-specific endochitinases isoforms or tobacco-specific endo-β-1,3-glucanases have anti-freezing activity or that post-translational modifications are required to form active proteins that posses anti-freezing activity (Antikainen & Griffith 1997a). AFPs have also been isolated from the dicot *Solanum dulcamara*, but these proteins are not similar in sequence to the AFPs of rye (Duman 1994; Hon *et al.* 1995).

1.3 Biochemical and physiological changes in response to cold

Cold-induced changes in gene expression lead to the global changes in biochemical and physiological that involved in adapting the plant to growth and development at low temperature and acquisition of freezing tolerance (Van Buskirk & Thomashow 2006). Changes in membrane lipid composition are critical to avoid freezing injury of cell membrane. Ultrastructural changes in the plasma membrane have been observed within 6 hours of the start of cold acclimation in *Arabidopsis* (Ristic & Ashworth 1993). Alterations in membrane lipid composition are correlated with membrane cryostability as observed during cold acclimation in all species examined (Steponkus 1984; Uemura & Steponkus 1994; Uemura, Joseph & Steponkus 1995). In cold acclimated plants, the amount of unsaturated fatty acyl groups in membrane lipids is increased compared to non-acclimated plants and higher amount of unsaturated fatty acids increase membrane fluidity that is required to tolerate chilling and survival at freezing temperatures (Welti *et al.* 2002; Los & Murata 2004).

Free proline content increases in plants in response to low temperature and other abiotic stresses (Delauney & Verma 1993). For example, cold-acclimated, wild-type

Arabidopsis plants show a ten fold increase in proline content. At warm temperatures, the proline content of the constitutively freezing-tolerant *esk1-1* mutant is 30-fold higher than in wild type (Xin & Browse 1998) suggesting that proline plays an important role in freezing tolerance. The present view is that this a consequence rather the cause of freezing tolerance since proline accumulation in wild-type *Arabidopsis* during cold acclimation lags behind the development of freezing tolerance (Wanner & Junttila 1999).

Several plant species accumulate betaines in response to low temperature (Kishitani *et al.* 1994). Although not normally accumulated in *Arabidopsis,* betaine does accumulate in transgenic lines expressing a bacterial choline oxidase that catalyzes the conversion of choline to betaine. These plants show significantly improved freezing tolerance, even though the accumulation of betaine is much less than that found in plant species that naturally accumulate betaines (Hayashi *et al.* 1997; Sakamoto & Murata 2000; Sakamoto *et al.* 2000).

The accumulation of sugars in response to low temperature and its association with low temperature acclimation is well documented (Wanner & Junttila 1999; Xin & Browse 2000; Cook *et al.* 2004; Kaplan & Guy 2004). Sucrose, which can accumulate in response to low temperature, has been proposed to serve as a cryoprotectant (Crowe *et al.* 1990). Thus, the *Arabidopsis* s*fr4* impaired in cold acclimation does not accumulate sugars in response to low temperature (McKown, Kuroki & Warren 1996), while the *esk1* mutant which exhibits constitutive freezing tolerance accumulates sugars at warm temperatures (Xin & Browse 1998).

Phenylpropanoid and flavonoid compounds induced in plants by abiotic stresses including low temperatures and ultra-violet radiation, have a protective function (Dixon & Paiva 1995; Weisshaar & Jenkins 1998; Winkel-Shirley 2001). Flavonoids can scavenge reactive oxygen species (Prasad 1996; Deng, Fang & Wu 1997; Swiderski, Muras & Koloczek 2004) that accumulate during exposure to low temperature (Prasad *et al.* 1994). Phenylpropanoids are important for cold acclimation. Reduction of phenylpropanoid content by inhibition of phenylalanine ammonia-lyase activity decreases the photochemical efficiency of photosystem II in low-temperature affected leaves and decreases the ability of these leaves to develop tolerance to the extracellular formation of ice. Surprisingly, reduced phenylpropanoid content is also associated with partial abrogation of the cold-induced growth effects, such as inhibition of leaf fresh weight increments and accumulation of dry matter, proteins and cell wall (Solecka & Kacperska 2003).

Transfer of warm-grown plants to chilling temperatures leads to a rapid inhibition of photosynthesis followed by a reduction in transcript levels for genes encoding photosynthetic-related proteins/enzymes (Strand *et al.* 1997). The combination of low

temperatures with high light intensity can induce chronic photoinhibition of photosystem II in temperature shifted leaves. Lowering the temperature reduces reaction rates and can therefore limit the electron-consuming sink activity for the absorbed excitation energy/light, particularly CO_2 fixation and photorespiration relative to electron transport (Huner *et al.* 1993; Huner, Oquist & Sarhan 1998). However, cold-developed leaves showed recovery in photosynthetic activity at low temperature, in part due to reprogramming of photosynthesis carbon metabolism (Strand *et al.* 1997; Strand *et al.* 1999; Stitt & Hurry 2002).

There are many aspects of general cellular functions affected by low temperatures. Studies of bacteria and yeast have shown that ribosomes play a crucial role in cold adaptation (Bayliss & Ingraham 1974; Broeze, Solomon & Pope 1978; Kondo, Kowalski & Inouye 1992; Dammel & Noller 1995; Jones & Inouye 1996). In the case of black locust (*Robinia pseudoacacia* L.) plants, there are approximately 17 ribosomal proteins that are differentially expressed at low temperatures in comparison to control. During cold acclimation, changes in ribosome structure detected by changes in thermal melting profiles have been observed (Bixby & Brown 1975). The quantity, polymerization, and melting points of polysomes are changed in winter rye seedlings (*Secale cereale*, cv Puma) during growth at low temperatures. In addition, differential expression of ribosome-associated proteins at low temperature were observed as well (Laroche & Hopkins 1987). It was found that polysomes isolated from cold-acclimated *Brassica napus* cell-suspension cultures translated mRNA more efficiently at low temperatures than do polysomes isolated from non-acclimated cell cultures (Johnson-Flanagan & Singh 1987; Johnson-Flanagen & Singh 1987).

Studies of cultured tobacco BY-2 cells show that exposure to cold stress for two weeks induced apoptotic changes of the nucleus and cytoplasm such as shrinking of the protoplast, chromatin condensation, migration of chromatin towards the nuclear membrane and appearance of DNA-free regions in nucleus (Koukalová *et al.* 1997).

Cold treatment can also alter the methylation of cytosines in DNA. Studies of *Arabidopsis* mutants altered in DNA methylation have shown that vernalization, i.e., the induction of a flowering-competent state by low-temperature treatment, depends on demethylation of DNA (Burn *et al.* 1993; Finnegan *et al.* 1998). In other cases, the physiological relevance of these effects is less clear. For example, cold treatment of maize seedlings reduces expression of *ZmMET1*, which encodes a DNA methyltransferase (Steward, Kusano & Sano 2000). This is associated with demethylation of Ac/Ds transposon sequences, primarily in the root quiescent center. Global demethylation of DNA has been observed in cold-stressed chicory (*Cichorium*

intybus L. var. *foliosum* cv. Flash) and depends on tissue type and the plant age (Demeulemeester, Van Stallen & De Proft 1999).

The most notable physiological changes during cold acclimation are reduction or cessation of plant growth that cannot be fully explained by the temperature dependence of enzymic reactions (Levitt 1980; Graham & Patterson 1982). Phytohormones seem to have a central role in regulating growth in cold environments. Transcript profiling experiments suggest that cold-repressed of auxin transport, auxin responsive genes, and the biosynthsis of gibberellins, ethylene, and brassinosteroid alter hormone homeostasis and contribute to reduced growth rate of plants in cold environments (Hannah, Heyer & Hincha 2005; Lee, Henderson & Zhu 2005). Accumulation of salicylic acid (SA) during cold acclimation has been reported to inhibit growth at chilling temperature in *Arabidopsis* (Scott *et al.* 2004). SA-deficient transgenic *Arabidopsis* overexpressing the bacteria SA hydroxylase gene *NahG* exhibit a 2.7-fold greater biomass than wild type after 2 months at 5 °C (Scott *et al.* 2004). It is known that ferulic acid can promote feruloylation of the matrix of pectins and hemicelluloses which increase cell wall stiffness and limit cell expansion (Fry 1986). It has been suggested that the increased content of cell-wall ferulic acid in response to cold (Solecka & Kacperska 2003) promotes cell wall rigidity that this might contributes to growth reduction in the cold (Solecka & Kacperska 2003).

Root and leaf growth are driven by the cell division and expansion restricted primarily to the root tip and the leaf base, respectively (Inzé & De Veylder 2006). Cell division and expansion are inhibited as part of the response of plants to abiotic stress (Tardieu *et al.* 2000; Sharp *et al.* 2004). However, the impact of abiotic stress on cell cycle gene regulation is limited. Low temperature is known to decrease the rate of cell division and hence organ size (Francis & Barlow 1988; Creber, Davis & Francis 1993) which appears to be compensated, at least in part, by an increase in the number of cells entering the cell cycle (Pollock & Eagle 1988; Creber *et al.* 1993; Rymen *et al.* 2007). A recent report showed that growth reduction caused by low (4 °C) night temperature is mainly due to prolonged cell cycle progression and reduced production of cells that subsequently enlarge in the elongation zone (Rymen *et al.* 2007). Cell cycle progression is governed by the regulation of the activity of different cyclins and cyclin-dependent kinases during the G1 to S (DNA replication) transition and the G2 to M (mitosis) transition (Inzé & De Veylder 2006). The mitotic index was unaffected in maize leaves by increased temperatures from 18 to 26 °C, although the cell division rate increased 2-fold within this range (Ben-Haj-Salah & Tardieu 1995). In maize leaves, the activity of the A-type cyclin-dependent kinase, one of the key regulators of cell cycle progression, is positively correlated with both cell division rates and rates of growth in response to

different temperatures and water supply. Therefore, temperature has the effects on the amount and the rate of the reaction (Granier, Inze & Tardieu 2000). With a genomics and bioinformatics approaches, Rymen et al. (2007) analysed the expression of a core set of 43 maize cell cycle genes in response to low night temperature. The investigators found that the majority of the positive regulators of cell cycle which include cyclins (A-, B-, and D-type) and cyclin-dependent kinases were down-regulated and differential regulation of cell cycle inhibitors in response to low night temeprature. These experimental results suggest that cell cycle regulation plays an important role in growth in response to stress.

1.4 Natural variation of *Arabidopsis thaliana*

The plant used in my dissertation work is the small, annual weed *Arabidopsis thaliana* (L.) Heyhn that is well-suited for molecular genetic studies and has been used by others as a model for cold acclimation and freezing tolerance (Zhang, Creelman & Zhu 2004). *Arabidopsis* is native to Europe and Central Asia and is widely distributed in different growth environments (Al-Shehbaz & O'Kane Jr 2002). It has been found in the latitudinal range from 68°N (North Scaninavia) to 0° (Mountains of Tanzania and Kenya) and from sea level up to 4250 m (Hoffmann 2002) which make it suitable for analyzing variation in adaptive traits. *Arabidopsis* has also been shown to exhibit considerable natural variation for potentially adaptive traits, such as resistance to biotic stresses, tolerance to abiotic stresses, flowering time, and growth rate (Alonso-Blanco & Koornneef 2000).

Besides mutant analysis, analysis of natural variation provides an important source of genetic variation that can be used to identify and characterize specific traits, and available powerful genomic tools could be employed to gain insight into the control of important biological processes in plants (Borevitz & Nordborg 2003).

Large variation has been reported for tolerance to abiotic stresses such as freezing temperatures (Hannah et al. 2006), drought (Meyer et al. 2001; McKay, Richards & Mitchell-Olds 2003), salt (Queseda et al. 2002), and UV light (Cooley et al. 2001). Genetic variation has also been described in the timing of flowering transition (Koornneef et al. 1998) and plant growth rate (Li, Suzuki & Hara 1998; Beemster et al. 2002; Pérez-Pérez, Serrano-Cartagena & Micol 2002).

1.5 Aim of the dissertation

Most studies of low-temperature responsiveness of plants have focused on cold acclimation and freezing tolerance induced by acute cold treatment. The main objective of this study is to use molecular approaches to identify genes important for growth, differentiation, and organogenesis at low, non-freezing temperatures in *Arabidopsis*. To achieve this objective I focused on the following specific aims:

1. To characterize the natural variation of root elongation in *Arabidopsis thaliana* across a wide range of latitude and altitude accessions. This allowed me to determine if there is ecotypic differentiation in the capacity of *Arabidopsis* accessions for root elongation in the cold.
2. To identify potential links between roots elongation in the cold and the expression of cell-cycle related genes and CBF-responsive genes following acute and chronic cold treatment. I studied a wide range of high altitude and latitude accession by using northern blot assay to examine the steady-state levels of limited number of CBF-pathway and cell cycle related genes to test my hypothesis.
3. To use a global, transcriptome approach to identify specific sets of genes associated with chronic cold responses and the adaptation of *Arabidopsis* accessions to growth in cold habitats. I used transcript profiling (GeneChip) method to study only two accessions (Col-0 and Sha) to have a global view of gene expression changes in response to chronic cold treatment.

2.0 Materials and methods

2.1 Plant materials

Seeds from 21 accessions of *Arabidopsis thaliana* (Table 2.1) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Two additional undocumented *Arabidopsis thaliana*, accessions 3661 and 3658, were collected from Simien Mountain, Ethiopia. Average summer temperatures from May to September were obtained from the nearest recording station (www.weatherbase.com; http://fallingrain.com/world/). Seeds were sterilized by incubating them for 2 min in 70% (v/v) ethanol, and then for 10 min in 10% (w/v) sodium hypochlorite, and then rinsing thoroughly in sterile water.

2.2 Growth conditions and low temperature treatments

2.2.1 Measurement of root elongation rate

Approximately 15 surface-sterilized seeds were plated approximately 6 mm from the edge of each of 6 replicate square (10 X 10 cm) Petri dishes (Greiner Bio-One, Germany) containing 1/2-strength MS salts and vitamins, 1 gL^{-1} sucrose, and 0.8 gL^{-1} Phytagel agar (Sigma-Aldrich, St Louis MO, USA). After sowing, seeds were stratified for 7 days at 4 °C in a cold room. The stratified seed were then incubated in Petri dishes at a near vertically position under long-day conditions (16-hours light/8-hours dark) at the temperature and light intensity indicated. Plants were raised at lower illumination, ~35 $\mu molm^{-2}s^{-1}$ (otherwise indicated), as recommended (Gilmour *et al.* 1988) to minimize photoinhibition and photooxidative stress (Huner *et al.* 1993; Gray *et al.* 1997). Ten degrees celsius was used for standard cold treatments, since growth of the *Arabidopsis* accessions was extremely slow at temperatures below 8 °C and reliable cooling was not feasible for technical reasons.

The positions of primary root tips were marked on the back of the Petri dishes at 7-day intervals for seedlings incubated continuously at 10 °C or after pre-incubation for 7 days at 21 °C (Shift 21 °C/10 °C). For controls, the positions of primary root tips were marked at 2-day intervals for seedlings incubated continuously at 21 °C. Root elongation rate is expressed as the average rate (mmd^{-1}) estimated by the least squares method from the linear part of the elongation curves of 15 replicate plants.

2.2.2 Acute and chronic low temperature treatments

In acute-cold experiments, plants raised for 18-21 days at 21 °C and illuminated with 100 $\mu molm^{-2}s^{-1}$ light, were incubated 0, 2, 4, and 24 hours in Petri dishes kept in a 4 °C cold room under constant illumination (cool-white fluorescent light at approximately 50 $\mu molm^{-2}s^{-1}$). For chronic cold treatment, we compared control plants maintained at 21 °C for 3 weeks with plants continuously grown at 10 °C for 5-6 weeks, and plants grown for 1 week at 21 °C and then for 5 weeks at 10 °C (21 °C/10 °C). These incubation times were selected to obtain plants at the comparable 8-10 leaf-stage of development.

2.2.3 Low temperature treatments for RNA profiling experiment

After stratification for 7 days at 4 °C, triplicate sets of plants in Petri dishes were treated in three conditions: 1) continuous incubation at 21 °C for 18 days (control); 2) continuous incubation at 10 °C for 5-6 weeks (chronic cold treatment); and, 3) pre-incubation for 18 days at 21 °C followed by incubation at 10 °C for 4 hours (acute cold treatment). These incubation times were selected to obtain plants at the comparable 8-10 leaf-stage of development.

Table 2.1. List of *Arabidopsis thaliana* accessions analyzed and their geographical origin.

			Location [b]			
NASC No.	Accession	Origin [a]	Altitude (m)	Longitude	Latitude	Temperature (°C) [c]
N1380	Mt-0	Martuba/Cyrenaika, Lybia	200	E22.77	N32.58	25
N1564	Tsu-0	Tsu, Japan	100	E136.52	N34.72	22
N0970	Bla-0	Blanes/Gerona, Spain	100	E02.80	N41.68	20.3
N1566	Tu-0	Turin, Italy	300	E07.67	N45.05	19.2
N1603	Ws-0	Wassilewskija, Belarus	100	E29.83	N52.25	16.2
NW20	Ler-0	Landsberg/Warthe, Poland [d]	100	E16.00	N53.00	16.1
N1093	Col-0	Landsberg/Warthe, Poland [e]	100	E16.00	N53.00	16.1
N0938	Ak-1	Achkarren/Freiburg, Germany	200	E07.85	N48.00	16
N0994	Br-0	Brunn, Czech	300	E16.63	N49.20	16
N1142	Er-0	Erlangen, Germany	300	E11.04	N49.59	16
N1114	Dr-0	Dresden, Germany	200	E13.75	N51.05	15.2
N1586	Wa-1	Warsaw, Poland	200	E21.00	N52.25	15.2
N1308	Le-0	Leiden, Netherlands	100	E04.50	N52.15	15.1
N1006	Bu-0	Burghaun, Germany	300	E09.72	N50.70	14.4
N1490	Rsch-0	Rschew/Starize, Russia	200	E34.33	N56.26	13.8
N1534	St-0	Stockholm, Sweden	100	E18.05	N59.33	13.6
N1148	Est-0	Estland, Estonia	200	E23/E28	N58.50	13.3
N1028	Bur-0	Burren, Ireland	100	W09.08	N53.15	13
N1436	Oy-0	Oystese, Norway	100	E06.22	N60.38	12.7
N1122	Edi-0	Edinburgh, UK	200	W03.21	N55.95	12.6
--	3661 [f]	Mt. Simien, Ethiopia	3100	E38.40	N13.15	11.4

--	3658 [f]	Mt. Simien, Ethiopia	4250	E38.40	N13.15	7.1
N0929	Sha	Pamiro-Alay, Tajikistan	3400	E68.48	N38.35	6

[a] Unless indicated, according to the NASC listing (www.arabidopsis.info).
[b] Altitude, longitude, and latitude of the habitat according to the NASC listing (www.arabidopsis.info) or at the recording station nearest to the collection site (http://fallingrain.com/world/).
[c] Average summer temperature from May to September at the recording station nearest to the collection site (www.weatherbase.com).
[d] Selected from an X-ray mutagenized population of Landsberg (La-0).
[e] Selected from the original population of Landsberg (isolate 5-13).
[f] Undocumented *Arabidopsis thaliana* accessions.

2.3 Molecular biology techniques

2.3.1 Isolation of high molecular weight of RNA

The entire root system of plants were harvested, immediately frozen in liquid N_2, pooled, and stored at –80 °C. Total RNA was extracted from pools of roots from 40-50 plants using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's protocol for plant RNA. About 100 mg of ground root tissue was used per milliliter of TRIzol® reagent. The RNA pellet was resuspended in 100 µl of DEPC-treated distilled water at 50 °C for 15 min. RNA concentration and purity were measured spectrophotometrically. Only high RNA purity (ratio of A_{260}/A_{280} > 1.8) was used in the subsequent experiments. The dissolved total RNA (100 µg) was purified with a RNeasy plant RNA extraction kit using the RNA clean-up protocol (QIAGEN, Germany). Purified RNA fractions were dissolved in 50 µl of DEPC-treated water and stored in a -80 °C freezer.

2.3.2 Primer design and preparation of double-stranded DNA probes

Primer pairs for preparing double-stranded DNA probes (Table 2.2) and real-time PCR (Table 2.3) were designed using Primer Express® v2.0 (Applied Biosystems, USA) and purchased from Microsynth AG, Balgach, Switzerland. Primers for amplifying gene specific DNA probes of *CBF* genes have been described (Medina *et al.* 1999). The other primers used are summarized in Table 2.2. The probes were designed to correspond to last exon and genes-specific regions of the 3'-UTR. To synthesize first-strand cDNA, about 2 µg of column-purified of high molecular weight of RNA was reverse-transcribed using SuperScript™ III reverse transciptase (Invitrogen, USA) according to the manufacturer's protocol. The first strand cDNA was used as template for the generation of double-stranded DNA using PCR method. PCR was carried out by incubating PCR mixture [mixture of 1 µl of RT product, 10 pmole of each oligo primer, 500 µmole of each dNTP, 1 X PCR buffer, and 1 unit of Taq polymerase, (Invitogen, USA)] in the PCR machine heated for 2 min at 95 °C, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55 °C and extension for 1 min at 72 °C. The PCR product was resolved in 1 % (w/v) of agarose gel electrophoresis and the corresponding DNA fragment was excised and purified with QIAquick gel extraction column (QIAGEN,

Germany) according to the manufacturer's protocol. The concentration and purity of column-purified DNA was measured using spectrophotometer. About 25 ng of purified DNA were labeled by random priming with ^{32}P-labeled dCTP according to the manufacturer's protocol (RadPrime DNA labeling system, Invitrogen, USA).

Table 2.2. Primers used in the generation of double-stranded hybridization probe sets.

Primer	Nucleotide sequence (5' - 3')	Product length (bp)	Accession
COR15a forward	ACTGGTATGGCTTCTTCTTTC	551	NM_129815
COR15a reverse	GGTGACTGTGGATACCATATCT		
RD29A forward	CTGACAACTGAAGAAGAAGACA	569	NM_124610
RD29A reverse	TTTACCCACTTTAGACCTAGTAGC		
CYCB1;1 forward	ACAAGTCTGTTGAGAGTGAATG	788	M80190
CYCB1;1 reverse	CTTAAAGGGTCCTAACTCCTAAG		
CYCD2;1 forward	AAGCTCTAACTCCATTCTCCTT	711	X83370
CYCD2;1 reverse	AAAGAGCTCTCTCTCTCTCTCTT		
CDKA;1 forward	GATTCTACTCCTGATTTCTCCA	772	M59198
CDKA;1 reverse	CACACACTCGAAATCTTCTTC		
β-TUB forward	GTGGATCACAGCAATACAGAG	503	AY081473
β-TUB reverse	CACTTCGTCTTCTTCATACTCA		

2.3.3 RNA blot hybridization

RNA gel electrophoresis, and blot hybridization was done by standard methods (Sambrook, Fritsch & Maniatis 1989). Approximately 10 μg of high molecular weight RNA in a volume of ~16 μl was mixed with 4 μl of 5 X RNA loading buffer consisting of 16 μl saturated aqueous bromophenol blue solution, 80 μl of 500 mM EDTA, pH 8.0, 720 μl of 37 % (v/v) formaldehyde, 2 ml of 100 % (v/v) glycerol, 3084 μl of formamide, 4 ml of 10 X MOPS buffer, 1 μl of 10 mgml^{-1} ethidium bromide, 100 μl DEPC-treated water]. 1 X MOPS buffer is 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, adjusted pH to 7.0 with NaOH. The samples of RNA were denatured by heating to 65 °C for 5 min and then chilling on ice. The denatured RNA samples were loaded onto the 1% (w/v) formaldehyde gel (1 g Agarose, 20 mM MOPS,

5 mM sodium acetate, 1 mM EDTA, 0.66 M formaldehyde) that had first been equilibrated for at least 30 min in 1 X MOPS running buffer. The loaded gel was electrophoresed in 1 X MOPS buffer until the bromophenol blue indicator had migrated two-third of the length of the gel. RNA was transfered overnight on a Hybond N+ Nylon membrane (Amersham, UK) by capillary transfer using 10XSSC as the transfer solution (1XSSC is 150 mM NaCl, 15 mM sodium citrate). The membrane was crosslinked using Stratalinker® (Stratagene, USA). Then briefly washed in 2XSSC, and allowed to dry at room temperature. The blot was pre-hybridized in the Techne hybridizer (HB-1D, Brouwer AG, Luzern, Switzerland) for 1 hr at 60 °C in 10 ml 1X PerfectHyb™ Plus Hybridization buffer (Sigma-Aldrich, USA). The ^{32}P-radiolabeled DNA probe was denatured at 95 °C for 10 min prior to add to the pre-hybridization buffer. After overnight hybridization, the blot was washed twice at 60 °C for 5 minutes each with 2 X SSC, 0.1% (w/v) SDS buffer and twice at 60 °C for 20 minutes each with 0.5 X SSC, 0.1% (w/v) SDS.

Images of blots were acquired after 2 hours exposure using a Typhoon Phosphoimager (Amersham, UK). Images were processed and quantified using ImageQuant TL, v2003.02 (Amersham, UK). If the blot was to be re-probed, the hybridized probe was removed by pouring of boiling 0.1 % (w/v) SDS on the blot and gently shaking until cool to room temperature.

2.3.4 Quantitative real-time PCR

RNA was extracted and purified as described in 2.3.1 from control and chronic cold treated root. Poly(dT) cDNAs were made by using the Invitrogen cDNA first-strand synthesis system as described in section 2.3.2 using 5 µg of purified RNA. Quantifications were performed on an ABI Prism 700 (Applied Biosystems, USA) real-time PCR machine with the SYBR® Green PCR Master Mix kit (Applied Biosystems, USA) to generate 60 – 130 bp amplicons according to the manufacturer's protocol. PCR was carried out in MicroAmp® optical 96-well reaction plates (Applied Biosystems, USA) heated for 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of denaturation for 15 sec at 95 °C and annealing-extension for 1 min at 60 °C. The primers used are shown in Table 2.3. The standard curve was made by plotting C_T (threshold cycle) value determined from the target amplification of each standard dilution (50-fold, 100-fold, and 200-fold) of mixed 1:1:1 ratio of acute, chronic cold-treated and control cDNAs of root

samples against the log transformed amounts of target cDNA (i.e., 50-fold dilution=4 units; 100-fold dilution=2 units; 200-fold dilution=1 unit). Data were analyzed using SDS 2.0 software (Applied Biosystems, USA). Baseline data were collected between cycles 3 and 15 to generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number. All amplification plots were analysed with an R_n threshold of 0.3 to obtain C_T values. C_T values for all target genes were normalized to the C_T value of Elongation Factor 1 α (At5g60390) to allow comparison of data from different PCR runs or cDNA samples.

Table 2.3. Primer pairs used in the quantitative real-time PCR.

Primer	Nucleotide sequence (5' - 3')	AGI No.
CBF2 forward	CGGAATCAACCTGTGCCAAGGAAA	At4g25470
CBF2 reverse	AGACCATGAGCATCCGTCGTCATA	
CBF3 forward	GAATGGAATCTTCATTATGTTTGTAAAACTGAG	At4g25480
CBF3 reverse	AACTGAATCAATTTAATTTACACTCGTTTCTC	
COR15a forward	AACAGTGAAACCGCAGATACATTGG	At2g42540
COR15a reverse	CTCCTCCACATACGCCGCAG	
RD29A forward	TTCTCCGATGGGCTTTGGTAGTG	At5g52310
RD29A reverse	TCCTAAACCGCCGTCAGATTCC	
marneral synthase forward	TCTGCTTGTTTCTTGACGACTTC	At5g42600
marneral synthase reverse	ATAGCATCAAACCGATGGTGATTC	
cell wall invertase 5 forward	TCACTGACGATGCTGTTGTTGTTC	At3g13784
cell wall invertase 5 reverse	GTTCGGTAAGGTTGGTTCTTGATATTATTG	
AtPEN forward	TAGACGGTTTCGATGATGATGTTGATG	At4g15370
AtPEN reverse	CGCCAGGAGGGTTCTCTGTAAC	
lipid transfer protein forward	CACATACGACGCACACAGTTT	At4g12490
lipid transfer protein reverse	CGGAAAAAGATAACGCCTCA	
CYCB1;1 forward	GTTAGGACCCTTTAAGAAGACGAAGAAG	At4g37490
CYCB1;1 reverse	GTTTCAAAGCGACTCATTAGACTTGTTC	
CYCD2;1 forward	GGATTGAATATGAGAGTTTTGTGAGAAAGG	At2g22490
CYCD2;1 reverse	TGGAGCAAGAACAGGGATGTCTC	
CDKA;1 forward	GCATGAATACTTCAAGGATCTTGGAGG	At3g48750
CDKA;1 reverse	TAGAATGAAGGAGATTACTGGTTTTATGCC	
EF1 alpha forward	TGAGCACGCTCTTCTTGCTTTCA	At5g60390
EF1 alpha reverse	GGTGGTGGCATCCATCTTGTTACA	

2.4 RNA profiling

2.4.1 GeneChip hybridization and raw data collection

Total RNA (5 µg) extracted from three independent pools of root of chronic cold-treated (5-6 weeks at 10 °C), acute (4 h 10°C), and 21 °C grown Col-0 and Sha-0 seedlings was reverse transcribed to synthesize double-stranded cDNA and then converted to biotin-labeled cRNA by *in vitro* transcription reaction according to manufacturer's protocols (Affymetrix, USA). The labeled target cRNA was purified, fragmented, and hybridized to ATH1 GeneChip array according to protocols provided by manufacturer (Affymetrix, USA) in a Hybridization Oven 640 (Affymetrix, USA). The chips were washed and stained with streptavidin phycoerythrin using a GeneChip Fluid Station 400 (Affymetrix, USA) and then scanned with a Gene Array Scanner. The raw images were condensed using GC_RMA using Refiner (Genedata, Switzerland).

2.4.2 Data processing and analyses

The output from all GeneChip hybridizations were scaled so that the arithmetic mean of the expressed genes (detection p-value ≤ 0.04) was set to 500. The normalized groups were made by dividing each gene by the median of its expressed values using Analyst (Genedata, Switzerland) software.

2.5 Statistical analysis

2.5.1 General

Root elongation rates were estimated by the least squares method from the linear part of curves of root length as a function of time using Microsoft Office Excel 2003. Statistical tests were performed with the regression analysis, correlation test, and Student's t-test of SigmaStat for Windows Version 3.11 package (Jandel Scientific Software, San Rafael, CA, USA).

2.5.2 RNA expression data

Expressionist v4.5.4 software (Genedata, Switzerland) was used for statistical analysis of expression data analysis. Statistically significant changes in mRNA abundance were determined from the t-test of means expression value of three biological replicates. The criterion for scoring genes (probe sets) as cold responsive was a ratio of mRNA abundance of cold-treated sample relative to non-treated sample ≥ 2-fold with *P*-value < 0.01. Distribution and overlapping of genes among cold treatments or accessions were performed with Venn diagram analysis tool (GeneData, Switzerland).

2.5.3 Enrichment of GO biological annotations

FatiGOPlus (Al-Shahrour, Diaz-Uriarte & Dopazo 2004; Al-Shahrour *et al.* 2005) was used to extract non-redundant over-represented/enrichment of GO biological annotations/terms that corresponding to GO hierarchy (level 3 – 9) for cold-responsive genes determined from t-test pairwise comparison with respect to a set of genes of reference/background (typically the rest of genes of the ATH1 GeneChip entry). Fisher's exact test was used to estimate the significant of the enrichment. The returned p-values from Fisher's exact test were adjusted by false discovery rate (FDR) method by multiple corrections as described (Al-Shahrour *et al.* 2004; Al-Shahrour *et al.* 2005). Enrichments of $p<0.05$ after FDR adjustment were judged to be significant.

2.5.4 DNA sequence alignment and hierarchical clustering

DNA sequence alignment was performed using BioEdit v7.0.5.3 (Hall 1999). A total of 89 cell cycle related genes which represented and passed the quality control (expressed, $P\leq0.04$) in the GeneChip array was use in the hierarchical clustering (Eisen *et al.* 1998) of expression changes of cell cycle genes. The expression ratios between cold treatment and control values were $\log_2$ transformed before clustering.

3.0 Variation in root growth rates and gene expression patterns

3.1 Introduction

The aim of initial growth experiments was to characterize the natural variation of root elongation in *Arabidopsis thaliana* across a wide range of latitude and altitude accessions. This allowed me to determine if there is ecotypic differentiation in the capacity of *Arabidopsis* accessions for root elongation in the cold. Previous investigations of plant growth at low temperatures suggested that most of the plants cease in growth between 2 °C and 8 °C (Pollock & Eagle 1988; Körner 2006) and some species begin to degenerate at lower than above mentioned chilling temperature such as apical abortion in calabrese (*Brassica oleracea* var. *italica*) at 4 °C (Forsyth *et al.* 1999). For technical reasons, 10 °C was chosen as the temperature for chronic, low temperature treatments. This is reasonable since 10 °C had been shown previously to be sufficient to induce cold acclimation process with the expression of key cold-responsive genes (Zarka *et al.* 2003).

Arabidopsis accessions are widely distributed throughout diverse growth environments and may retain genetic variations in important traits that required for adaptation to various conditions. It is a common approach to identify cold-tolerance traits in *Arabidopsis* plants that search for natural variations among accessions in their response to low temperature (Koornneef, Alonso-Blanco & Vreugdenhil 2004; Hannah *et al.* 2006). At present, it is unclear to what extent the CBF response associated with acute cold-acclimation plays a role in long-term (chronic) cold adaptation or how plants grow and develop in a cold environment. To address these issues, I studied the effects of long-term cold treatment on the growth and expression of well-characterized cold- and cell-cycle related genes in *Arabidopsis* roots. Cell division in meristems co-determines growth by producing the cells that will subsequently expand and differentiate to reach a given mature size (Fiorani *et al.* 2000). Temperature is known to alter rates of cell division, and hence organ size (Francis & Barlow 1988; Creber *et al.* 1993). Progression through the cell cycle and the influence of environmental factors on division rate depend on cyclin-dependent kinases associated with their cyclins (Meijer & Murray 2001). For example, the overall growth rate of different *Arabidopsis* accessions appears to depend on A-type cyclin dependent kinase (CDKA1At/Arath;CDKA;1) activity (Beemster *et al.* 2002). Ectopic expression of *Arabidopsis* D-type cyclin CYCD2At (Arath;CYCD2;1) in

transgenic tobacco accelerates the rate of cell cycling and results in an overall increase in growth rate (Cockcroft *et al.* 2000; Boucheron *et al.* 2005). Similarly, ectopic expression of B-type cyclin 1 (CYC1At/Arath;CYCB1;1) under the control of *CYCD2;1* promoter in *Arabidopsis* markedly accelerates root growth without altering the pattern of lateral root development suggesting that CYCB1;1 is associated with growth limitation in roots (Doerner *et al.* 1996).

3.2 Root elongation rates and habitat temperatures are not correlated

For all accessions tested, the length of the primary root increased in a linear fashion starting 6-7 days after sowing of seeds (data not shown). Thus, for quantitative comparisons, results are expressed as the increase in primary root length estimated from the linear part of the curves. The results show that there is a natural variation in root elongation rate at 21 °C among *Arabidopsis* accessions ranging from 3.3 ± 0.4 mmd^{-1} for Er-0 to 13.9 ± 0.4 mmd^{-1} for Est-0 (Fig. 3.1). Depending on accession, elongation rates were greatly reduced at 10 °C by 10 to 45 % of the rates at 21 °C of the same accession (Fig. 3.1, 3.2) For most accessions, growth of warm pre-treated plants was slightly faster than for plants grown continuously at 10 °C (Fig. 3.1 and Fig. 3.2). Accession Ws-0 (2.15 ± 0.07 mmd^{-1}) and Rsch-0 (2.11 ± 0.06 mmd^{-1}) were among the fastest growing while accession 3658 was the slowest growing (0.67 ± 0.03 mmd^{-1}) when raised continuously at 10 °C. Of the accessions tested, Col-0 showed the highest rate of growth (2.80 ± 0.04 mmd^{-1}) in the temperature shift experiment and accession 3658 was again the slowest growing (0.72 ± 0.05 mmd^{-1}) in the same cold treatment (Fig. 3.1).

I also expressed the data as the per cent elongation rate at 10 °C relative to 21 °C to estimate growth potential in the cold and correct for intrinsic differences in elongation rates (Fig. 3.2). Based on this parameter, the potential for growth in the cold was similar for most accessions, with the exception of the accession Er-0 from Erlangen, Germany, with a growth potential of 45 %. Thus, it appears that the differences in low-temperature growth among accessions reflect intrinsic variation rather than accession-specific responses to low temperature. Higher capacity of growth of accession Er-0 may be due to genetic-determined of roots branching (secondary rooting) instead of primary root elongation as found in other accessions. The elongation rates at 10 °C were positively correlated with growth rate at 21 ºC (df=21, 10 °C, $p<0.01$; 21 °C/10 °C, $p<0.001$) (Fig. 3.3). However, neither the rates of growth at 10 °C and 21 °C, nor the relative growth rate at 10 °C were significantly correlated (df=21, $P>0.05$) with the average growing season (May to September) habitat temperature from where these accessions originated. For instant, growth rate of highland, Sha accession was comparable to lowland common laboratory strains i.e. Col-0, Ler-0, and Ws-0. This suggests that effects on root elongation of habitat temperatures during the growing season are not a major factor in the adaptation of accessions to cold habitats.

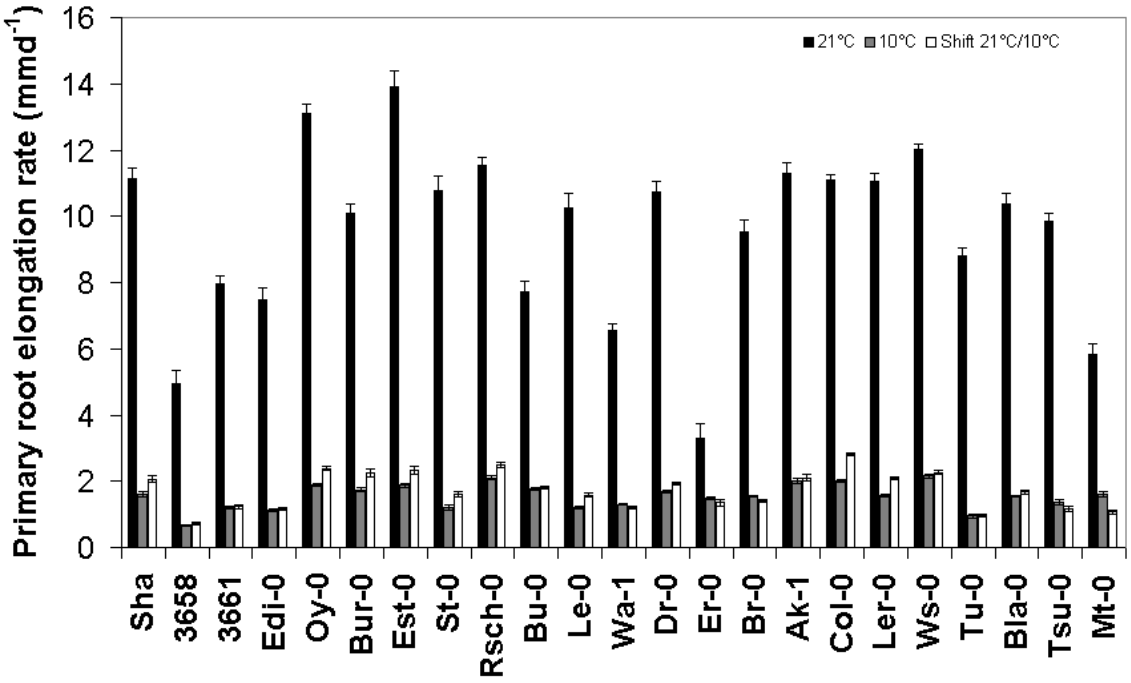


Figure 3.1. Comparison of root growth rates of *Arabidopsis* accessions at 21 °C and 10 °C. Root growth is expressed as the average elongation rate of primary roots that either continuous grown at 21 °C (black bars), 10 °C (gray bars) or at 10 °C following pre-incubation for 7 days at 21 °C (white bars). Error bars: ± SEM for 15 plants. Accessions are ranked by habitat temperature from coldest (left) to warmest (right).

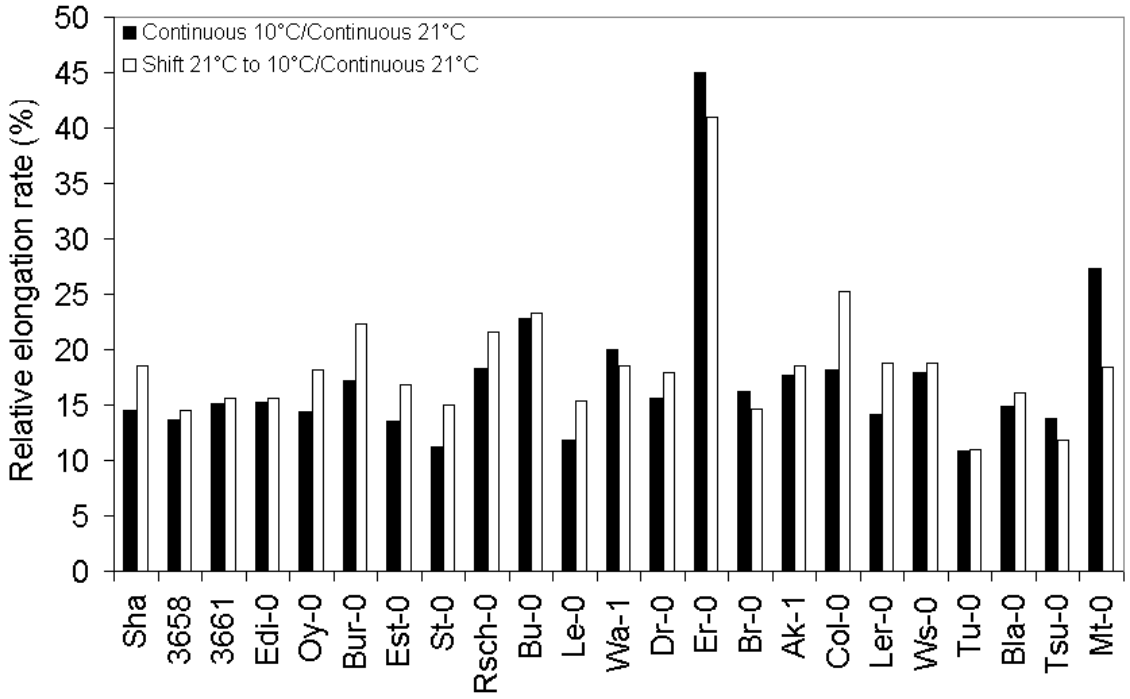


Figure 3.2. Comparison of growth rates of various *Arabidopsis* accessions in response to low temperature treatments expressed as percent of 21 °C controls. Root growth is expressed as percent of 21 °C controls either continuous grown at 10 °C (black bars) or at 10 °C following pre-incubation for 7 days at 21 °C (white bars). Accessions are ranked by habitat temperature from coldest (left) to warmest (right).

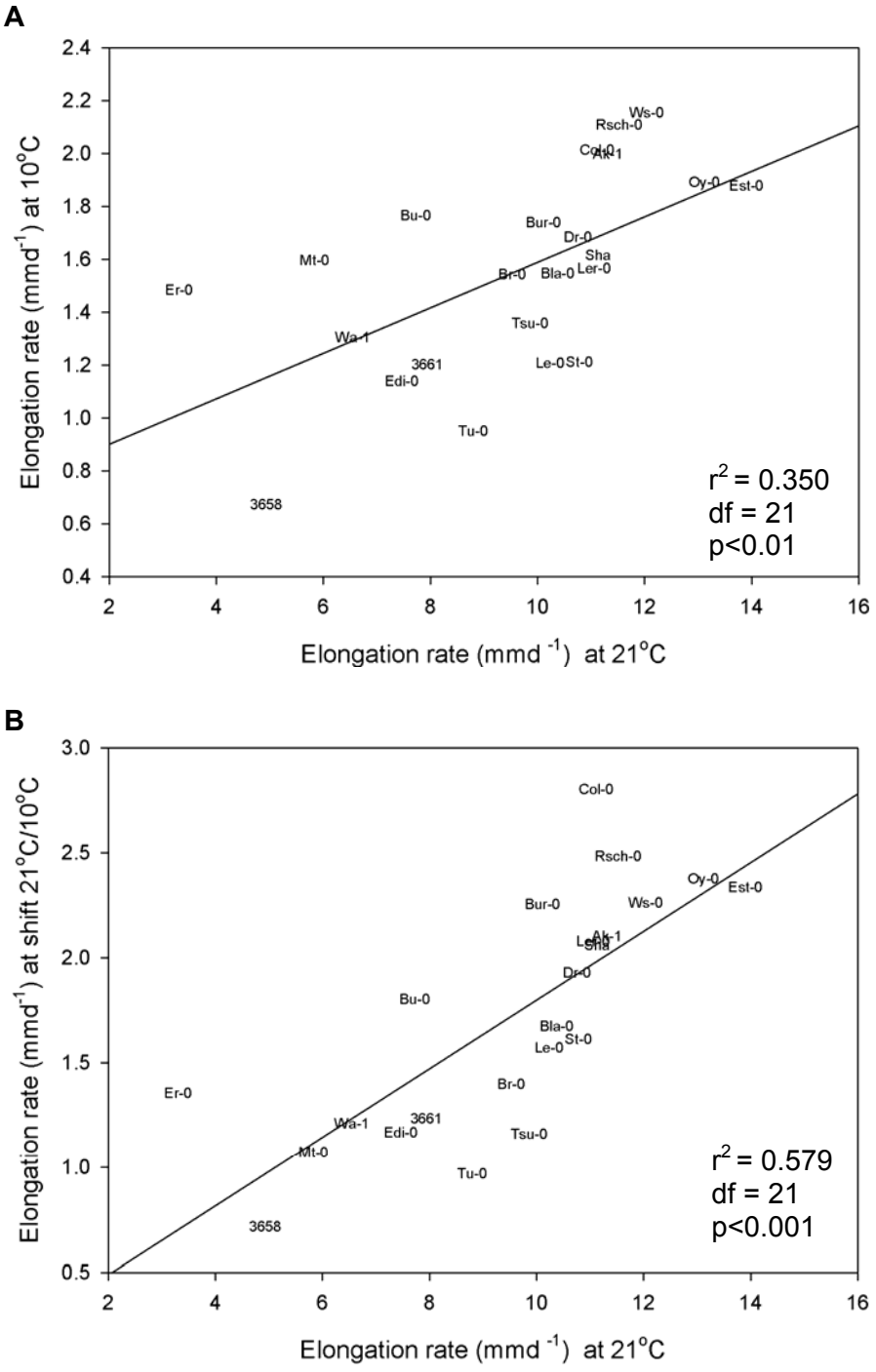


Figure 3.3. The positive correlation between elongation rates of roots of plants grown continuously at 10 °C (**A**) or pre-incubated for 7 days at 21 °C and then grown at 10 °C (**B**) with those of plants grown continuously at 21 °C. Results shown for the 23 *Arabidopsis* accessions indicated.

3.3 Acute cold-induction of CBF-pathway genes in roots

I measured the time course for expression of *CBF1*, *CBF2*, *CBF3*, *COR15a* and *RD29A* mRNAs in roots of *Arabidopsis* plants incubated for up to 24 hours at 4 °C (Fig. 3.4). Although the magnitude and timing varied among the accessions, all of the accessions tested showed an early induction of *CBF2* and *CBF3* that peaked at 2-4 hours and a later induction of *COR15a* and *RD29A* (Fig. 3.4). Similar results were obtained in two independent experiments. *CBF1* was not detected in any of the accessions tested. Interestingly, while the upstream genes *CBF2/3* were induced in the high-altitude accessions 3661 and 3658, the downstream genes *COR15a* and *RD29A* showed very weak induction relative to Col-0 (Fig. 3.4A, 3.4C). These effects were not due to genetic differences in the coding region recognized by the Col-0 hybridization probe. The coding regions of 3661, 3658 and Col-0 were ca. 99% and 75% identical in nucleotide sequence for *COR15a* and *RD29A*, respectively (Fig. 3.5). The patterns of induction other than for 3661 and 3658 were very similar to those reported earlier for common laboratory strains (Gilmour *et al.* 1998; Ishitani *et al.* 1998; Liu *et al.* 1998; Zarka *et al.* 2003). Thus, the canonical CBF response pathway functions in most of the diverse high- and low-altitude accessions tested.

For RNA blots in which hybridization signals were above background, we compared the maximum expression level of genes in response to acute cold with the elongation rates of the accession at 21 °C and at 10 °C. No consistent correlations at the 5% level were found in the two independent experiments with the high-altitude set of plants (df=2) or with the low-altitude set of plants (df=3). There was also no obvious correlation of maximum expression levels with the summer temperatures of the source habitats of high- or low-altitude accessions. For example, Col-0 and Sha, with average summer temperatures of 16.1 °C and 6.0 °C respectively, exhibited roughly similar expression levels of the downstream *COR15a* and *RD29A* genes.

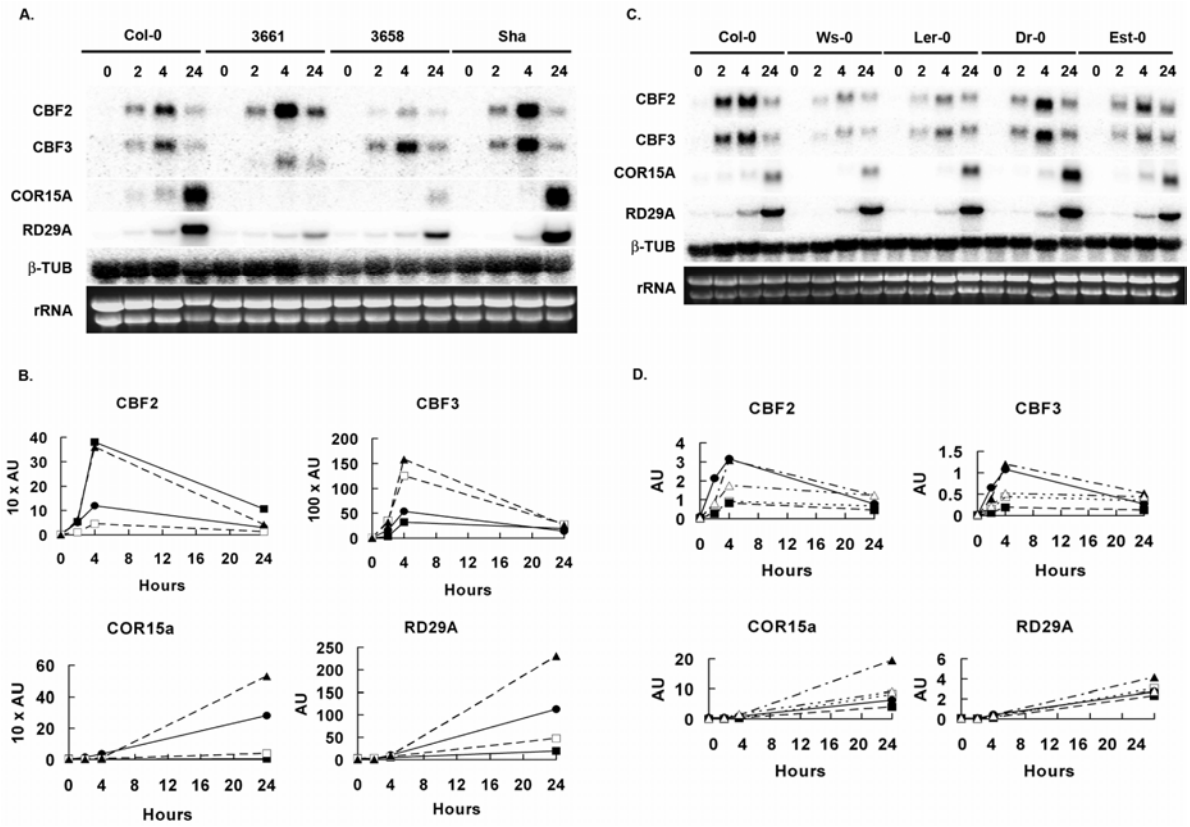


Figure 3.4. Effects of acute cold treatment at 4 °C on expression of *CBF*-pathway mRNAs in roots. RNA-blot hybridization of total RNA from high-altitude (**A**) and low-altitude (**C**) accessions with the laboratory strain Col-0 as standard. Relative accumulation of mRNAs obtained with the high-altitude (**B**): Col-0 (filled circles), 3661 (filled squares), 3658 (open squares), and Sha-0 (filled triangles), and low-altitude (**D**): Col-0 (filled circles), Ws-0 (filled squares), Ler-0 (open squares), Dr-0 (filled triangles), and Est-0 (open triangles) accessions. Seedlings 21 days after germination with 8 to 10 true leaves were incubated at 4 °C and total RNA prepared from roots at the times indicated (0, 2, 4, or 24 hours). RNA blots were hybridized with probes for *CBF2*, *CBF3*, *COR15A*, *RD29A*, and *β-tubulin* (*β-TUB*). *β-tubulin mRNA* and ethidium bromide-stained rRNA served as loading controls. Accumulation of mRNA is expressed in arbitrary units (AU) normalized for expression of the *β-tubulin* loading standard.

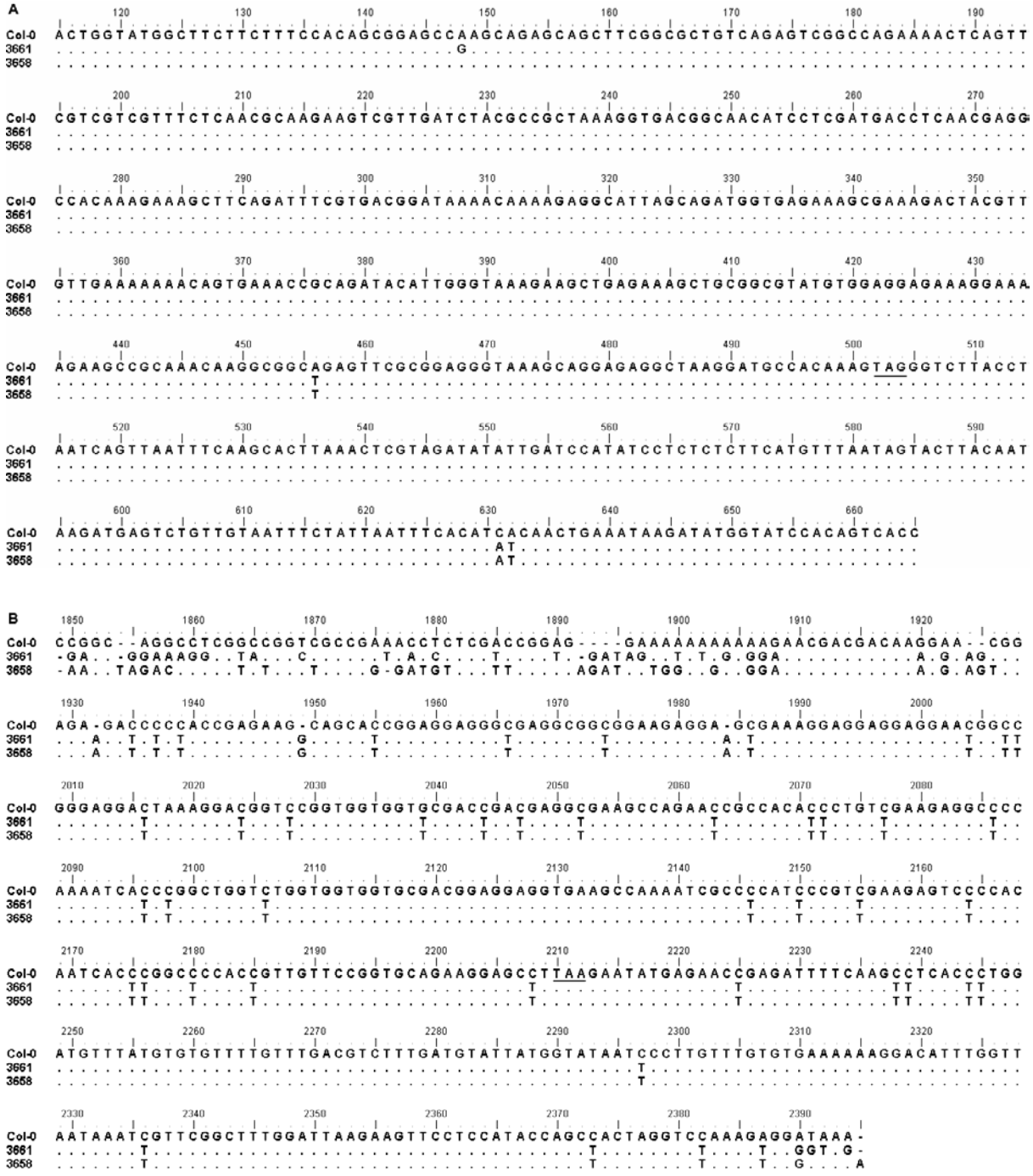


Figure 3.5. Nucleotide sequence alignment of DNA probes of three *Arabidopsis* accessions represented by part of the last exon and 3'-UTR region that hybridize with *COR15a* (**A**) and *RD29A* (**B**) genes of the northern blot. The stop codon of the last exon is underlined in the reference DNA sequences of Col-0. DNA sequence after the stop codon is the fragment of the 3'-UTR. Dashed lines indicate gaps that are introduced to maximize alignment. Dots indicate nucleotides that are identical to DNA sequences originated from Col-0.

3.4 Chronic cold-induction of CBF-pathway genes

Roots of plants grown in cold conditions showed higher levels of *CBF2* mRNA than did warm-grown controls (Fig. 3.6). This cold induction was most pronounced in Col-0, 3661 and Sha. *CBF3* was barely detected and no signals for *CBF1* were detected. The downstream genes *RD29A* and *COR15A* showed consistent, strong cold induction in Col-0 and Sha, weak cold induction in 3661 and 3658, and variable cold induction in the other accessions tested. Cold induction was usually more pronounced in 21 °C/10 °C plants than for plants grown continuously at 10 °C. These results show that the *CBF* genes expressed in response to acute cold treatment are also expressed in plants grown at 10 °C; and, that under both conditions the accessions Col-0 and Sha show strong responses, while the accessions 3661 and 3658 show weak responses. The magnitude of induction of *RD29A* was consistently correlated ($p < 0.05$, $df = 2$) with the elongation rate of cold-grown roots in the two independent experiments performed with the high-altitude set of plants.

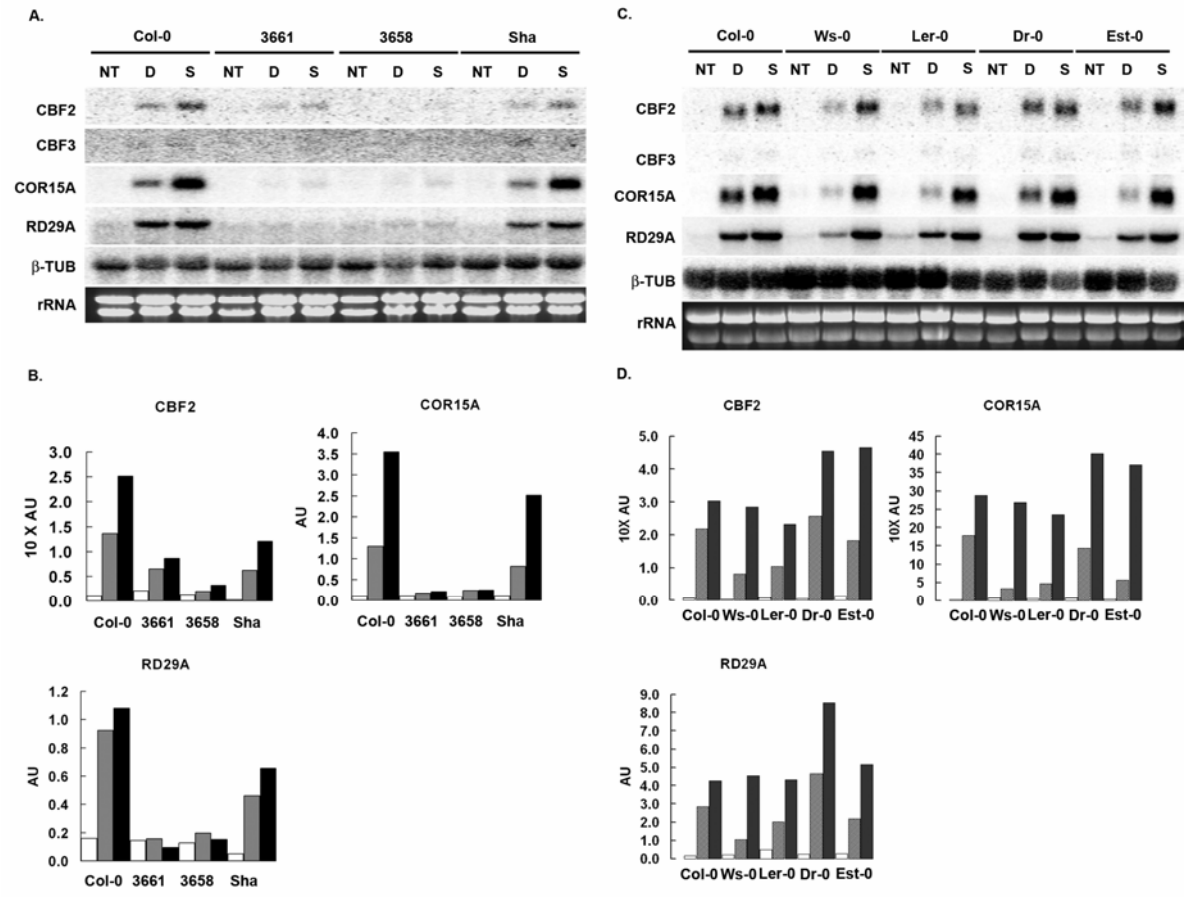


Figure 3.6. Effects of chronic cold treatment at 10 °C on expression of *CBF*-pathway mRNAs in roots. RNA-blot hybridization of total RNA from high-altitude (**A**) and low-altitude (**C**) accessions with the laboratory strain Col-0 as standard. Relative accumulation of mRNAs obtained with the high-altitude (**B**) and low-altitude (**D**) accessions. RNA was extracted from roots of plants raised for 3 weeks at 21 °C (NT, white bars), from roots of plants raised for 5-6 weeks at 10 °C (D, gray bars), or from roots of plants raised for 1 week at 21 °C and then raised for an additional 5 weeks at 10 °C (S, black bars). Plants in the different treatments were harvested at the 8-10 leaf-stage. RNA blots were hybridized with probes for *CBF2*, *CBF3*, *COR15A*, *RD29A*, and *β-tubulin* (*β-TUB*). *β-tubulin mRNA* and ethidium bromide-stained rRNA served as loading controls. Accumulation of mRNA is expressed in arbitrary units (AU) normalized for expression of the *β-tubulin* loading standard.

3.5 Cold induction of cell-cycle-related genes

Hybridization signals for *CYCB1;1*, and *CYCD2;1* mRNAs were very weak and relatively higher for *CDKA;1* for acute cold treatment at 4 °C of high-altitude accessions (Fig. 3.7A). Quantification of the normalized signals (Fig. 3.7B, 3.7D) suggest that mRNA levels changed slightly—less than ca. 30%-- with increasing time of cold treatment. These results and those obtained in the experiment with low-altitude accessions that gave stronger hybridization signals (Fig. 3.7C), show that acute cold treatment usually resulted in a small, time-dependent decrease in expression of cell-cycle related genes. In contrast, chronic cold treatment substantially increased *CYCB1;1* expression and to a lesser extent *CDKA;1* expression (Fig. 3.8A, 3.8C). Quantification of the normalized signals (Fig. 3.8B, 3.8D) reveal that all accessions tested showed cold induction of *CYCB1;1* expression that ranged from approximately 1.5- to 2-fold in several low-altitude accessions to 4-5 folds in the high-altitude accessions 3661 and 3658. Expression of *CDKA;1* was also induced by ca. 1.5 to 3.0 fold in 3661 and 3658, but was not induced in the other accessions. A similar pattern of expression was obtained for *CYCD2;1*, which showed a slight ca. 1.5-fold induction, but only in the accessions 3661 and 3658. Taken together, these results show that *CYCB1;1* expression is substantially induced in response to chronic, but not acute cold treatment (Compare Fig. 3.8 with Fig. 3.7), in several different *Arabidopsis* accessions. No consistent correlations at the 5% level were found between inductions of cell-cycle related genes and either the rate of root elongation or the habitat temperature of the accessions.

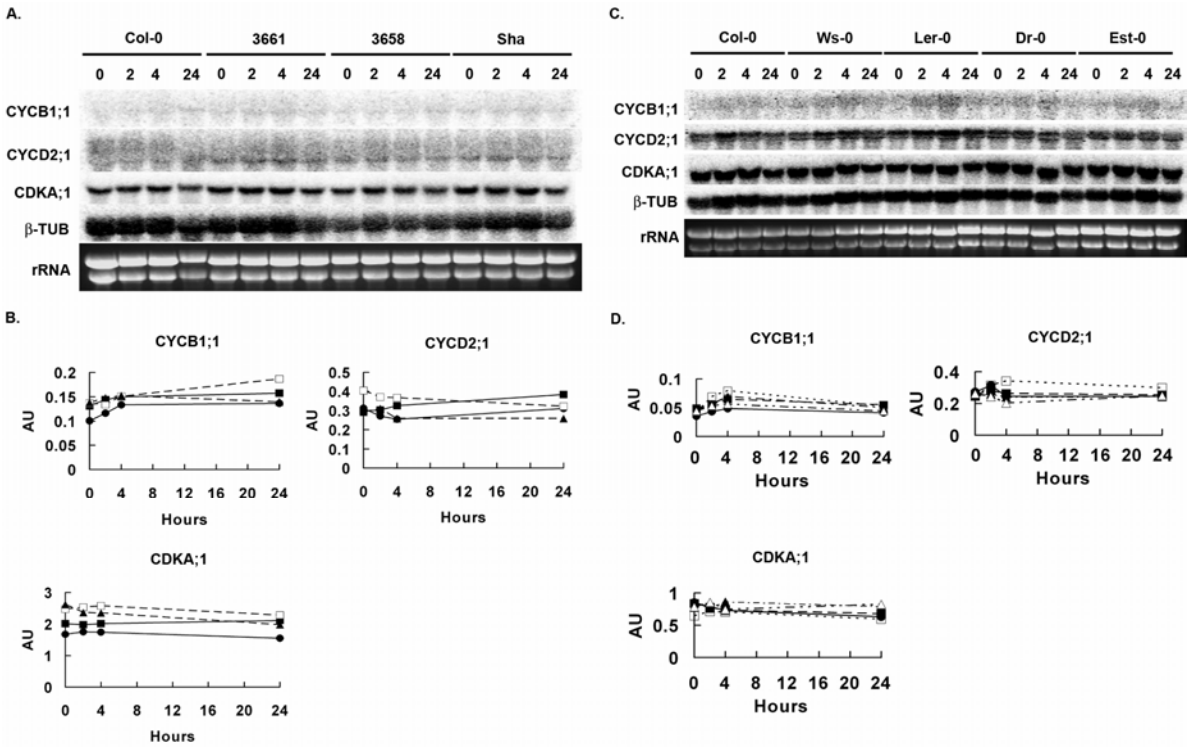


Figure 3.7. Effects of acute cold treatment at 4 °C on expression of cell-cycle related mRNAs in roots. RNA-blot hybridization of total RNA from high-altitude (**A**) and low-altitude (**C**) accessions with the laboratory strain Col-0 as standard. Relative accumulation of mRNAs obtained with the high-altitude (**B**): Col-0 (filled circles), 3661 (filled squares), 3658 (open squares), and Sha-0 (filled triangles), and low-altitude (**D**): Col-0 (filled circles), Ws-0 (filled squares), Ler-0 (open squares), Dr-0 (filled triangles), and Est-0 (open triangles) accessions. Seedlings 21 days after germination with 8 to 10 true leaves were incubated at 4 °C and total RNA prepared from roots at the times indicated (0, 2, 4, or 24 hours). RNA blots were hybridized with probes for *CYCB1;1*, *CYCD2;1*, *CDKA;1* and *β-tubulin* (*β-TUB*). *β-tubulin mRNA* and ethidium bromide-stained rRNA served as loading controls. Accumulation of mRNA is expressed in arbitrary units (AU) normalized for expression of the *β-tubulin* loading standard.

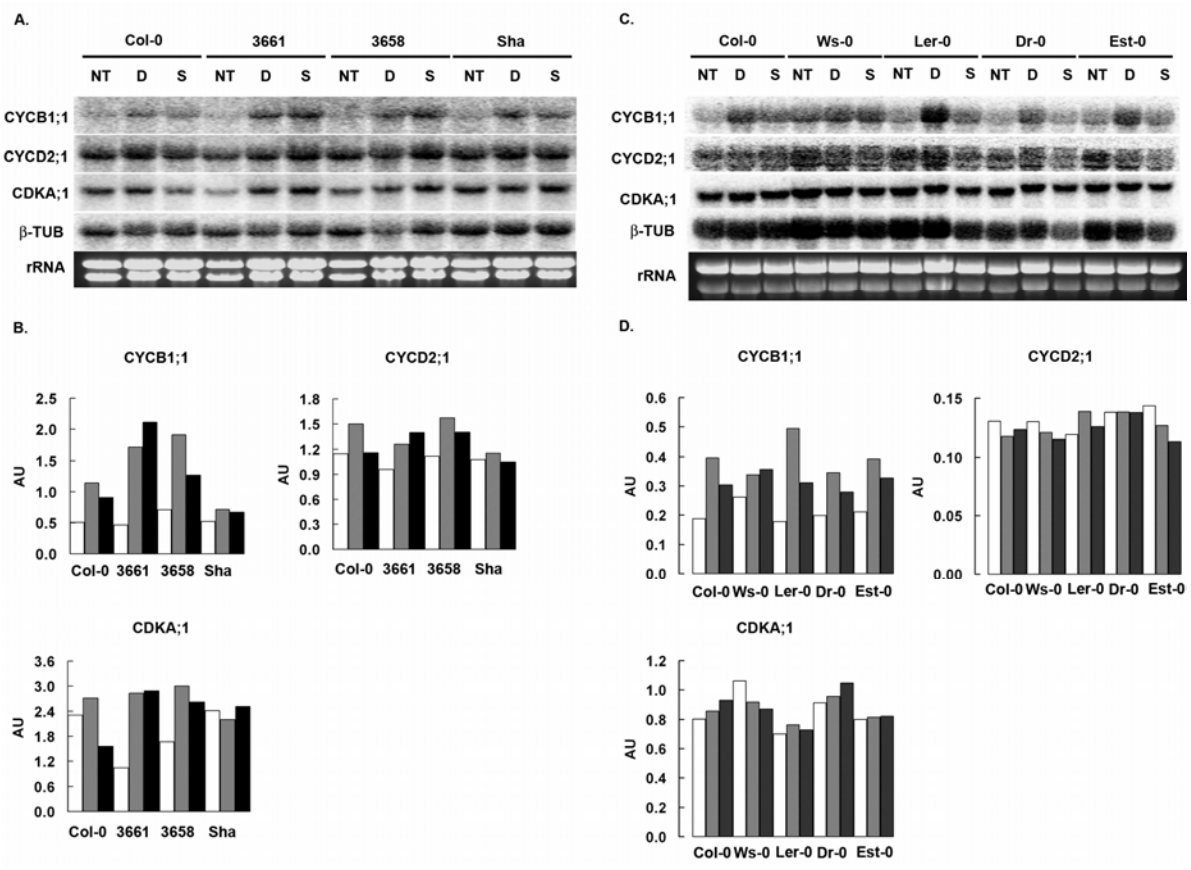


Figure 3.8. Effects of chronic cold treatment at 10 °C on expression of cell-cycle related mRNAs in roots. RNA-blot hybridization of total RNA from high-altitude (**A**) and low-altitude (**C**) accessions with the laboratory strain Col-0 as standard. Relative accumulation of mRNAs obtained with the high-altitude (**B**) and low-altitude (**D**) accessions. RNA was extracted from roots of plants raised for 3 weeks at 21 °C (NT, white bars), from roots of plants raised for 5-6 weeks at 10 °C (D, gray bars), or from roots of plants raised for 1 week at 21 °C and then raised for an additional 5 weeks at 10 °C (S, black bars). Plants in the different treatments were harvested at the 8-10 leaf-stage. RNA blots were hybridized with probes for *CYCB1;1*, *CYCD2;1*, *CDKA;1* and *β-tubulin* (*β-TUB*). *β-tubulin mRNA* and ethidium bromide-stained rRNA served as loading controls. Accumulation of mRNA is expressed in arbitrary units (AU) normalized for expression of the *β-tubulin* loading standard.

3.6 Conclusions

There is natural variation in primary root elongation rates at warm and low temperature and depending on accessions. However, elongation rates were not significantly correlated with the average temperature of the habitats during the growing season indicating that there is no ecotypic differentiation for this trait.

CBF-pathway genes induced by acute cold-treatment are also induced in roots of plants grown at 10 °C of our chronic cold-treatment. The expression of the down-stream *COR15a* and *RD29A* under chronic cold treatment is associated with the capacity of roots to grow in the cold environments. Neither these expression patterns nor the capacity of roots to grow in the cold are likely to be an important factor in the adaptation of *Arabidopsis* to cold habitats.

Chronic-cold induced of *CBF2* may be required for maintaining slow growth rates at low temperature in addition for cold tolerance activity. Chronic-cold induction of *CYCB1;1* mRNA could facilitate root growth by maintaining cell division and proliferation activities under chronic cold environments. Our results are consistent with the hypothesis that chronic-cold inductions of *CBF2*, *COR15a* and *RD29A* and the B-type cyclin *CYCB1;1* have a role in the growth and development of *Arabidopsis* and possibly other temperate species at low temperature.

4.0 Transcript profiling of cold-exposed *Arabidopsis* accessions

4.1 Introduction

Low temperature induces a wide range of biochemical and physiological responses in plants including those implicated in cold tolerance such as alterations in lipid composition; accumulation of compatible osmolytes (e.g. soluble sugars, proline, betaine, and polyols); transient increases in abscisic acid (ABA); increased levels of antioxidants; reduced water content; and, reduced rates of enzymatic reactions (Thomashow 1999; Xin & Browse 2000). These responses are regulated, at least in part, by alterations in steady-state mRNA levels resulting from altered transcription, altered mRNA stability, or both processes (Guy, Niemi & Brambl 1985; Guy 1999; Pearce 1999).

The *Arabidopsis* CBF cold-responsive pathway (Gilmour *et al.* 1998; Liu *et al.* 1998; Shinwari *et al.* 1998) as well as CBF-independent pathways (Xin & Browse 1998; Zhu *et al.* 2004; Zhu *et al.* 2005) have important roles in low temperature acclimation and freezing tolerance (Thomashow 1999; Shinozaki & Yamaguchi-Shinozaki 2000; Thomashow 2001). Microarray studies suggest that low temperature acclimation is associated with the activation of additional, multiple low-temperature regulatory pathways (Seki *et al.* 2001; Fowler & Thomashow 2002; Kreps *et al.* 2002; Vogel *et al.* 2005).

Microarray-based analyses have been made of *Arabidopsis* (Fowler & Thomashow 2002; Kreps *et al.* 2002; Seki *et al.* 2002; Hannah *et al.* 2005) and rice (Rabbani *et al.* 2003) that response to acute low temperatures in the timeframe hours to 2 weeks. However neither global changes in gene expression associated with chronic (long-term) cold treatment nor their correlation with root growth in different ecotypes have been studied. In this chapter I describe microarray experiments aimed at identifying transcriptome differences associated with acute and chronic cold treatment of the common laboratory *Arabidopsis* accession Col-0, and the Sha accession from a cold, high-altitude habitat.

4.2 Identification of cold-responsive genes and validation of microarray results

I used the Affymetrix ATH1 GeneChip array to survey global gene expression in response to acute cold treatment for 4 h at 10 °C and chronic cold treatment of plants for approximately 6 weeks at 10 °C. The control for both cold treatments was plants grown at 21 °C. Measurements were made of biological triplicate of pooled total RNA (~50 seedlings for each replicate) prepared from the entire root system harvested from plants at the comparable 8 – 10 leaf-stage of development. Genomic sequence comparisons have shown that Col-0 and Sha are distantly related and, hence, likely to show considerable genetic polymorphism (Nordborg *et al.* 2005). Because the Affymetrix ATH1 GeneChip was designed for the Col-0 genome, signal strengths for the same gene do not necessarily provide a reliable measure of expression differences when comparing different accessions. To correct for possible polymorphism effects, I calculated the fold changes between cold treatments and warm controls. Fold changes were calculated by dividing median expression values to minimize outlier effects (McKillup 2005).

Cold-responsive genes were identified on the basis of two criteria: First, mean expression values of the cold treated and control plants must be significant at the 1% level as judged by the t-test of means. Second, fold changes must be equal or greater than 2. Based on these criteria, 1127 genes were up-regulated and 1657 genes were down-regulated by acute or chronic cold treatment in Col-0. Under the same conditions and in the same experiment 1011 genes were up-regulated and 1600 genes were down-regulated in Sha (Fig. 4.1).

To validate the microarray data, I measured RNA levels by RT-qPCR of eleven genes that showed increased, decreased, or approximately constant expression in response to cold in microarray assays. The expression ratios obtained from the microarray and by RT-qPCR showed a highly significant positive, linear correlation (Table 4.1) suggesting that the microarray assay provides reliable estimates of transcript abundance.

The FatiGOPlus algorithm (Al-Shahrour *et al.* 2004; Al-Shahrour *et al.* 2005) was used to screen for Gene Ontology (GO) terms enriched in a test dataset of annotated genes relative to the rest of genes represented on the ATH1 GeneChip. Enrichments of $p<0.05$ by Fisher's exact test and after false discovery rate (FDR) adjustment were judged to be significant. This method reveals differential enrichment of genes and

differences in cold-responsive genes in datasets from acute- and chronic-cold treated plants. Because products of the same gene can be involved in more than one biological process, a single gene can be classified by more than one GO term.

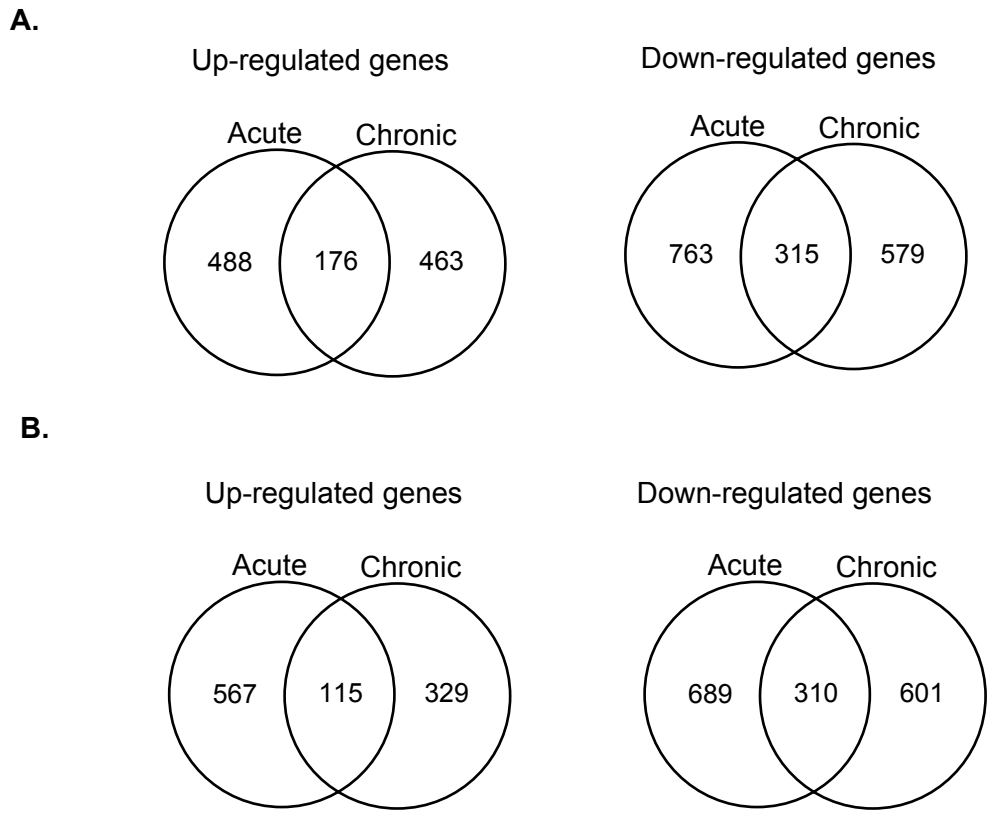


Figure 4.1. Overlapping of acute and chronic cold responsive genes identified (T-test, $p<0.01$, ≥2-fold) in Col-0 (**A**) or Sha (**B**) accession.

Table 4.1. Correlation between microarray and qPCR expression ratios for selected genes of Col-0 or Sha accession.

		Col-0				Sha-0			
		Acute		Chronic		Acute		Chronic	
AGI No.	Annotation	microarray [a]	qPCR [b]	Microarray [a]	qPCR [b]	Microarray [a]	qPCR [b]	Microarray [a]	qPCR [b]
At4g25470	CBF2	9.384	6.482	6.538	4.896	6.610	7.515	3.118	2.892
At4g25480	CBF3	6.926	6.625	3.642	3.997	19.381	8.072	2.411	2.149
At2g42540	COR15a	5.240	5.675	22.627	21.707	3.815	3.125	10.900	5.919
At5g52310	RD29A	2.197	1.607	11.447	11.601	5.590	4.766	12.848	4.333
At5g42600	marneral synthase	0.316	0.174	0.004	0.005	0.131	0.376	0.018	0.021
At3g13784	cell wall invertase 5	0.873	0.249	60.860	21.976	1.206	1.308	71.571	39.220
At4g15370	pentacyclic triterpene synthase, putative	1.182	0.708	31.683	11.618	1.068	1.093	2.509	4.449
At4g12490	lipid transfer protein (LTP)	3.134	2.324	223.590	163.188	3.132	2.027	16.735	0.330
At4g37490	CYCB1;1	1.965	1.565	2.515	2.338	1.508	1.766	2.378	1.849
At2g22490	CYCD2;1	1.161	1.023	1.090	0.915	1.181	1.308	1.123	0.931
At3g48750	CDKA;1	0.993	0.841	1.104	0.822	1.066	1.191	1.242	0.947
	Pearson correlation coefficient, r [c]	0.962		0.986		0.868		0.963	
	p value	2.13E-06		2.34E-08		5.27E-04		2.01E-06	

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[b] Ratios of median of expression values of cold-treated to warm controls that were normalized to transcript levels of elongation factor 1 α (At5g60390).
[c] Correlation coefficient values from the comparison of expression ratios obtained from microarray and qPCR methods with *p* value indicated, df=10.

4.3 Enrichment of genes affected by cold treatment

There were substantial differences in the distribution of genes responsive to acute- and chronic-cold in Col-0 and Sha (Fig. 4.1). Less than 18% of cold-responsive genes were shared in datasets obtained with the two cold treatments. Table 4.2 shows the non-redundant and significant GO term over-represented in each cold treatment of the Col-0 and Sha accessions. The biological functions of genes up-regulated by acute-cold treatment differed from up-regulated by chronic-cold treatment. For both Col-0 and Sha, genes responsive to signalling molecules such as the phytohormones abscisic acid, jasmonic acid, ethylene, and salicyclic acid were frequently over-represented in the acute response relative to the chronic response (Table 4.2A). Synergistic and antagonistic actions of these signals are known to play important roles in protecting plants from biotic and abiotic stress (Bostock 2005; Lorenzo & Solano 2005; Mauch-Mani & Mauch 2005). Similarly, genes responsive to UV irradiation or genes involved in disaccharide biosynthesis were over-represented in acute cold treatment relative to chronic cold treatment.

Table 4.3 shows dramatically higher induction of transcripts encoding enzymes involved in disaccharide biosynthesis by acute cold than by chronic cold. Thus, for example, transient expression of genes encoding trehalose-6-phosphate phosphatase (At4g22590; At4g12430; At5g65140; At5g10100; At5g51460), β-amylase (At4g17090), sucrose-phosphatase (At3g52340, At1g51420), and raffinose synthase (At5g40390) were likely involved in the disaccharide biosynthetic process (Fig. 4.2) which were up-regulated more than 2-fold in the acute cold treatment but reduced to less than 2-fold for most of the diasaccharide biosynthesis related genes (Table 4.3). This suggests that accumulation of sugars as compatible osmolytes are likely found in the acute cold exposed *Arabidopsis* plants. Genes encoding putative trehalose-6-phosphate phosphatase (At4g12430, At5g10100) and β-amylase (At4g17090) were chronic cold-induced more than 2-fold in Col-0. Similary, transcripts encoding putative sucrose-phosphatase (At1g51420) and β-amylase (At4g17090) were chronic cold-induced more than 2-fold in Sha. This suggests that accumulation of trehalose, sucrose and maltose are also important as compatible osmolytes for chronic cold exposed plants.

Table 4.2. Non redundant terms of biological processes significantly over-represented in sets of either up- (**A**) or down-regulated (**B**) genes that response to acute- or chronic-cold treatment in Col-0 or Sha.

A. Up-regulated genes

Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Col-0, acute cold (664)		
response to salicylic acid stimulus	1.18E-03	4
regulation of transcription, DNA-dependent	7.37E-03	8
response to abscisic acid stimulus	8.21E-03	5
response to jasmonic acid stimulus	8.40E-03	4
response to ethylene stimulus	8.40E-03	5
response to UV	2.42E-02	6
disaccharide biosynthetic process	2.49E-02	7
phenylpropanoid metabolic process	3.50E-02	6
Col-0, chronic cold (639)		
flavonoid biosynthetic process	3.65E-03	8
lipid transport	1.13E-02	5
regulation of transcription, DNA-dependent	2.44E-02	8
circadian rhythm	4.95E-02	3
Sha, acute cold (682)		
regulation of transcription	1.30E-05	7
response to salt stress	1.19E-04	5
response to jasmonic acid stimulus	1.71E-04	4
response to cadmium ion	2.47E-04	6
response to ethylene stimulus	1.00E-03	5
flavonoid biosynthetic process	2.07E-03	8
response to abscisic acid stimulus	2.85E-03	5
response to salicylic acid stimulus	2.85E-03	4
regulation of timing of meristematic phase transition	4.47E-03	9
response to gibberellic acid stimulus	5.33E-03	5
response to UV-B	8.48E-03	7
diasaccharide biosynthetic process	2.20E-02	7
Sha, chronic cold (444)		
cell wall organization and biogenesis (sensu Magnoliophyta)	1.62E-04	6
inorganic anion transport	7.26E-04	7
phenylpropanoid biosynthetic process	3.09E-02	7
choline biosynthetic process	4.82E-02	9

B. Down-regulated genes Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Col-0, acute cold (1078)		
photosynthesis	2.23E-08	3
electron transport	9.95E-04	5
response to heat	3.29E-02	5
Col-0, chronic cold (894)		
electron transport	5.86E-08	5
secondary cell wall biosynthetic process (sensu Magnoliophyta)	7.78E-06	8
photosynthesis, light harvesting in photosystem II	9.92E-05	7
oligopeptide transport	2.37E-04	6
terpenoid biosynthetic process	5.36E-04	9
photosynthesis, light harvesting in photosystem I	1.71E-02	7
photosynthetic water oxidation	2.06E-02	6
Sha, acute cold (999)		
photosynthesis	3.23E-05	3
circadian rhythm	3.03E-03	3
starch catabolic process	3.84E-03	9
Sha, chronic cold (911)		
secondary cell wall biosynthetic process (sensu Magnoliophyta)	2.28E-05	8
electron transport	3.60E-05	5
regulation of transcription, DNA-dependent	1.24E-02	8
triterpenoid metabolic process	3.50E-02	9
photosynthesis, light harvesting in photosystem I	3.51E-02	7
vitamin K biosynthetic process	3.51E-02	8
xylem histogenesis	3.51E-02	8
terpenoid biosynthetic process	4.10E-02	9

[a] p-value adjusted for false discovery rate (FDR).
[b] Gene ontology biological process level.
Values in parenthesis are the number of genes in the dataset indicated.

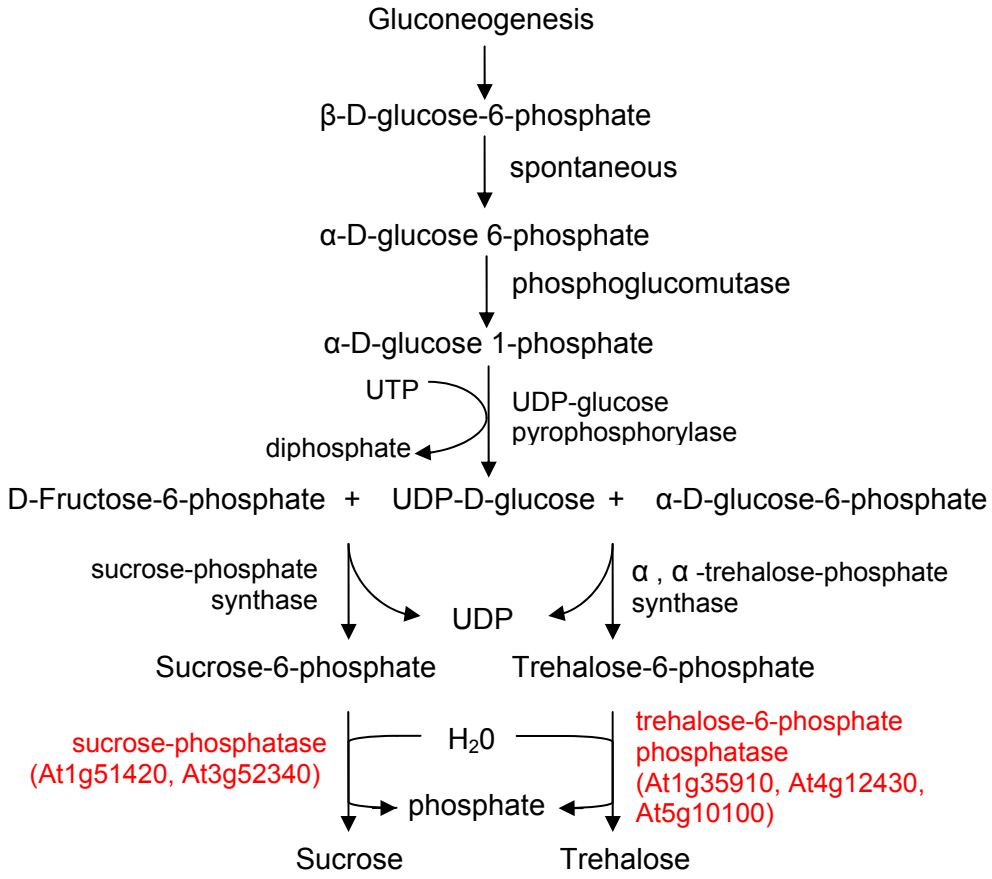
Table 4.3. Expression values of enriched genes related to disaccharide biosynthetic process in acute cold-treated Col-0 (**A**) or Sha (**B**) accession.

A. Col-0		Median expression value			Ratio *	
Annotation	AGI.	warm	acute	chronic	acute	chronic
trehalose-6-phosphate phosphatase (TPPA)	At5g51460	80.17	215.46	112.60	2.69	1.40
trehalose-6-phosphate phosphatase, putative	At4g12430	68.32	252.82	149.70	3.70	2.19
trehalose-6-phosphate phosphatase, putative	At4g22590	221.12	472.18	347.03	2.14	1.57
trehalose-6-phosphate phosphatase, putative	At5g10100	181.71	1144.18	555.97	6.30	3.06
trehalose-6-phosphate phosphatase, putative	At5g65140	65.97	138.90	85.53	2.11	1.30
β-amylase (CT-BMY)	At4g17090	29.23	761.94	166.89	26.06	5.71
raffinose synthase	At5g40390	1857.09	3821.79	2796.10	2.06	1.51

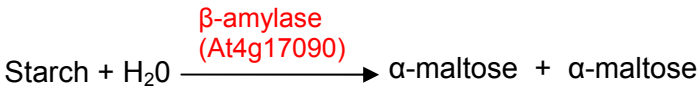
B. Sha		Median expression value			Ratio *	
Annotation	AGI.	warm	acute	chronic	acute	chronic
trehalose-6-phosphate phosphatase, putative	At5g10100	193.48	1304.98	264.05	6.74	1.36
trehalose-6-phosphate phosphatase, putative	At4g12430	154.60	627.58	258.30	4.06	1.67
trehalose-6-phosphate phosphatase, putative	At1g35910	17.54	59.22	23.79	3.38	1.36
sucrose-phosphatase 2 (SPP2)	At3g52340	113.43	418.91	163.13	3.69	1.44
sucrose-phosphatase, putative	At1g51420	94.46	196.77	238.63	2.08	2.53
β-amylase (CT-BMY)	At4g17090	19.26	451.63	71.13	23.45	3.69
raffinose synthase	At5g40390	1501.33	3714.22	2251.08	2.47	1.50

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

A.



B.



C.

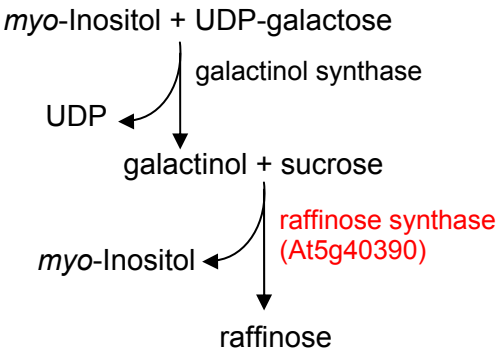


Figure 4.2. Simplified biosynthetic pathways for sucrose or trehalose (**A**), α-maltose (**B**), and raffinose (**C**). Genes that labeled in red were induced in acute cold treatments of root of Col-0 or Sha accession.

Genes grouped under “phenylpropanoid metabolic process” were over-represented among acute cold up-regulated genes in Col-0 (p=3.5E-02) but not in Sha. On the other hand, genes responsive to salt stress (p=1.19E-04) and gibberellic (p=5.33E-03) were over-represented in acute-cold treated Sha but not in Col-0. The “regulation of transcription” group was the most significantly (p=1.3E-5) over-represented group in the acute cold responsive genes of Sha. Genes involved in flavonoid biosynthetic process were over-represented in the chronic cold treated Col-0 (p=3.65E-03) and acute cold-treated Sha (2.07E-03), while those grouped under “phenylpropanoid biosynthetic process” (3.09E-02) were found in chronic cold-treated Sha (Table 4.2A). Genes encoding enzymes involved in the synthesis of phenylpropanoids and flavonoids that serve as protectants against biotic and abiotic stresses (Dixon & Paiva 1995; Weisshaar & Jenkins 1998; Winkel-Shirley 2001) were frequently found in cold treated plants (Table 4.2A), suggesting that they function in the cold responses.

Genes encoding enzymes involved in flavonoid biosynthetic process were up-regulated in response to cold (Fig. 4.3, Table 4.4). These include 4-coumarate:CoA ligase 3 (4CL3) (At1g65060), chalcone synthase (At5g13930), chalcone isomerase/Transparent Testa 5 (At3g55120), flavanone 3-hydroxylase/Transparent Testa 6 (At3g51240), flavonol synthase 1 (At5g08640, At5g63580), flavonol 3’-hydroxylase/Transparent Testa 7 (At5g07990), dihydroflavonol 4-reductase family / dihydrokaempferol 4-reductase family (At2g45400), UDP-rhamnose:flavonol-3-O-rhamnosyltransferase (UGT78D1) (At1g30530), flavonol 7-O-glucosyltransferase (At4g34135), and flavonoid metabolism transcription factor MYB domain protein 12 (At2g47460) (Mehrtens *et al.* 2005) (Table 4.4).

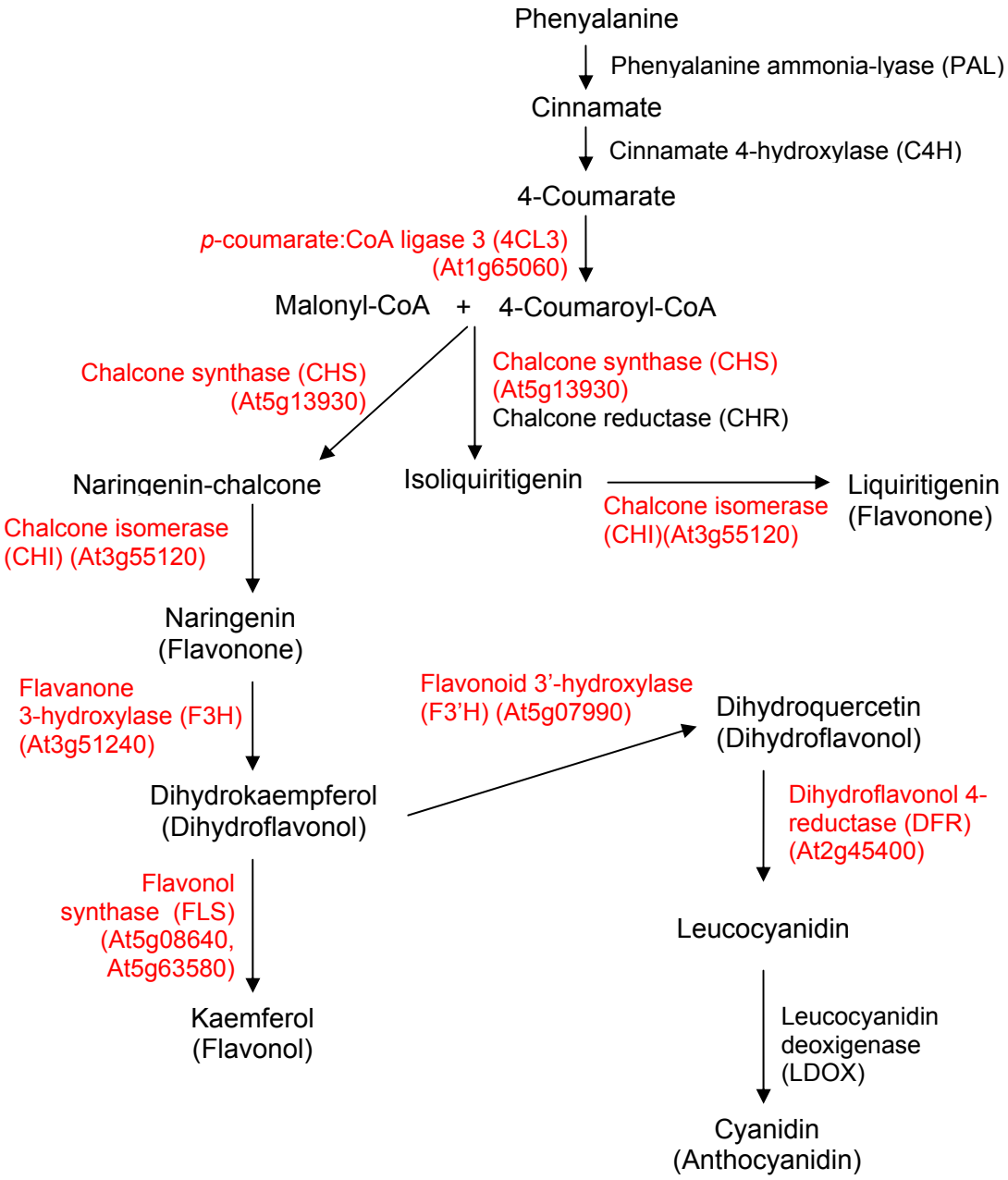


Figure 4.3. Flavonoid biosynthetic pathway of higher plants. Genes that labeled in red were induced in cold treatments of root of Col-0 or Sha accession.

Table 4.4. Expression values of cold-induced genes grouped under “phenylpropanoid metabolic process” in acute cold treated Col-0 (**A**); “flavonoid biosynthetic process” in chronic cold treated Col-0 (**B**), or acute cold treated Sha (**C**); or “phenylpropanoid biosynthetic process” in chronic cold treated Sha (**D**) accession.

		Median expression value			Ratio[a]	
Annotation	AGI.	warm	acute	chronic	acute	chronic
A. phenylpropanoid metabolic process, Col-0, acute cold						
disease resistance-responsive family protein	At1g64160	6.73	17.98	8.81	2.67	1.31
dihydroflavonol 4-reductase	At2g45400	124.23	249.98	147.7	2.01	1.19
cinnamoyl-CoA reductase-related	At2g23910	61.43	514.47	879.34	8.38	14.32
cinnamoyl-CoA reductase-related	At4g30470	394.03	1390.03	979	3.53	2.48
cinnamoyl-CoA reductase-related	At5g14700	18.85	152.14	25.56	8.07	1.36
chorismate mutase, chloroplast (CM1)	At3g29200	362.45	1014.62	575.54	2.8	1.59
myb family transcription factor (MYB12)	At2g47460	108.07	1071.67	407.78	9.92	3.77
O-methyltransferase family 2 protein	At1g33030	6.35	18.31	17.13	2.88	2.7
p-coumarate--CoA ligase 3 (4CL3)	At1g65060	191.79	399.16	993.43	2.08	5.18
flavonol synthase, putative	At5g63580	91.82	190.43	37.4	2.07	0.41
flavonoid 3'-hydroxylase (F3'H)	At5g07990	51.17	354.3	880.49	6.92	17.21
flavanone 3-hydroxylase (F3H)	At3g51240	2217.12	4898.76	5133.61	2.21	2.32
ribonuclease 1 (RNS1)	At2g02990	105.84	69.75	396.3	0.66	3.74
B. flavonoid biosynthetic process, Col-0, chronic cold						
myb family transcription factor (MYB12)	At2g47460	108.07	1071.67	407.78	9.92	3.77
flavonoid 3'-hydroxylase (F3'H)	At5g07990	51.17	354.3	880.49	6.92	17.21
flavanone 3-hydroxylase (F3H)	At3g51240	2217.12	4898.76	5133.61	2.21	2.32
flavonol synthase 1 (FLS1)	At5g08640	776.6	1542.79	2904.8	1.99	3.74
chalcone synthase	At5g13930	4005.72	3792.71	8229.13	0.95	2.05
chalcone isomerase (CHI)	At3g55120	613.36	1085.09	2576.37	1.77	4.2
UDP-rhamnose:flavonol-3-O-rhamnosyltransferase	At1g30530	127.06	288.53	508.55	2.27	4
C. flavonoid biosynthetic process, Sha, acute cold						
myb family transcription factor (MYB12)	At2g47460	194.82	1164.41	297.06	5.98	1.52
flavonoid 3'-hydroxylase (F3'H)[b]	At5g07990	31.91	371.41	420.83	11.64	13.19

flavanone 3-hydroxylase (F3H)[b]	At3g51240	1699.21	5392.74	3693.49	3.17	2.17
flavonol synthase 1 (FLS1)[b]	At5g08640	1025.38	2529.11	2568.04	2.47	2.5
chalcone isomerase (CHI)[b]	At3g55120	963.96	2473.53	2529.41	2.57	2.62
UDP-rhamnose:flavonol-3-O-rhamnosyltransferase[b]	At1g30530	63.53	499.22	331.48	7.86	5.22
flavonol 7-O-glucosyltransferase	At4g34135	1527.71	3158.72	2282.01	2.07	1.49
oxidoreductase, 2OG-Fe(II) oxygenase family protein	At4g25300	15.34	31.29	26.78	2.04	1.75
D. phenylpropanoid biosynthetic process, Sha, chronic cold						
cinnamoyl-CoA reductase-related	At2g23910	164.73	766.72	572.23	4.65	3.47
flavonoid 3'-hydroxylase (F3'H)[b]	At5g07990	31.91	371.41	420.83	11.64	13.19
flavanone 3-hydroxylase (F3H)[b]	At3g51240	1699.21	5392.74	3693.49	3.17	2.17
flavonol synthase 1 (FLS1)[b]	At5g08640	1025.38	2529.11	2568.04	2.47	2.5
chalcone isomerase (CHI)[b]	At3g55120	963.96	2473.53	2529.41	2.57	2.62
UDP-rhamnose:flavonol-3-O-rhamnosyltransferase[b]	At1g30530	63.53	499.22	331.48	7.86	5.22
cinnamyl-alcohol dehydrogenase, putative	At1g72680	366.38	536.61	743.16	1.46	2.03
caffeoyl-CoA 3-O-methyltransferase, putative	At1g67980	144.94	338.12	341.84	2.33	2.36

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[b] 5 cold-induced genes in group “flavonoid biosynthetic process” that shared between acute and chronic cold responses as indicated in Table 4.8A.

Genes encoding cinnamoyl-CoA reductase-related (At2g23910, At4g30470, At5g14700), S-adenosyl-L-methionine:transcaffeoyl CoA 3-O-methyltransferase (At1g67980), and a putative cinnamyl-alcohol dehydrogenase/oxidoreductase (At1g72680) concerned with phenylpropanoid metabolism were also cold induced. These genes are of interest because they encode enzymes important for synthesis of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig. 4.4) that are lignin precursors (Boerjan, Ralph & Baucher 2003).

Lipid transfer protein (*LTP*) genes, which are grouped under the "lipid transport" GO term and encode potential cell-wall loosening activities (Nieuwland *et al.* 2005), were over-represented in the chronic cold treated Col-0 (Table 4.2A). *LTP* gene (At4g12490) was induced by more than 223 fold in chronic-cold treated Col-0 (Table 4.1, 4.9), and its expression level was confirmed by RT-qPCR (Table 4.1). Genes grouped under "cell wall organization and biogenesis (Sensu Magnoliophyta)" were highly over-represented primarily in the set of genes induced by chronic cold in Sha (1.62E-04) but not in Col-0 (Table 4.2A). This group includes genes encoding proline-rich extensin-like family proteins (At5g49080, At4g08410, At5g35190, At5g06630, At1g26250, At3g54580, At2g24980, At5g06640), which are cell wall structural proteins (Tierney & Varner 1987; Cassab 1998); α-expansin 18 (At1g62980) and β-expansin 2 (At1g65680), which have the cell wall loosening activity (Cosgrove 1998; Cosgrove 2001); the cell-wall structural constituent of ATHRGP1 (At3g54590); and, the ROP-Interactive CRIB motif-containing protein 1 (RIC1) (At2g33460) were among the cell wall related genes up-regulated in the chronic cold but not in acute cold treated Sha. *β-expansin 2* was the only exception in which *β-expansin 2* gene was up-regulated more than 2-fold in acute cold treated Sha, in addition to chronic cold treatment (Table 4.5). *Phosphoethanolamine N-methyltransferase 1*/ *XIPOTL1* (At3g18000), the only gene enriched in the choline biosynthetic process group, was induced more than 2-fold, but only in Sha treated by chronic cold (Table 4.9).

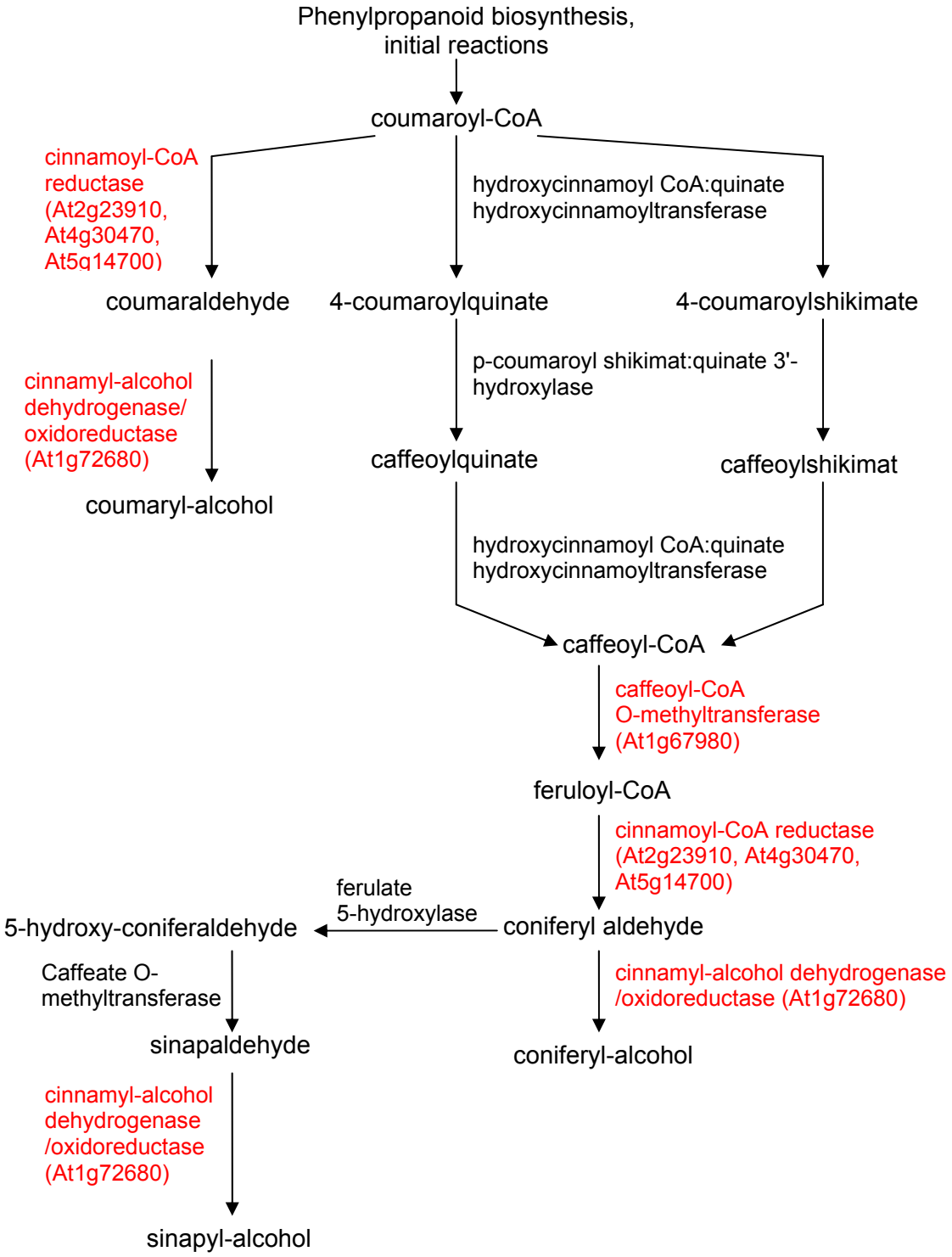


Figure 4.4. A simplified biosynthesis pathway of monolignols. Genes that labeled in red were induced in response to acute or chronic cold treatment of root of Col-0 or Sha that led to the synthesis of coumaryl-, sinapyl-l or coniferyl-alcohol.

Table 4.5. Expression values of chronic cold-induced Sha genes grouped under “cell wall organization and biogenesis (sensu Magnoliophyta)”.

		Median expression value			Ratio *	
Annotation	AGI	warm	acute	chronic	acute	chronic
proline-rich extensin-like family protein	At5g49080	82.64	96.55	298.21	1.17	3.61
proline-rich extensin-like family protein	At4g08410	37.86	44.30	144.01	1.17	3.80
proline-rich extensin-like family protein	At5g35190	322.60	247.03	831.40	0.77	2.58
proline-rich extensin-like family protein	At5g06640	191.42	117.52	612.42	0.61	3.20
proline-rich extensin-like family protein	At3g54580	111.56	57.15	340.91	0.51	3.06
proline-rich extensin-like family protein	At2g24980	217.98	103.78	575.83	0.48	2.64
proline-rich extensin-like family protein	At5g06630	224.46	119.29	882.00	0.53	3.93
proline-rich extensin, putative	At1g26250	44.24	15.47	99.06	0.35	2.24
α-expansin 18	At1g62980	376.59	397.66	854.90	1.06	2.27
β-expansin 2	At1g65680	107.78	232.95	512.15	2.16	4.75
ATHRGP1, structural constituent of cell wall	At3g54590	260.93	251.39	746.73	0.96	2.86
ROP-Interactive CRIB motif-containing protein 1	At2g33460	29.41	40.85	76.62	1.39	2.61

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

The seedlings used for transcriptome analyses were grown under conditions in which roots were exposed to light. Roots exposed to light are known to green and develop chloroplasts (Heltne & Bonnett 1970; Oliveira 1982; Usami *et al.* 2004). Althought not intend to survey transcript abundance of photosynthetic related genes in root, however, greening of roots provide an opportunity to study the cold effects on photosynthesis and detect any enrichment of photosynthetic related genes in the root system. A number of photosynthesis-related genes were significantly over-represented in the set of genes down-regulated by cold treatment in Col-0 and Sha (Table 4.2B). Genes grouped under the function "electron transport" were also affected by cold treatment, with the exception of acute-cold treated Sha plants (Table 4.2B). Almost half of these genes were down-regulated by cold treatment, which probably reflects the decreased reduction of oxidative metabolism in the cold (Appendix Table VII, VIII, IX).

Genes related to terpenoid biosynthesis and metabolism were affected by cold treatment (Tables 4.2B). For example, transcript abundance of genes encoding pentacyclic triterpene synthase 1 (AtPEN1) (At4g15340), lanosterol synthase 1 (At3g45130), marneral synthase (At5g42600), and lupeol synthase 1 (At1g78970) were repressed by chronic cold treated Col-0 or Sha (Table 4.6). The terpenoid pathway is required for the biosynthesis of numerous biologically active molecules including some phytoalexins gibberellins (GA), abscisic acid (ABA), brassinosteroids, and cytokinins (Aharoni, Jongsma & Bouwmeester 2005). Expression of genes encoding β-carotene hydroxylase (At4g25700) and zeaxanthin epoxidase/ABA1 (At5g67030), which are the key genes responsible for converting carotene to ABA (Nambara & Marion-Poll 2005) (Fig. 4.5), were repressed by chronic-cold treatment of Col-0, whereas only β-carotene hydroxylase was repressed by chronic-cold treatment of Sha (Table 4.6). In contrast, acute cold-treatment of Col-0 and Sha resulted in a ca. 3-fold induction of β-carotene hydroxylase expression, but less than 2-fold regulation of zeaxanthin epoxidase/ABA1.

2,3-Oxidosqualene is the key precursor in triterpenoid pathways leading to plant sterols and brassinosteroids (Fig. 4.6) (Phillips *et al.* 2006). Genes encoding enzymes catalyzing the first step in these pathways were down-regulated by chronic cold treatment of Col-0 or Sha. The most dramatic effect was on expression of the marneral synthase, which was down-regulated ca. 224 fold in Col-0 and 54-fold in Sha and confirmed by RT-qPCR (Table 4.1, 4.6). The gene encoding pentacyclic triterpene synthase 1/AtPEN1, which catalyzes the first step in the cycloartenol pathway leading to the membrane component β-sitosterol and brassinosteroids, was repressed by more than 3 and 5 fold in Col-0 and Sha, respectively (Table 4.6).

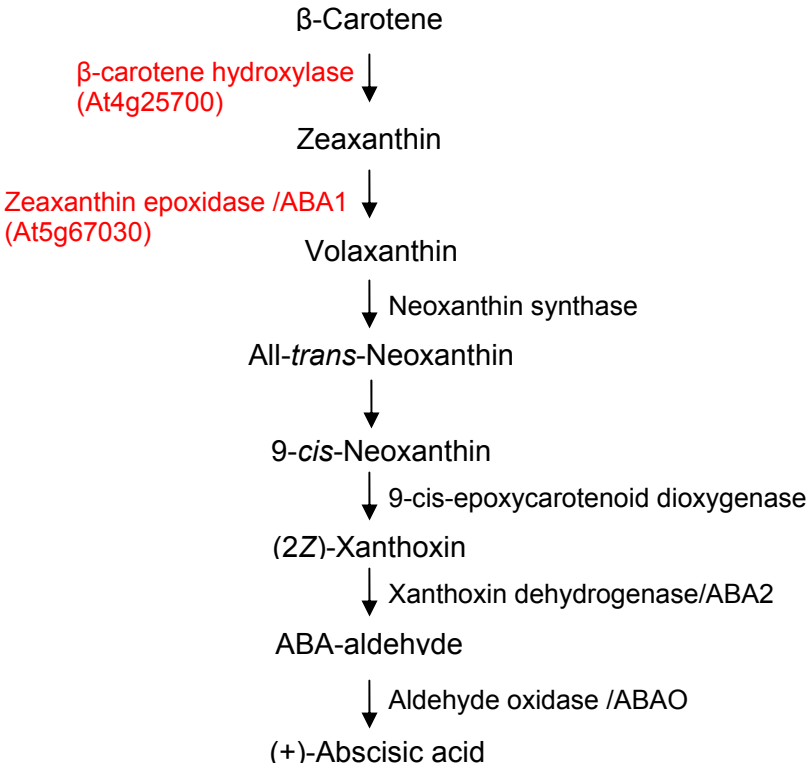


Figure 4.5. A simplified pathway for the biosynthesis of (+)-abscisic acid in plants. Genes labeled in red were chronic-cold repressed in root of Col-0 or Sha accession.

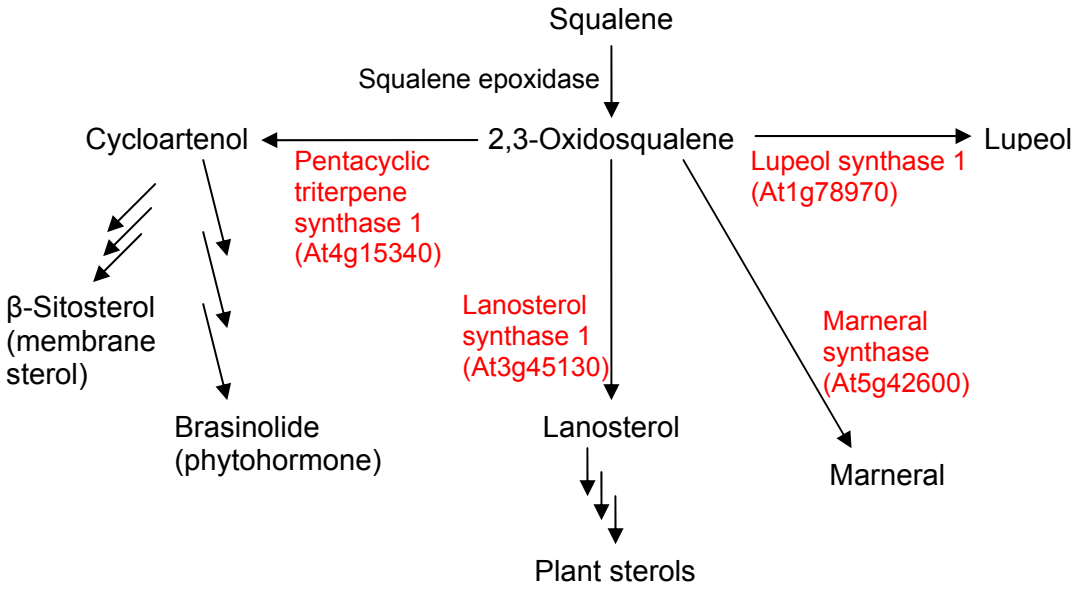


Figure 4.6. A simplified plant triterpenoid biosynthetic pathways. Genes labeled in red were chronic-cold repressed in root of Col-0 or Sha accession. Multiple arrows indicate products can be further modified by multiple enzymes to form membrane sterols, brassinosteroids, and other compounds.

Table 4.6. Expression values of chronic cold-repressed genes that were grouped under "terpenoid biosynthetic process" in Col-0 or Sha accession.

		Col-0					Sha				
		Median expression value			Ratio[a]		Median expression value			Ratio[a]	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
terpene synthase 12 [b, c]	At4g13280	849.017	178.056	159.301	0.210	0.188	493.241	192.446	170.532	0.390	0.346
terpene synthase-like sequence-1,8-cineole [b]	At3g25830	5387.069	3545.205	2537.319	0.658	0.471	4021.337	2595.685	2697.966	0.645	0.671
terpene synthase-like sequence-1,8-cineole [b]	At3g25820	1227.682	834.727	324.204	0.680	0.264	835.823	719.795	623.390	0.861	0.746
pentacyclic triterpene synthase 1/AtPEN1 [b,c , d]	At4g15340	190.961	203.216	58.690	1.064	0.307	22.530	16.669	4.439	0.740	0.197
marneral synthase [b, c, d]	At5g42600	1055.554	333.044	4.695	0.316	0.004	449.477	59.017	8.227	0.131	0.018
lanosterol synthase 1 [c, d]	At3g45130	34.492	19.100	56.190	0.554	1.629	184.179	35.086	86.529	0.191	0.470
lupeol synthase 1 (LUP1) [c, d]	At1g78970	68.542	79.128	37.618	1.154	0.549	20.225	18.809	6.352	0.930	0.314
β-carotene hydroxylase [b, c]	At4g25700	258.414	766.782	99.984	2.967	0.387	171.289	614.755	70.225	3.589	0.410
zeaxanthin epoxidase/ABA1 [b]	At5g67030	1414.831	724.336	661.464	0.512	0.468	661.951	918.100	469.776	1.387	0.710
gibberellin 3 beta-hydroxylase (GA4) [b, c]	At1g15550	912.882	169.015	418.326	0.185	0.458	820.295	186.939	320.346	0.228	0.391
gibberellin 2-oxidase / GA2-oxidase (GA2OX2) [c]	At1g30040	58.369	48.379	15.507	0.829	0.266	140.727	96.924	16.812	0.689	0.119
cytochrome p450-type monooxygenase 97a3 [b]	At1g31800	210.376	105.447	87.689	0.501	0.417	184.179	35.086	86.529	0.191	0.470

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[b] 9 genes in group "terpenoid biosynthetic process" in set of chronic-cold repressed genes of Col-0 as shown in Table 4.2B.
[c] 8 genes in group "terpenoid biosynthetic process" in set of chronic-cold repressed genes of Sha as shown in Table 4.2B.
[d] 4 genes in group "triterpenoid metabolic process" in set of chronic cold-repressed genes of Sha as shown in Table 4.2B.

Genes in the group of “secondary cell wall biosynthetic process (sensu Magnoliophyta)” showed a highly significant enrichment in the set of genes repressed by chronic cold treatment of Col-0 (p=7.78E-6) and Sha (p=2.28E-05), but essentially unaffected by acute cold treatment (Table 4.2B). For example, genes in a series of *irregular xylem* genes encoding cellulose synthase catalytic subunits were chronic cold-repressed more than 2 – 5 fold in both accessions. The “xylem histogenesis” group of genes was enriched in chronic-cold treated Sha. Included in this group are Vascular Related NAC-Domain Protein 7 (VND7) (At1g71930), Altered Phloem Development (APL) (At1g79430), and ATHB-8 (At4g32880) which were significantly down-regulated 2 – 3 fold (Table 4.7). These genes have been implicated in regulating protoxylem vessel formation (Kubo *et al.* 2005), determining the organization of phloem poles (Bonke *et al.* 2003), and promoting vascular cell differentiation (Baima *et al.* 2001) in roots.

Table 4.7. Expression values of chronic cold-repressed of secondary cell wall biosynthesis or xylem histogenesis related genes in Col-0 or Sha accession.

		Col-0					Sha				
		Median expression value			Ratio[a]		Median expression value			Ratio[a]	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
secondary cell wall biosynthetic process [b]											
cellulase synthase 8 (CESA8)/IRX1[c]	At4g18780	154.11	158.95	27.16	1.03	0.18	176.55	180.86	35.03	1.02	0.20
cellulase synthase 7 (CESA7)/IRX3[c]	At5g17420	241.67	247.85	49.40	1.03	0.20	273.99	226.41	69.82	0.83	0.25
cellulase synthase 4 (CESA4)/IRX5	At5g44030	238.31	124.70	48.98	0.52	0.21	161.63	55.24	40.88	0.34	0.25
phytochelatin synthetase /COBL4/IRX6[c]	At5g15630	76.29	58.14	16.68	0.76	0.22	102.43	68.94	20.74	0.67	0.20
glycosyl transferase family 43 protein/IRX9[c]	At2g37090	182.60	143.08	51.80	0.78	0.28	176.69	130.54	77.35	0.74	0.44
laccase 4/diphenol oxidase, putative/IRX12[c]	At2g38080	193.42	214.51	37.01	1.11	0.19	241.56	263.47	60.49	1.09	0.25
xylem histogenesis [d]											
Altered Phloem Development (APL)	At1g79430	148.26	106.58	82.88	0.72	0.56	220.00	150.51	97.54	0.68	0.44
homeobox-leucine zipper (HB-8)/AtHB-8	At4g32880	269.54	220.12	111.46	0.82	0.41	456.06	316.95	172.87	0.69	0.38
Vascular Related Nac-Domain 7 (VND7)	At1g71930	16.81	13.88	7.51	0.83	0.45	17.52	12.61	5.60	0.72	0.32

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[b] Significantly over-represented in Col-0 or Sha accession as indicated in Table 4.2B.
[c] These genes were grouped under “cellulose and pectin-containing secondary cell wall biogenesis” and significantly over-represented in repressed genes specific to chronic response in Sha as indicated in Table 4.8B.
[d] Identical set of genes that were significantly over-represented in both chronic-cold-repressed Sha dataset and chronic-cold-repressed-specific Sha dataset as indicated in Table 4.2B and Table 4.8B, respectively.

4.4 Shared and cold-treatment-specific gene expression

Section 4.3 described biological functions or genes which were affected by acute or chronic cold treatment. The same sets of genes were sorted to identify shared and cold-treatment-specific genes in response to acute or chronic cold treatment (Fig. 4.1). The numbers of up-regulated genes shared by both cold treatments were 176 in Col-0 and 115 in Sha. The numbers of down-regulated genes shared by both cold treatments were 315 and 310 in Col-0 and Sha, respectively (Fig. 4.1A, 4.1B). At least 82% of cold-regulated genes were cold-treatment-specific in either Col-0 or Sha (Fig 4.1A, 4.1B).

The results of screens for enriched GO terms are summarized in Table 4.8 and were similar to the results summarized in Table 4.2. Genes in the group “regulation of transcription” (p=8.98E-03) and genes related to hormonal and salicylic acid responses such as ”response to salicyclic acid stimulus” (8.98E-03), “response to abscisic acid stimulus” and “response to ethylene stimulus”, were over-represented and induced specifically by acute cold treatment of Col-0. Similar hormone responses were found in Sha as well, and with additional groups including “response to jasmonic acid stimulus”, “response to gibberellin stimulus” and “response to auxin stimulus”. Genes grouped under the GO terms “regulation of transcription” (p=4.67E-04), response to “cadmium ion”, “salt stress”, and “PSII associated light-harvesting complex II catabolic process” were also enriched specifically in the set of acute cold-induced genes in Sha (Table 4.8A).

The only functional group enriched in the set of genes up-regulated specifically by chronic cold-treatment of Col-0 was “lipid transport” (2.01E-02) (Table 4.8A, 4.9). In contrast, genes in the groups “cell wall organization and biogenesis”, “inorganic anion transport”, “cellular response to starvation”, “root development”, “choline biosynthetic process”, and “glycolipid biosynthetic process” were over-represented in the set of genes specifically induced by chronic-cold treatment of Sha (Table 4.8A, 4.9).

Table 4.8. Non redundant terms of biological processes significantly over-represented in sets of either up- (**A**) or down-regulated (**B**) genes that specific to acute- or chronic-cold treatment in Col-0 or Sha accession.

(**A**) Up-regulated genes Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Col-0, acute specific (488)		
response to salicylic acid stimulus	8.98E-03	4
regulation of transcription	8.98E-03	7
response to abscisic acid stimulus	1.13E-02	5
response to ethylene stimulus	3.89E-02	5
Col-0, shared (176)		
No significant terms	NA[c]	NA
Col-0,chronic specific (463)		
lipid transport	2.01E-02	5
Sha, acute specific (567)		
regulation of transcription	4.67E-04	7
response to cadmium ion	4.67E-04	6
response to salt stress	1.58E-03	5
response to jasmonic acid stimulus	1.68E-03	4
response to ethylene stimulus	2.01E-03	5
response to salicylic acid stimulus	8.93E-03	4
response to abscisic acid stimulus	2.35E-02	5
response to gibberellin stimulus	2.91E-02	5
PSII associated light-harvesting complex II catabolic process	3.96E-02	8
response to auxin stimulus	3.96E-02	5
Sha, shared (115)		
flavonoid biosynthetic process	1.97E-03	8
Sha, chronic specific (329)		
cell wall organization and biogenesis	1.10E-04	5
inorganic anion transport	8.68E-04	7
cellular response to starvation	1.62E-02	7
root development	2.30E-02	6
choline biosynthetic process	2.30E-02	9
glycolipid biosynthetic process	2.99E-02	8

(**B**) Down-regulated genes		
Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Col-0, acute specific (763)		
No significant terms	NA[c]	NA
Col-0, shared (315)		
photosynthesis, light harvesting	7.29E-04	6
electron transport	7.29E-04	5
Col-0,chronic specific (579)		
No significant terms	NA	NA
Sha, acute specific (689)		
starch catabolic process	1.42E-03	9
circadian rhythm	2.72E-03	3
Sha, shared (310)		
photosynthesis, light harvesting in photosystem I	5.65E-03	7
response to heat	1.54E-02	5
Sha, chronic specific (601)		
cellulose and pectin-containing secondary cell wall biogenesis	1.92E-04	8
xylem histogenesis	2.44E-02	8
electron transport	3.38E-02	5

[a] p-value adjusted for false discovery rate (FDR).
[b] Gene ontology biological process level.
[c] Not applicable.
Values in parenthesis are the number of genes in the dataset indicated.

Genes in group “glycolipid biosynthetic” encoding phospholipase D Zeta 2 (PLDZ2) (At3g05630), monogalactosyldiacylglycerol synthase type C (MGD3) (At2g11810), and UDP-sulfoquinovose:DAG sulfoquinovosyltransferase/sulfolipid synthase (SQD2) (At5g01220), while phosphoethanolamine N-methyltransferase 1 (PEAMT)/XIPOTL1 (AT3g18000) in group “choline biosynthetic”. PLDZ2 and PEAMT are involve in the biosynthesis of choline (Fig. 4.7) (Cruz-Ramirez *et al.* 2004). MGD3 is catalyzes the conversion of 1,2-diacylglycerol for digalactosyldiacylglycerol (DGDG) (Fig 4.8A). SQD2 is responsible for the conversion of UDP-sulfoquinovose to sulfoquinovosyldiacylglycerol (SQDG) (Fig. 4.8B). Both MGDG and DGDG are part of the non-phosphorus lipid composition of plastid or plasma membrane (Benning 1998; Awai *et al.* 2001). The expression levels of these genes in both accessions are shown in Table 4.9 and Appendix Table III.

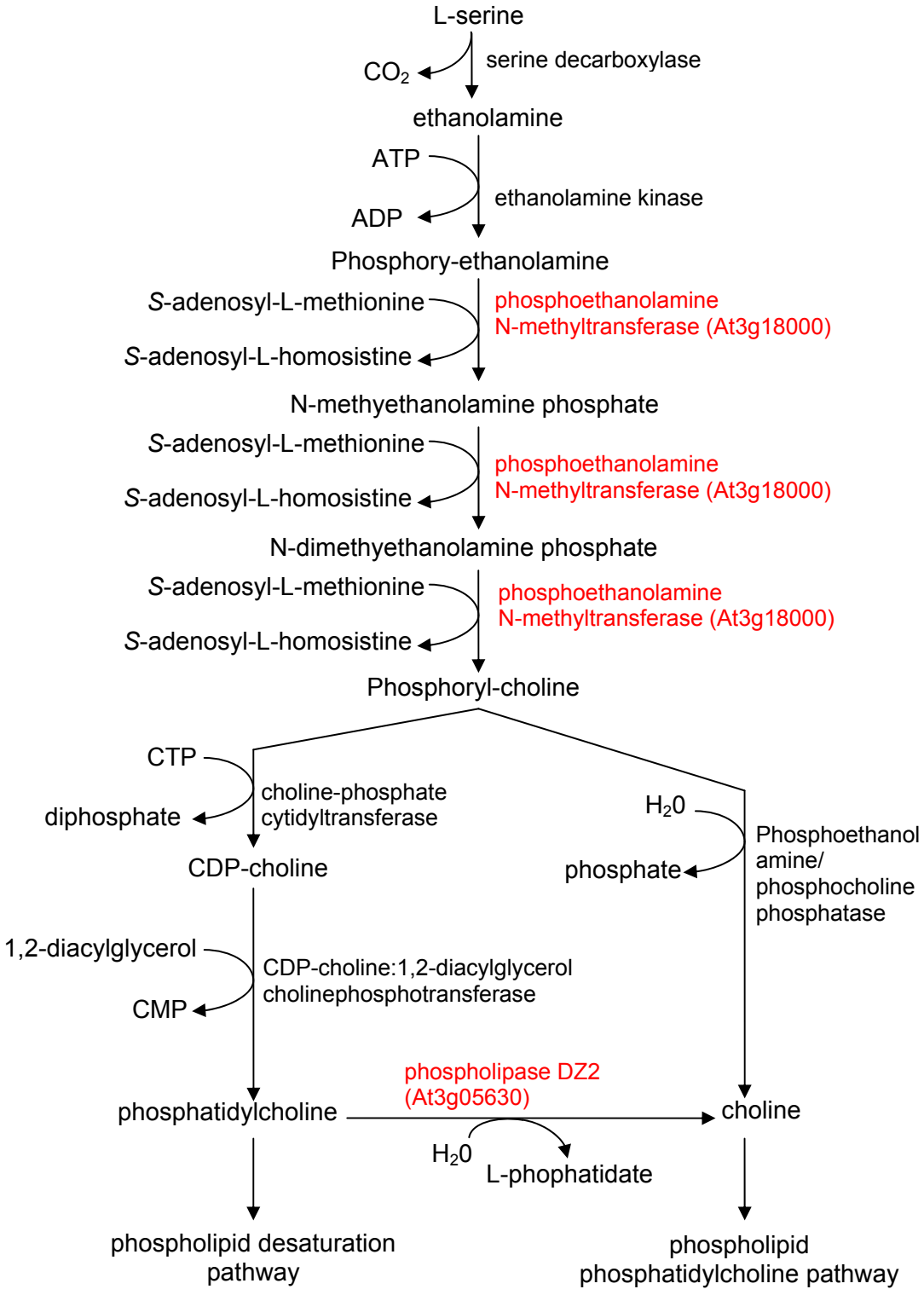
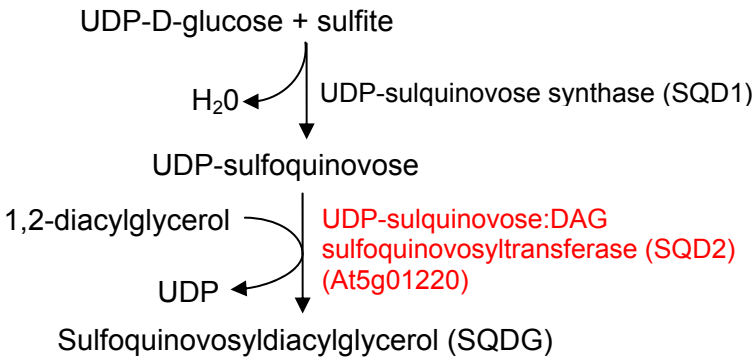


Figure 4.7. A simplified choline biosynthetic pathway using serine as initial precursor. Genes labeled in red were specific to chronic cold-induced of Sha dataset.

A.



B.

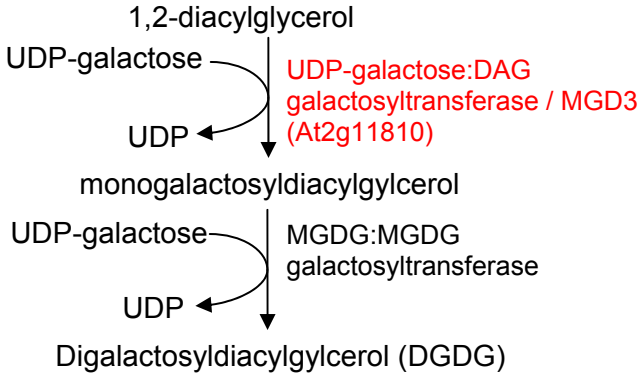


Figure 4.8. Simplified biosynthetic pathways for sulfoquinovosyldiacylglycerol (**A**) or digalactosyldiacylgylcerol (**B**). Genes labeled in red were specific chronic cold-induced of Sha dataset.

"Flavonoid biosynthetic process" genes were significantly over-represented and shared in up-regulated genes in Sha (Table 4.8A). Similar genes were annotated under "flavonoid biosynthetic process" including flavonoid 3'-hydroxylase (At5g07990), flavanone 3-hydroxylase (At3g51240), flavonol synthase 1 (At5g08640), chalcone isomerase (At3g55120), and UDP-rhamnose:flavonol-3-O-rhamnosyltransferase (At1g30530) (Table 4.4C, 4.4D). However, no enrichment of genes in particular GO terms in up-regulated genes which shared between acute and chronic cold treatments in Col-0.

Genes concerned with photosynthesis were over-represented in sets of genes down regulated by both cold treatments in both accessions (Table 4.8B). Genes grouped

under “photosynthesis, light harvesting” (7.29E-04) and “electron transport” (7.29E-04) were significantly over-represented and down-regulated by acute and chronic cold treatments of Col-0. Genes grouped under “photosynthesis, light harvesting in photosystem I” and “response to heat” were over-represented and down-regulated by acute and chronic cold treatments of Sha (Table 4.8).

Although no function groups of genes were over-represented in the sets of genes specifically repressed by acute- or chronic- cold treatment of Col-0, the functional groups “cellulose and pectin-containing secondary cell wall biogenesis” (p=1.92E-04), “xylem histogenesis” (2.44E-02), and “electron transport” (3.38E-02), were over-represented in the set of genes specifically repressed by chronic-cold treatment of Sha (Table 4.8B). Similar set of genes in the groups of “cellulose and pectin-containing secondary cell wall biogenesis”, and “xylem histogenesis” were were over-represented in the set of genes specifically repressed by chronic-cold treatment of both accessions (Table 4.7).

Table 4.9. Expression values of chronic cold induced-specific genes in Col-0 or chronic cold induced genes that specific to Sha that grouped in various GO terms.

		Col-0[a]					Sha[b]				
		Median expression value			Ratio[c]		Median expression value			Ratio[c]	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
Col-0, chronic specific											
lipid transport[d]											
lipid transfer protein, putative	At5g01870	1237.29	1219.73	3317.00	0.99	2.68	768.64	1057.35	2450.65	1.38	3.19
lipid transfer protein 5 (LTP5)	At3g51600	215.17	241.77	443.94	1.12	2.06	182.96	219.29	234.44	1.20	1.28
protease inhibitor/lipid transfer protein	At5g64080	182.69	338.35	499.35	1.85	2.73	204.64	208.26	288.05	1.02	1.41
protease inhibitor/lipid transfer protein	At4g12490	25.54	80.06	5711.32	3.13	223.59	18.94	59.31	316.91	3.13	16.73
protease inhibitor/lipid transfer protein	At4g12480	1311.44	2316.52	5679.19	1.77	4.33	50.24	76.40	550.04	1.52	10.95
protease inhibitor/lipid transfer protein	At4g15160	65.95	21.57	132.62	0.33	2.01	126.54	29.65	266.27	0.23	2.10
protease inhibitor/lipid transfer protein	At4g22470	6.63	7.74	137.56	1.17	20.73	5.52	5.38	12.07	0.98	2.19
protease inhibitor/lipid transfer protein	At4g12500	27.10	68.46	2589.64	2.53	95.54	5.93	7.62	50.23	1.29	8.47
protease inhibitor/lipid transfer protein[e]	At4g12470	1371.83	3074.09	9358.44	2.24	6.82	196.49	935.32	2751.40	4.76	14.00
Sha, chronic specific											
cell wall organization and biogenesis											
proline-rich extensin-like family protein[f]	At5g49080	243.21	216.00	430.94	0.89	1.77	82.64	96.55	298.21	1.17	3.61
proline-rich extensin-like family protein[f]	At4g08410	68.42	84.13	98.33	1.23	1.44	37.86	44.30	144.01	1.17	3.80
proline-rich extensin-like family protein[f]	At5g06630	214.70	145.39	328.24	0.68	1.53	224.46	119.29	882.00	0.53	3.93
proline-rich extensin-like family protein[f]	At5g06640	205.91	180.78	276.26	0.88	1.34	191.42	117.52	612.42	0.61	3.20
proline-rich extensin-like family	At3g54580	153.30	97.97	180.05	0.64	1.17	111.56	57.15	340.91	0.51	3.06

protein[f]											
proline-rich extensin-like family protein[f]	At2g24980	263.10	176.88	332.65	0.67	1.26	217.98	103.78	575.83	0.48	2.64
proline-rich extensin-like family protein[f]	At5g35190	331.36	293.47	557.15	0.89	1.68	322.60	247.03	831.40	0.77	2.58
proline-rich extensin, putative	At1g26250	51.84	33.52	148.50	0.65	2.86	44.24	15.47	99.06	0.35	2.24
α-expansin 18[f]	At1g62980	389.45	514.60	573.38	1.32	1.47	376.59	397.66	854.90	1.06	2.27
ATHRGP1, structural constituent of cell wall[f]	At3g54590	339.07	420.24	409.91	1.24	1.21	260.93	251.39	746.73	0.96	2.86
Rop-Interactive Crib Motif-Containing Protein 1	At2g33460	21.17	42.14	42.55	1.99	2.01	29.41	40.85	76.62	1.39	2.61
pectinesterase family protein	At2g45220	552.61	707.24	1284.25	1.28	2.32	613.18	535.96	1336.08	0.87	2.18
pectinesterase family protein	At5g04960	801.32	956.99	955.94	1.19	1.19	826.44	774.77	1770.06	0.94	2.14
pectinesterase family protein	At3g10710	273.72	392.78	415.18	1.43	1.52	254.86	319.65	716.23	1.25	2.81
inorganic anion transport											
sulfate transporter[g]	At4g08620	21.54	26.73	22.41	1.24	1.04	56.03	59.75	276.50	1.07	4.93
sulfate transporter (Sultr1;3)[g]	At1g22150	5.49	7.46	6.81	1.36	1.24	6.65	11.23	77.41	1.69	11.63
sulfate transporter, putative[g,h]	At1g23090	19.49	71.25	41.36	3.65	2.12	52.96	122.21	118.14	2.31	2.23
phosphate transporter (PT2)	At2g38940	2133.22	1653.35	4636.21	0.78	2.17	681.23	656.57	3166.84	0.96	4.65
high-affinity nitrate transporter (ACH1)	At1g08090	12.01	7.57	19.21	0.63	1.60	10.18	8.01	43.10	0.79	4.23
inorganic phosphate transporter (PHT3)	At5g43360	13.62	23.77	18.51	1.75	1.36	61.55	114.09	1497.31	1.85	24.33
expressed protein	At5g50200	430.12	358.71	1776.29	0.83	4.13	523.72	433.88	1152.82	0.83	2.20
cellular response to starvation											
phospholipase D Zeta 2 (PLDZ2)[i]	At3g05630	75.50	47.47	99.72	0.63	1.32	26.59	16.03	129.73	0.60	4.88
monogalactosyldiacylglycerol synthase type C (MGD3)[i]	At2g11810	196.05	122.58	263.81	0.63	1.35	29.71	43.46	298.16	1.46	10.04
UDP-sulfoquinovose:DAG sulfoquinovosyltransferase (SQD2)[i]	At5g01220	1448.87	777.29	1388.65	0.54	0.96	592.03	424.10	1744.82	0.72	2.95
2-oxoacid-dependent oxidase, putative	At3g49620	6.26	7.89	9.10	1.26	1.45	6.83	6.61	18.01	0.97	2.64

root development											
phospholipase D Zeta 2 (PLDZ2)[j]	At3g05630	75.50	47.47	99.72	0.63	1.32	26.59	16.03	129.73	0.60	4.88
Morphogenesis of Root Hair 2 (MRH2)[j]	At3g54870	58.60	43.38	78.74	0.74	1.34	60.93	43.64	158.80	0.72	2.61
Morphogenesis of Root Hair 6 (MRH6)[j]	At2g03720	317.02	365.43	479.66	1.15	1.51	541.41	463.48	1258.57	0.86	2.32
leucine-rich repeat/extensin 1 (LRX1)[j]	At1g12040	23.40	34.93	34.19	1.49	1.46	21.12	26.75	92.43	1.27	4.38
Nitrate Transporter 2.1[j]	At1g08090	12.01	7.57	19.21	0.63	1.60	10.18	8.01	43.10	0.79	4.23
copper transporter 1 (COPT1)[j]	At5g59030	68.82	80.47	78.51	1.17	1.14	51.07	69.06	111.31	1.35	2.18
homeobox-leucine zipper family protein	At5g66700	65.22	65.55	166.34	1.01	2.55	11.91	14.03	30.68	1.18	2.58
choline biosynthetic process											
phosphoethanolamine N-methyltransferase 1[k]	At3g18000	376.28	554.34	580.12	1.47	1.54	487.17	462.72	1058.12	0.95	2.17

[a] Only “lipid transport” terms was significantly enriched in chronic-specific dataset in Col-0 ($p<0.05$).
[b] All functional groups/GO terms were significantly enriched in chronic-specific dataset in Sha ($p<0.05$).
[c] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[d] Identical annotations grouped under “lipid transport” in chronic cold-induced transcripts of Col-0 as in Table 4.2A.
[e] Not included in the GO term “lipid transport” of chronic cold-induced specific response of Col-0 in Table 4.8A.
[f] These genes were grouped under “cell wall organization and biogenesis (sensu Magnoliophyta)” for chronic cold response and Sha specific genes in Table 4.10A.
[g] These genes were grouped under “sulfate transport” for chronic cold response of Sha specific genes in Table 4.10A.
[h] Not included in the GO term “inorganic anion transport” of chronic cold-induced specific response of Sha in Table 4.8A.
[i] These genes were also grouped under “glycolipid biosynthetic process” in chronic cold-induced specific response of Sha in Table 4.8A. These genes were Identical to genes grouped under “glycolipid biosynthetic process” or “cellular response to phosphate starvation” for chronic cold response and Sha specific genes in Table 4.10A.
[j] These genes were grouped under “root development” for chronic cold response of Sha specific genes in Table 4.10A.
[k] An identical gene that grouped under “choline biosynthetic process” for chronic cold-induced genes in Sha and Sha-specific as indicated in Table 4.2A and Table 4.10A, respectively.

4.5 Shared and accession specific gene expression

The set of cold-responsive genes was also sorted on the basis of accession to detect shared and accession-specific gene expression (Fig. 4.9). The results show that 34% of cold-responsive genes were specific for Col-0; that 29% were specific for Sha; and, that 37% were shared by both accessions. The results of screens for enriched GO terms are summarized in Table 4.10, 4.11, 4.12, and Appendix Table I-IX.

There was considerable overlap in the functional groups identified in datasets sorted on the basis of cold treatment (Table 4.8) and accession (Table 4.10). In general, however, there was poor agreement in the enrichment for GO terms in a geneset from the same accession sorted for a specific cold-treatment and in the corresponding geneset from the same cold treatment sorted for a specific accession. The one major exception was the functional groupings concerned with cell enlargement, cell-wall organization, and, membrane lipids, ion transport, which were very similar in the response of Sha to chronic-cold treatment. Genes in these functional groups show a robust specific association with the chronic cold response of Sha. Interestingly, no GO terms were enriched in the sets of genes induced by acute-cold treatment that were specific to either Col-0 or Sha.

I also compared the cold responsiveness of genes in several of the functional groups shown to be enriched in Col-0 specific, Sha specific and shared genesets (Table 4.11, 4.12, and Appendix Table I-IX). The results show that, in general, transcripts for photosynthetic-related and electron transport-related genes were strongly repressed by 2 to 12 fold and 2 to 25 fold, respectively, in both accessions (Table 4.10B, Appendix Table V, VII, VIII, and IX).

Screens of cold-responsive genesets for the GO terms “regulation of transcription” and “regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic processes” identified numerous transcription factors with diverse functions. Substantial numbers of genes encoding transcription factors were cold-induced and shared in both accessions (Table 4.11). Major classes of transcription factors in this group were members of the AP2/EREBP, MYB-R2R3, bHLH, bZIP, zinc-finger, and WRKY families. The only transcription factors consistently showing accession-specific down-regulation were in the functional grouping enriched in the chronic cold-repressed dataset specific to Sha (Table 4.12). Included in this group were genes encoding the MADS family [AGL21 (At4g37940), AGL72 (At5g51860), the squamosa promoter-binding

protein, putative (At3g57920)], brassinosteroid enhanced expression 2 (BEE2) (At4g36540), auxin-responsive factor (ARF4) (At5g60450), cytokinin response factor 5 (CRF5) (At2g46310), ethylene-responsive element-binding factor 5 (ERF5) (At5g47230), ethylene-responsive element-binding family protein (At5g07580), and scarecrow-like transcription factor 7 (SCL7) (At3g50650) (Table 4.12). These results shows that expression of numerous transcription factors concerned with growth and development are specifically repressed by chronic-cold treatment of Sha.

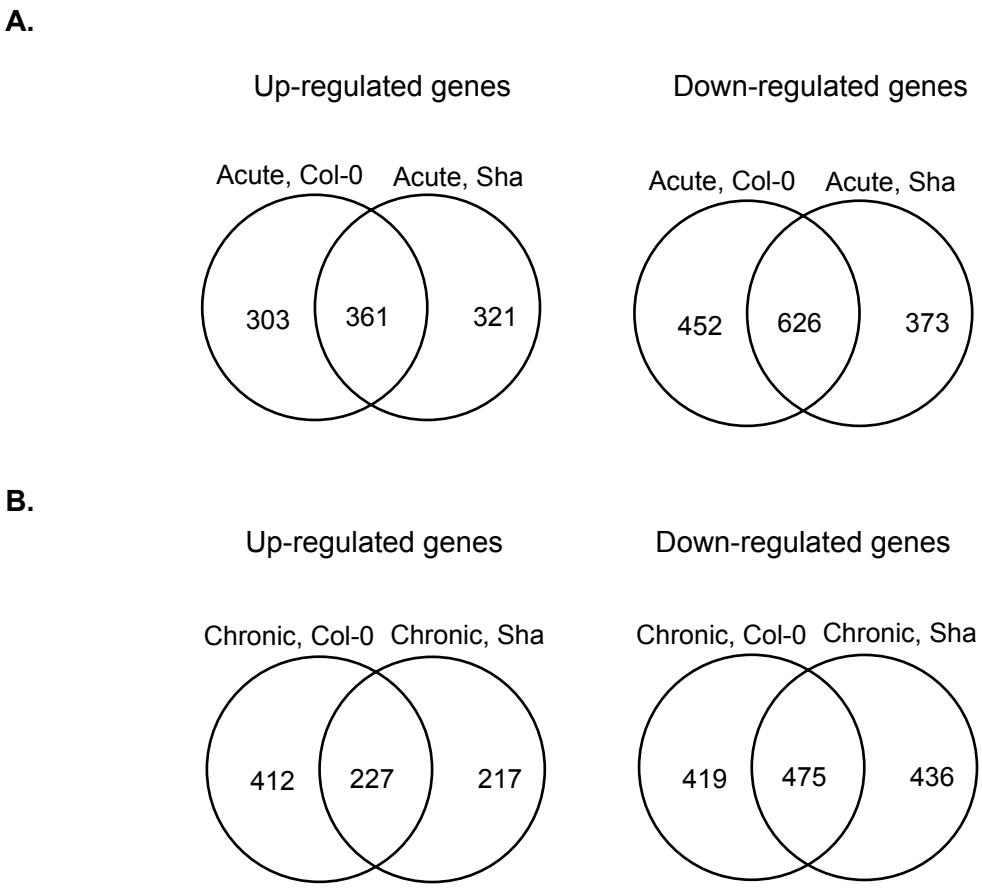


Figure 4.9. Overlapping of cold responsive genes between Col-0 and Sha accessions in response to acute (**A**) or chronic (**B**) cold treatment.

Table 4.10. Non redundant terms of biological processes significantly over-represented in sets of accession-specific or shared between Col-0 and Sha genes in either up- (**A**) or down- (**B**) regulated dataset in response to acute or chronic cold treatment.

A. Up-regulated genes Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Acute cold , Col-0 specific (303)		
No significant terms	NA[c]	NA
Acute cold , Sha specific (321)		
No significant terms	NA	NA
Acute cold , shared (361)		
response to ethylene stimulus	4.07E-03	5
response to salt stress	4.84E-03	5
response to jasmonic acid stimulus	4.84E-03	4
response to cadmium ion	1.35E-02	6
response to salicylic acid stimulus	1.35E-02	4
response to gibberellic acid stimulus	1.35E-02	5
response to UV-B	1.58E-02	7
regulation of transcription, DNA-dependent	3.78E-02	8
phenylpropanoid metabolic process	4.30E-02	6
response to auxin stimulus	4.30E-02	5
Chronic cold , Col-0 specific (412)		
circadian rhythm	4.97E-02	3
Chronic cold , Sha specific (217)		
cell wall organization and biogenesis (sensu Magnoliophyta)	2.80E-04	6
glycolipid biosynthetic process	1.68E-02	8
choline biosynthetic process	2.21E-02	9
sulfate transport	2.43E-02	8
cellular response to phosphate starvation	3.15E-02	8
root development	3.26E-02	6
Chronic cold , shared (227)		
flavonoid biosynthetic process	2.08E-02	8
regulation of transcription	3.32E-02	7

B. Down-regulated genes Biological process, non-redundant terms	FDR p-value[a]	Level[b]
Acute cold, Col-0 specific (452)		
photosynthesis	4.26E-03	3
Acute cold, Sha specific (373)		
circadian rhythm	4.36E-03	3
Acute cold, shared (626)		
photosynthesis	8.88E-04	3
cellular polysaccharide catabolic process	4.51E-02	7
electron transport	4.71E-02	5
Chronic cold, Col-0 specific (419)		
electron transport	2.04E-03	5
photosynthesis, light harvesting in photosystem II	2.79E-02	7
monoterpenoid metabolic process	4.04E-02	9
oligopeptide transport	4.41E-02	6
Chronic cold, Sha specific (436)		
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.99E-02	6
Chronic cold, shared (475)		
secondary cell wall biosynthetic process (sensu Magnoliophyta)	9.14E-07	8
electron transport	3.94E-03	5
photosynthesis, light harvesting in photosystem I	9.60E-03	7

[a] p-value adjusted for false discovery rate (FDR).
[b] Gene ontology biological process level.
[c] Not applicable.
Values in parenthesis are the number of genes in the dataset indicated.

To obtain a global comparison of the magnitude of cold-responsive gene expression in Col-0 and Sha, I generated a list comprising the 987 unique genes responsive to acute-cold treatment and the 702 unique genes responsive to chronic-cold treatment shared by both accessions. I then estimated the correlation of the fold response of Sha versus Col-0 for both cold treatments. There was a highly significant (P=6.321E-273) positive correlation in the response to acute cold treatment. The slope of the linear regression line (least squares method) was 1.06, indicated that for the magnitude of the response to acute cold is essentially identical in Col-0 and Sha. A similar, highly significant (P=4.6E-44) positive correlation was found for the response to chronic cold treatment. In this case, however, the slope of the linear regression line was 0.18. These results show that the magnitude of the response of Sha to chronic cold is 5 fold lower than that of Col-0. Thus, the two accessions exhibit quantitative, global, differences in response that are cold-treatment specific.

Table 4.11. Enrichment of transcription factor-related genes in sets of acute- or chronic-cold-responsive genes shared between Col-0 and Sha accessions.

		Col-0					Sha				
		Median expression value			Ratio[a]		Median expression value			Ratio[a]	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
regulation of transcription, DNA-dependent (acute cold)											
AP2 domain-containing transcription factor, putative /SCHLAFMUTZE (SMZ)[b]	At3g54990	80.2	230.93	290.46	2.88	3.62	86.51	190.34	237.04	2.2	2.74
AP2 domain-containing transcription factor family protein[b]	At2g33710	31.72	263	99.4	8.29	3.13	34.51	171.41	136.96	4.97	3.97
AP2 domain-containing protein[b]	At5g52020	14.6	254.17	31.91	17.41	2.19	22.21	207.66	46.66	9.35	2.1
AP2 domain-containing transcription factor, putative / TINY2	At5g11590	5.84	19.23	33.6	3.29	5.75	4.27	9.75	6.12	2.28	1.43
DRE-binding protein (DREB1C) / CRT/DRE-binding factor 2 (CBF2)[b]	At4g25470	13.03	122.3	85.2	9.38	6.54	9.63	63.63	30.01	6.61	3.12
DRE-binding protein (DREB1A) / CRT/DRE-binding factor 3 (CBF3)	At4g25480	114.98	796.32	418.71	6.93	3.64	17.72	343.43	42.73	19.38	2.41
bZIP protein / elongated hypocotyl 5 (HY5)	At5g11260	249.31	517.71	294.22	2.08	1.18	249.33	607.57	286.05	2.44	1.15
cytokinin response factor 3 (CRF3)	At5g53290	41.08	92.96	50.5	2.26	1.23	47.54	106.87	56.24	2.25	1.18
ethylene-responsive element-binding protein, putative (EREBP)	At1g72360	193.7	445.39	236.26	2.3	1.22	136.63	300.17	163.22	2.2	1.19
ethylene-responsive factor, putative	At4g18450	8.11	17.64	7.82	2.17	0.96	9.51	21.08	14.19	2.22	1.49
bZIP transcription factor family protein (BZIP58)	At1g13600	152.56	675.05	106.17	4.42	0.7	56.41	448.94	75.1	7.96	1.33
bZIP transcription factor family protein	At1g68880	314.65	900.44	198.02	2.86	0.63	292.37	750.49	222.97	2.57	0.76
homeobox-leucine zipper family protein / HOMEOBOX PROTEIN 54	At1g27050	27.06	84.68	35.29	3.13	1.3	33.44	94.7	36.47	2.83	1.09
myb family transcription factor (MYB12)	At2g47460	108.07	1071.67	407.78	9.92	3.77	194.82	1164.41	297.06	5.98	1.52
myb family transcription factor (MYB13)[b]	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.7
myb family transcription factor (MYB41)	At4g28110	18.08	68.7	9.26	3.8	0.51	7.61	30.72	8.29	4.04	1.09
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor (MYB74)	At4g05100	35.95	144.15	18.56	4.01	0.52	27.5	96.45	19.28	3.51	0.7
myb family transcription factor / enhancer of	At1g01380	19.72	57.57	15.44	2.92	0.78	27.13	109.08	39.73	4.02	1.46

TRY and CPC 1											
myb-related protein CAPRICE (CPC)	At2g46410	64.6	321.7	69.45	4.98	1.08	60.59	176.57	51.49	2.91	0.85
myb family transcription factor	At1g01520	5.15	46.96	6.91	9.12	1.34	4.65	38.28	5.13	8.24	1.1
WRKY DNA-binding protein 57	At1g69310	287.47	879.27	542.67	3.06	1.89	177.14	580.39	268.42	3.28	1.52
zinc finger (GATA type) family protein / (ZIM-LIKE 2)	At1g51600	71.13	166.86	129.68	2.35	1.82	64.63	136.5	110.99	2.11	1.72
RNA polymerase sigma subunit SigE (sigE) / sigma-like factor (SIG5)	At5g24120	450.17	1782.86	369.25	3.96	0.82	256.74	1556.87	337.82	6.06	1.32
PHD finger family protein	At3g14740	81.01	178.8	103.2	2.21	1.27	82.09	226.15	142.57	2.75	1.74
AT hook motif-containing protein	At5g62260	111.04	232.9	125.93	2.1	1.13	122.35	259.86	129.15	2.12	1.06
heat shock transcription factor C1 (HSFC1)	At3g24520	269.94	1441.21	331.92	5.34	1.23	104.77	525.33	182.81	5.01	1.74
heat shock transcription factor 2 (HSTF2) / heat shock transcription factor A1E[b]	At3g02990	34.2	150.23	157.8	4.39	4.61	65.8	230.34	187.75	3.5	2.85
regulation of transcription (chronic cold)[c]											
AP2 domain-containing transcription factor family protein/RAP2.6L	At5g13330	111.75	166.84	259.42	1.49	2.32	139.24	233	295.06	1.67	2.12
AP2 domain-containing transcription factor/target of EAT1-2	At5g60120	102.85	89.5	286.47	0.87	2.79	107.45	116.12	264.87	1.08	2.46
basic helix-loop-helix family protein (BHLH101)	At5g04150	3.61	5.47	13.7	1.52	3.8	4.02	4.97	13.36	1.24	3.32
myb family transcription factor (MYB55)	At4g01680	84.75	177.16	203.48	2.09	2.4	79.53	193.63	177.27	2.43	2.23
myb family Circadian 1 (CIR1)	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
WRKY DNA-binding protein 28	At4g18170	10.23	12.12	49.46	1.18	4.83	7.97	18.19	37.02	2.28	4.65
WRKY DNA-binding protein 54	At2g40750	7.72	8.03	223.34	1.04	28.93	7.99	8.23	59.99	1.03	7.51
WRKY DNA-binding protein 60	At2g25000	98.62	253.24	329.93	2.57	3.35	85.52	139.68	221.11	1.63	2.59
Dof-type zinc finger domain-containing protein	At1g29160	219.02	303.26	629.63	1.38	2.87	211.71	212.96	428.08	1.01	2.02
zinc finger (B-box type) family protein	At5g54470	43.44	914.17	104.64	21.04	2.41	7.28	478.41	31.8	65.72	4.37
zinc finger (C2H2 type) family protein	At4g16610	12.94	29.27	34.45	2.26	2.66	30.13	47.46	73.68	1.58	2.45
zinc finger (B-box type) family protein	At3g21890	32.46	535.55	116.6	16.5	3.59	20.08	459.81	109.68	22.9	5.46
squamosa promoter-binding protein, putative	At5g50570	98.64	91.67	264.02	0.93	2.68	35.73	31.98	106.11	0.89	2.97
homeobox-leucine zipper family protein (HB53)	At5g66700	65.22	65.55	166.34	1.01	2.55	11.91	14.03	30.68	1.18	2.58
E2F transcription factor-3 (E2F3)	At2g36010	21.21	44.25	48.6	2.09	2.29	37.96	50.69	82.21	1.34	2.17

RWP-RK domain-containing protein	At2g43500	65.25	106.62	132.74	1.63	2.03	98.96	283.43	271.72	2.86	2.75
maternal effect embryo arrest 3	At2g21650	23.38	23.85	72.48	1.02	3.1	7.43	8.89	21.65	1.2	2.91
DNA-binding protein, putative	At3g11580	33.38	59.34	146.61	1.78	4.39	55.06	91.02	187.64	1.65	3.41
DNA-binding protein, putative	At2g36080	136.92	261.89	326.35	1.91	2.38	143.84	232.38	299.08	1.62	2.08

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.
[b] Six genes in group "regulation of transcription"that were chronic cold-induced, shared between Col-0 and Sha.
[c] Altogether 25 genes including genes shared with acute cold response as labeled ([b]).

Table 4.12. Enrichment of transcription factor-genes in sets of chronic cold-repressed genes specific to Sha accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process											
AP2 domain-containing transcription factor TINY, putative	At4g32800	7.62	9.88	7.26	1.30	0.95	13.89	10.86	6.35	0.78	0.46
AP2 domain-containing transcription factor, putative	At1g64380	137.33	187.24	117.35	1.36	0.85	110.83	237.80	34.55	2.15	0.31
AP2 domain-containing transcription factor, putative	At5g51190	51.25	71.97	18.92	1.40	0.37	50.92	25.62	15.31	0.50	0.30
CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	At1g54160	6.91	10.63	4.24	1.54	0.61	23.16	10.16	6.56	0.44	0.28
DNA-binding protein RAV1 (RAV1)	At1g13260	404.98	46.61	312.09	0.12	0.77	371.10	130.77	128.83	0.35	0.35
basic helix-loop-helix (bHLH) family protein	At2g42280	210.15	196.19	154.00	0.93	0.73	292.52	169.28	124.54	0.58	0.43
basic helix-loop-helix (bHLH) family protein	At5g57150	469.91	116.07	192.14	0.25	0.41	330.56	103.26	156.28	0.31	0.47
bZIP transcription factor family protein	At4g37730	484.12	294.86	327.04	0.61	0.68	577.16	305.71	267.41	0.53	0.46
bZIP family transcription factor	At3g51960	36.51	25.23	20.58	0.69	0.56	38.73	27.48	16.59	0.71	0.43
basic leucine zipper O2 homolog 3 (BZO2H3)	At5g28770	246.16	125.01	160.43	0.51	0.65	230.57	47.16	52.04	0.20	0.23

MADS-box protein (AGL72)	At5g51860	5.53	7.22	5.49	1.31	0.99	19.76	46.69	4.94	2.36	0.25
AGAMOUS-LIKE 21 (AGL21)	At4g37940	489.12	376.89	244.67	0.77	0.50	493.90	404.09	189.34	0.82	0.38
squamosa promoter-binding protein, putative	At3g57920	11.68	13.31	8.61	1.14	0.74	40.71	19.42	15.45	0.48	0.38
myb family transcription factor (MYB46)	At5g12870	38.13	21.23	20.49	0.56	0.54	57.30	30.68	21.00	0.54	0.37
myb family transcription factor (MYB48) / myb domain protein 111 (MYB111)	At3g46130	437.14	281.60	301.76	0.64	0.69	480.09	427.41	153.14	0.89	0.32
myb family transcription factor (MYB51)	At1g18570	54.96	13.21	16.03	0.24	0.29	44.26	13.43	12.54	0.30	0.28
myb family transcription factor (MYB68)	At5g65790	274.69	269.22	190.65	0.98	0.69	319.15	286.70	153.37	0.90	0.48
myb family transcription factor	At5g08520	173.38	50.02	114.22	0.29	0.66	168.11	37.04	75.06	0.22	0.45
myb family transcription factor	At3g11280	678.36	630.21	675.62	0.93	1.00	925.17	539.65	433.64	0.58	0.47
myb family transcription factor	At5g01200	12.94	7.25	7.71	0.56	0.60	19.05	10.93	8.52	0.57	0.45
altered phloem development (APL)	At1g79430	148.26	106.58	82.88	0.72	0.56	220.00	150.51	97.54	0.68	0.44
WRKY DNA-binding protein 2 (WRKY2)	At5g56270	65.39	47.72	35.29	0.73	0.54	111.72	54.96	38.79	0.49	0.35
WRKY DNA-binding protein 7 (WRKY7)	At4g24240	348.33	190.47	260.31	0.55	0.75	394.67	269.56	179.67	0.68	0.46
WRKY DNA-binding protein 27 (WRKY27)	At5g52830	83.21	23.76	66.55	0.29	0.80	138.72	53.69	66.02	0.39	0.48
Dof-type zinc finger domain-containing protein	At4g24060	265.41	227.15	196.33	0.86	0.74	398.17	248.79	188.87	0.62	0.47
Dof-type zinc finger domain-containing protein/OBF binding protein 1	At3g50410	39.94	65.19	26.24	1.63	0.66	47.16	46.53	10.66	0.99	0.23
zinc finger (C2H2 type) protein 3 (ZFP3)	At5g25160	521.31	688.72	415.98	1.32	0.80	546.67	593.66	242.12	1.09	0.44
zinc finger (C2H2 type) family protein	At2g28710	64.62	65.70	64.79	1.02	1.00	166.77	178.87	69.93	1.07	0.42
br enhanced expression 2 (BEE2)	At4g36540	16.38	8.43	7.56	0.51	0.46	18.82	6.32	5.89	0.34	0.31
auxin-responsive factor (ARF4)	At5g60450	150.01	116.83	87.74	0.78	0.58	166.07	105.57	74.20	0.64	0.45
cytokinin response factor 5 (CRF5)	At2g46310	29.87	54.75	13.63	1.83	0.46	51.37	60.51	10.37	1.18	0.20
ethylene-responsive element-binding factor 5 (ERF5)	At5g47230	175.24	237.68	168.06	1.36	0.96	224.59	186.72	87.99	0.83	0.39
ethylene-responsive element-binding family protein	At5g07580	346.29	179.27	197.39	0.52	0.57	321.79	118.26	103.53	0.37	0.32
scarecrow-like transcription factor 7 (SCL7)	At3g50650	31.01	14.63	30.75	0.47	0.99	35.45	14.46	15.52	0.41	0.44
antitermination NusB domain-containing protein	At4g26370	60.97	15.91	51.50	0.26	0.84	104.64	29.36	36.57	0.28	0.35

maternal effect embryo arrest 47 (MEE47)	At4g00950	14.08	14.71	11.38	1.04	0.81	17.89	12.33	8.54	0.69	0.48
similar to meristem protein - like reproductive meristem protein 1, broccoli	At5g32460	3.47	6.20	4.09	1.79	1.18	39.07	18.68	7.93	0.48	0.20
unknown transcription factor	At3g10040	41.66	12.97	15.72	0.31	0.38	84.23	14.37	28.35	0.17	0.34

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

4.6 Expression of cell cycle genes in response to cold

The results in Chapter 3 showed for several different *Arabidopsis* accessions that expression of *CYCB1;1* was substantially induced in response to chronic, but not acute cold treatment. To gain a more comprehensive view of how cold treatment affects expression of cell-cycle related genes, I examined the expression patterns of the 89 core cell-cycle genes represented on the ATH1 GeneChip. Ratios of the median expression values of cold-treated relative to warm controls were calculated using the set of genes that passed the quality control, i.e., expressed $p \leq 0.04$. Hierarchical clustering of the $\log_2$ transformed values indicated that the ratios obtained for most genes fell into 7 groups (Fig. 4.10):

Group I: Genes were weakly expressed or repressed by acute cold treatment, but up-regulated by chronic cold treatment.

Group II: Genes were induced by roughly the same magnitude by acute and chronic cold treatment.

Group III: Genes were induced by acute-cold treatment and weakly induced by chronic-cold treatment of Sha, but not responsive to chronic-cold treatment of Col-0.

Group IV: Genes were induced by acute-cold treatment and weakly induced by chronic-cold treatment of Col-0 and weakly repressed by chronic-cold treatment of Sha.

Group V: Genes were repressed by chronic-cold treatment, but weakly induced by acute-cold treatment in both accessions.

Group VI: Genes were repressed by acute-cold treatment and weakly induced by chronic-cold treatment of Col-0 and repressed by chronic-cold treatment of Sha.

Group VII: Genes were repressed in both cold treatments in both Col-0 and Sha.

Table 4.13 shows the expression ratios obtained for the clustered genes. Included in Group I and Group II were the the B-type cyclins that govern the G2-to-M progression (Inzé & De Veylder 2006), namely, *CYCB1;4*, *CYCB2;1* and *CYCB2;2* in Group I, and *CYCB1;1*, *CYCB1;3*, *CYCB2;3*, *CYCB2;4* and *CYCB3;1* in Group II. Only

CYCB1;1, *CYCP4;*3 and *E2Fa* were induced by chronic cold treatment approximately 2-3 fold in both Col-0 and Sha. *CYCB2;3* and *CYCB3;1* were induced approximately 2-fold by both cold treatments in Col-0. D-type cyclins, which have been shown to govern the G1-to-S progression during cell division and responded to external stimulus (Inzé & De Veylder 2006), clustered in several different groups, namely, *CYCD4;1* in group I; *CYCD2;1*, *CYCD5;1* and *CYCD6;1* in Group III; *CYCD4;2* in Group V; *CYCD3;2* and *CYCD3;3* in Group VI; and *CYCD1;1* in group VII. None of the D-type cyclins exhibited >2-fold regulation by cold with the exception of *CYCD1;1* in Group VII, which showed a 2-3 fold repression in response to acute or chronic cold treatment.

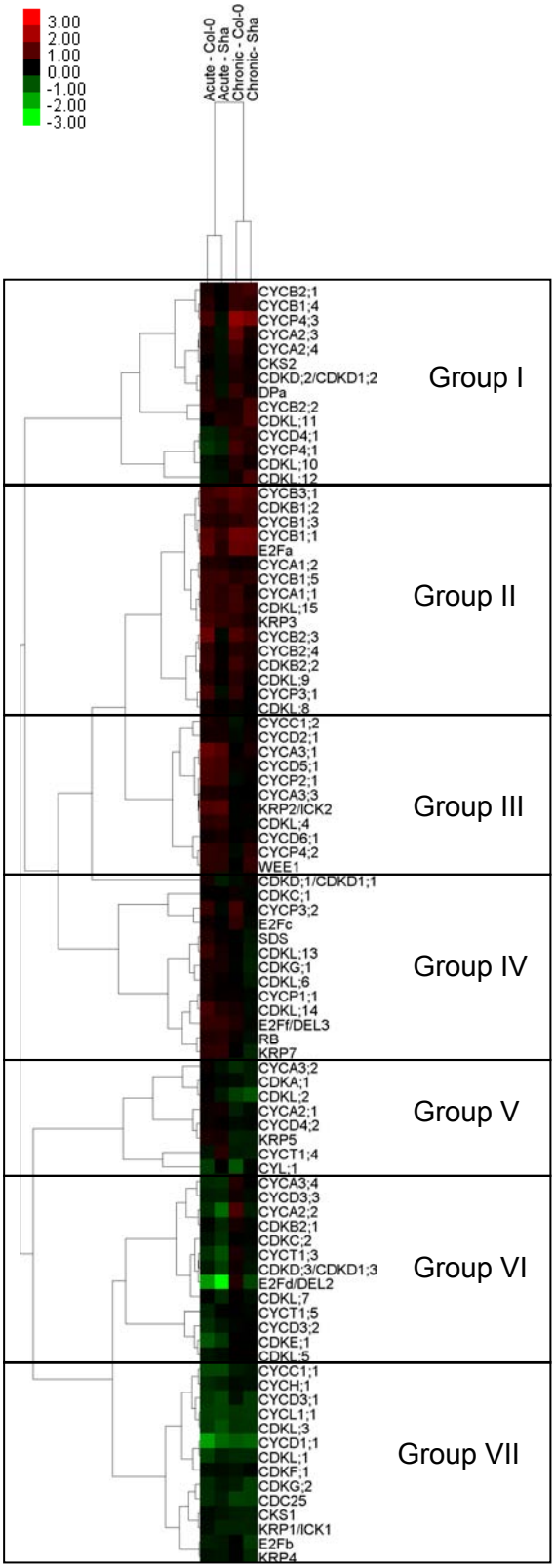


Figure 4.10. Hierarchical clustering of 89 core cell-cycle genes represented in the GeneChip arrays. Color code shows the $\log_2$ transformed expression ratios of cold-treated relative to warm controls. Red indicates up-regulation, green indicates down-regulation, and black indicates unchanged expression.

Table 4.13. Expression values of cell cycle related genes in response to acute or chronic cold in Col-0 or Sha accession.

		Col-0					Sha				
		Median expression value			Ratio[a]		Median expression value			Ratio[a]	
Annotation	AGI	warm	Acute	Chronic	Acute	Chronic	warm	Acute	Chronic	Acute	Chronic
Group I											
CYCA2;3	At1g15570	13.26	15.38	31.42	1.16	**2.37**	25.98	22.41	32.29	0.86	1.24
CYCA2;4	At1g80370	30.84	37.00	53.08	1.20	1.72	39.13	34.19	44.31	0.87	1.13
CYCB1;4	At2g26760	184.01	246.56	283.58	1.34	1.54	222.00	221.13	313.44	1.00	1.41
CYCB2;1	At2g17620	30.83	37.01	46.85	1.20	1.52	25.75	25.82	44.57	1.00	1.73
CYCB2;2	At4g35620	19.71	27.73	23.62	1.41	1.20	14.99	17.30	26.28	1.15	1.75
CYCD4;1	At5g65420	11.88	8.74	17.59	0.74	1.48	11.45	9.16	15.38	0.80	1.34
CYCP4;1	At2g44740	26.30	17.31	45.38	0.66	1.73	25.57	19.26	33.67	0.75	1.32
CYCP4;3	At5g07450	24.95	43.22	82.64	1.73	**3.31**	71.97	63.68	196.56	0.88	**2.73**
CDKD;2	At1g66750	155.77	191.43	195.70	1.23	1.26	285.21	255.93	329.01	0.90	1.15
CDKL;10	At1g57700	84.86	72.88	116.62	0.86	1.37	94.90	88.61	105.60	0.93	1.11
CDKL;11	At1g09600	7.94	7.90	9.95	1.00	1.25	14.93	19.61	27.38	1.31	1.83
CDKL;12	At1g71530	29.98	24.60	34.34	0.82	1.15	56.10	47.74	100.04	0.85	1.78
CKS2	At2g27970	269.16	281.72	373.27	1.05	1.39	345.59	307.76	370.28	0.89	1.07
DPa	At5g02470	92.78	118.60	141.59	1.28	1.53	141.01	121.04	159.37	0.86	1.13
Group II											
CYCA1;1	At1g44110	131.85	209.16	213.89	1.59	1.62	101.16	140.38	115.82	1.39	1.14
CYCA1;2	At1g77390	4.99	6.63	5.55	1.33	1.11	5.48	6.94	6.29	1.27	1.15
CYCB1;1	At4g37490	59.28	116.46	149.09	1.96	**2.52**	83.00	125.20	197.38	1.51	**2.38**
CYCB1;3	At3g11520	72.84	99.82	102.21	1.37	1.40	87.48	111.73	138.21	1.28	1.58
CYCB1;5	At1g34460	58.19	91.92	76.40	1.58	1.31	46.03	73.16	65.71	1.59	1.43
CYCB2;3	At1g20590	31.22	73.78	59.03	**2.36**	1.89	65.16	61.03	93.44	0.94	1.43
CYCB2;4	At1g76310	60.06	89.54	79.13	1.49	1.32	70.98	74.15	89.03	1.04	1.25
CYCB3;1	At1g16330	25.38	41.73	54.14	1.64	**2.13**	32.94	60.57	60.48	1.84	1.84
CYCP3;1	At2g45080	10.44	17.87	15.28	1.71	1.46	11.10	9.90	11.23	0.89	1.01
CDKB1;2	At2g38620	102.33	165.38	190.75	1.62	1.86	113.99	160.86	182.42	1.41	1.60
CDKB2;2	At1g20930	169.68	259.77	277.74	1.53	1.64	193.69	205.48	238.06	1.06	1.23
CDKL;8	At3g05050	243.72	283.45	262.58	1.16	1.08	286.92	287.14	283.35	1.00	0.99

CDKL;9	At1g54610	714.06	938.37	961.46	1.31	1.35	858.40	883.52	935.27	1.03	1.09
CDKL;15	At1g53050	292.36	458.05	475.56	1.57	1.63	289.78	408.89	368.94	1.41	1.27
E2Fa	At2g36010	21.21	44.25	48.60	**2.09**	**2.29**	37.96	50.69	82.21	1.34	**2.17**
KRP3	At5g48820	27.20	47.55	37.91	1.75	1.39	42.12	63.54	48.96	1.51	1.16
Group V											
CYCA2;1	At5g25380	5.66	6.30	4.43	1.11	0.78	5.31	6.07	4.78	1.14	0.90
CYCA3;2	At1g47210	165.08	181.73	114.58	1.10	0.69	180.02	153.88	144.54	0.85	0.80
CYCD4;2	At5g10440	6.80	7.56	6.18	1.11	0.91	8.62	9.04	8.11	1.05	0.94
CYCT1;4	At4g19600	312.59	267.46	258.19	0.86	0.83	12.46	17.16	9.76	1.38	0.78
CYL;1	At4g34090	17.38	10.79	8.78	0.62	0.51	12.29	12.68	11.51	1.03	0.94
CDKA;1	At3g48750	969.09	962.77	877.73	0.99	0.91	1205.11	1130.41	970.16	0.94	0.81
CDKL;2	At1g74330	22.54	23.89	13.84	1.06	0.61	26.34	21.48	13.54	0.82	0.51
KRP5	At3g24810	21.70	26.92	16.88	1.24	0.78	39.87	47.60	31.22	1.19	0.78
Group VII											
CYCC1;1	At5g48640	152.82	87.45	121.96	0.57	0.80	146.15	82.61	122.51	0.57	0.84
CYCD1;1	At1g70210	131.17	35.17	63.09	**0.27**	**0.48**	105.32	44.74	46.63	**0.42**	**0.44**
CYCD3;1	At4g34160	426.16	274.64	374.29	0.64	0.88	441.01	252.11	278.38	0.57	0.63
CYCH;1	At5g27620	114.17	79.88	104.34	0.70	0.91	125.40	97.28	110.09	0.78	0.88
CYCL1;1	At2g26430	600.98	368.09	395.82	0.61	0.66	632.42	373.31	403.63	0.59	0.64
CDKF;1	At4g28980	320.93	288.36	277.53	0.90	0.86	291.69	264.59	275.95	0.91	0.95
CDKG;2	At1g67580	334.01	239.55	266.86	0.72	0.80	344.92	290.20	209.53	0.84	0.61
CDKL;1	At5g39420	8.46	5.13	6.06	0.61	0.72	8.05	6.28	5.79	0.78	0.72
CDKL;3	At1g18670	145.62	92.53	97.75	0.64	0.67	187.51	96.70	123.79	0.52	0.66
CKS1	At2g27960	340.83	314.30	269.79	0.92	0.79	212.08	181.79	164.16	0.86	0.77
KRP1/ICK1	At2g23430	90.26	80.49	68.40	0.89	0.76	85.75	65.23	65.22	0.76	0.76
KRP4	At2g32710	228.98	200.86	221.67	0.88	0.97	240.21	203.08	173.08	0.85	0.72
E2Fb	At5g22220	22.40	17.65	21.95	0.79	0.98	24.20	20.04	15.27	0.83	0.63
CDC25	At5g03455	960.66	672.04	577.14	0.70	0.60	858.19	686.58	521.55	0.80	0.61

[a] Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates. Bold numbers are mRNA changed in abundance for more than 2-fold, either up- or down-regulation in response to cold treatments.

4.7 Conclusions

Acute- and chronic-cold treatments had major effects on the expression of *Arabidopsis* genes at the mRNA level: 11% of the 24,000 genes represented on the ATH1 GeneChip responded by at least 2-fold to either or both cold treatments. Genes concerned with disaccharide biosynthesis, transcriptional regulation and genes responsive to signaling molecules such as ABA, jasmonic acid, ethylene, and salicyclic acid were often induced as part of the acute-cold response of both accessions. These results are consistent with earlier microarray experiments focused exclusively on responses of *Arabidopsis* accessions to acute-cold treatment (Fowler & Thomashow 2002; Kreps *et al.* 2002; Seki *et al.* 2002; Lee *et al.* 2005). By comparing the two cold treatments in the same experiment, I found that at least 35% of cold-responsive genes responded specifically to chronic cold treatment. This suggests there are fundamental differences in the response of plants to acute-cold treatment and growth at low, nonfreezing temperatures.

GO-term analyses applied to genesets sorted on the basis of cold-treatment or accession identified cold-treatment and accession specific functional groupings. The assignment of GO terms obtained by the two types of sorting often differed, and the significance-level of enrichments was often near the statistical cutoff of P=0.05. Thus, some of these discrepancies are likely to be statistical artifacts resulting from differences in sample size. Nevertheless, there was substantial agreement in the types of functional groupings obtain by the two sorting methods (Table 4.8 and Table 4.10). Genes were specifically enriched for each grouping and could be confirmed by measuring expression levels. The results obtained lead to the important conclusion that natural variation in the accessions, the nature of the cold-treatment, and the interaction of these parameters contribute to the pattern of cold-responsive gene expression. The known functions of the identified genes and the possible biological significance of their expression patterns are discussed in Chapter 5.

5.0 Discussion

5.1 Effect of chronic cold on root elongation

My initial study focused on how long-term cold affects root elongation and its possible role in determining the natural range of *Arabidopsis*. I surveyed accessions from diverse habitats and compared root-elongation rates with cold-induced alterations of selected genes associated with cold responsiveness and cell-cycle regulation. Root elongation rather than leaf expansion was chosen as the model because the growth of primary roots is basically linear (Baskin *et al.* 1995; Beemster *et al.* 2002) and, from the ecophysiological point of view, soil temperature is less affected by diurnal temperature fluctuations than is the ambient temperature experienced by the aerial parts of plants (Bliss 1956). A second important consideration was that expression of genes well-established as cell-cycle markers in *Arabidopsis* roots could be used to monitor effects on cell production (Birnbaum *et al.* 2003; Beemster *et al.* 2005). There was natural variation in root elongation rates at 21 °C among the accessions that ranged by up to 4 fold. A similar range has been reported for 18 accessions raised under similar conditions at 22 °C (Beemster *et al.* 2002). Raising plants at 10 °C reduced the rate of root elongation to roughly 10 to 45% of the 21°C control (Fig. 3.2), and depended on the accession.

My findings showed that root elongation rates at 10 °C were not significantly correlated with the average temperature during the growing season suggests that the rate of primary root growth under such temperatures is not a major factor in adaptation to cold habitats. Similarly, in a comparative study of *Arabidopsis* accessions grown at 6 and 14 °C, Hasdai et al. (2006) were unable to find a significant correlation of freezing tolerance with parameters of aerial growth including leaf length, hypocotyl length, and height of inflorescence (Hasdai *et al.* 2006). In contrast, several parameters related to the growth of leaves and production of aerial parts of *Arabidopsis* at 22 °C, have been shown to be negatively correlated with latitude and, hence, average spring and autumn temperatures in different *Arabidopsis* accessions (Li *et al.* 1998), and temperate lowland/alpine grasses have been found to exhibit clear thermal adaptation of growth (Körner & Woodward 1987). One possible explanation for the lack of ecotypic differentiation for root elongation in the accessions tested is the very short life cycle and

small size of *Arabidopsis*, which together permit a restriction of growth to short warm spells and/or sheltered microhabitats.

5.2 Effects of cold treatment on the expression of CBF-pathway and cell-cycle related genes

Chapter 3 shows that the acute cold induction of key components in the *CBF* pathway described earlier for laboratory strains of *Arabidopsis* (Gilmour *et al*. 1998; Ishitani *et al*. 1998; Liu *et al*. 1998; Jaglo *et al*. 2001; Zarka *et al*. 2003) occurs in diverse natural accessions (Fig. 3.4) and that this pathway is also active in roots of plants raised at low temperature (Fig. 3.6). Evidence suggests that cold acclimation in laboratory strains of *Arabidopsis* depends on the activation of multiple low-temperature regulatory pathways (Kreps *et al*. 2002; Seki *et al*. 2001, 2002; Vogel *et al*. 2005). Downstream CBF-pathway genes are not expressed in the constitutively freezing-tolerant *eskimo1* mutant (Xin & Browse 1998) and certain cold responsive genes do not depend on the *CBF* pathway (Fowler & Thomashow 2002). The function of additional cold perception pathways might provide an explanation for the weak cold induction of downstream *COR15a* and *RD29A* genes in the high-altitude accessions 3661 and 3658.

Cold induction of the CBF-pathway genes (Fig 3.6A, 3.6C) and root (Fig. 3.1) elongation was usually more rapid and pronounced in 21 °C/10 °C plants than in plants grown continuously at 10 °C. This indicates that temperature early in seedling development affects the subsequent response of roots to chronic cold treatment. Similar effects of temperature shifts have been reported for physiological responses of *Arabidopsis* leaves. Whereas mesophyll cells of leaves formed at low temperature showed increased cytoplasmic volume and density, these changes were minimal in temperature shifted leaves. Leaves formed at a low temperature exhibited greater changes in the activity of Calvin Cycle enzymes and accumulation of sucrose than did temperature shifted leaves(Strand *et al.* 1999).

CBF2 was the only *CBF* gene appreciably induced in roots in response to chronic cold (Fig. 3.6A, 3.6C). *CBF2* is believed to serve a dual function: it appears to down regulate *CBF1* and *CBF3*, and transcriptionally activates genes downstream in the *CBF* pathway ((Novillo *et al.* 2004). Growth is inhibited by overexpression of CBF1, CBF2, or CBF3 in transgenic *Arabidopsis* (Gilmour *et al.* 2004). These findings together with my results suggest that expression of *CBF2* in chronic cold regulates the expression of

CBF1 and *CBF3* and that this affects the balance between growth and cold tolerance. Chronic-cold induced of *CBF2* may thus, not only explain cold tolerance but also contribute to the overall slow growth rate at low temperature.

Root elongation at low temperature was correlated with expression of *RD29A* mRNA suggests that chronic activation of the *CBF* pathway could help facilitate growth in cold environments. Nevertheless, we found neither appreciable constitutive expression of *CBF* genes nor a correlation of expression levels in accessions from cold habitats. Therefore, while there is natural variation in different accessions, constitutive activity of the *CBF*-pathway and chronic cold activation of this pathway do not appear to play a major role in determining the distribution of the accessions tested in cold habitats.

I also examined the possibility that cold-induced expression of cell-cycle genes has a role in regulating root growth at low temperatures. The elongation of primary roots depends on a combination of cell production in the meristem and expansion of cortical cells in the elongation zone (Beemster *et al.* 2002). *CDKA;1* and *CYCD2;1* mRNAs are constitutively expressed in the meristem, the elongation zone, and mature zone of warm grown Col-0 root (Birnbaum *et al.* 2003; Beemster *et al.* 2005). Cell production rates in different warm-grown *Arabidopsis* accessions are strongly correlated with the activity of CDKA;1 in roots suggesting that CDKA;1 activity, but not *CDKA;1* mRNA is a factor limiting root growth in natural populations. In most accessions I studied, including the Col-0, Ler-0, and Est-0 accessions investigated by Beemster et al. (2002), neither acute or chronic cold treatment increased *CDKA;1* or *CYCD2;1* mRNA expression. The only exception was the high altitude accessions 3661 and 3658, which exhibited a consistent induction of *CDKA;1* mRNA by chronic cold treatment (Fig 3.8A). In no case, however, were expression levels of *CDKA;1* or *CYCD2;1* mRNA correlated either with root elongation rates or the habitat temperature of the accessions. Thus, expression of *CDKA;1* and *CYCD2;1* mRNAs are not likely to be growth-limiting factors in the accessions I studied.

CYCB1;1 mRNA is an established marker for cell proliferation and a limiting factor for growth of *Arabidopsis* roots (Doerner *et al.* 1996; Beemster *et al.* 2002; Inzé & De Veylder 2006). My most interesting finding was that *CYCB1;1* mRNA consistently induced in roots by chronic but not acute cold in all the accessions studied (compare Fig. 3.8 with Fig. 3.7). Levels of *CYCB1;1* mRNA were not, however, significantly correlated with the rate of root elongation at either 10 °C or 21 °C. This is in contrast to recent studies of developing maize leaves showing that low (4 °C) night temperature reduced levels of *CYCB1;1* mRNA and transcripts of several other positive regulators of the cell

cycle in association with reduced growth and cell production (Rymen *et al.* 2007). While organ-specific differences in cold response cannot be ruled out, one possible explanation is that maize is a chilling-sensitive plant, whereas *Arabidopsis* has the capacity to acclimatize to cold (Allen & Ort 2001). Accumulation of *CYCB1;1* mRNA in the entire root system depends on the expression level of *CYCB1;1* in proliferating cells and the size of the meristematic compartment. *CYCB1;1* mRNA accumulation was not correlated with the elongation rate of cold-grown roots relative to that of warm-grown roots. Thus, it is unlikely that the cold induction I observed reflects solely an increase in the relative amount of meristematic tissue in shorter, cold-grown roots. Growth at low, non-freezing temperatures is known to prolong the duration of the cell cycle (Francis & Barlow 1988), which appears to be compensated, at least in part, by an increase in the number of cells entering the cell cycle (Creber *et al.* 1993; Rymen *et al.* 2007). We speculate that cold is perceived by a signaling pathway that acts down stream of CYCB1;1 to inhibit root elongation. This increases CYCB1;1 expression by an as yet unknown mechanism, and, hence the size of the meristematic compartment. According to this view, induction of *CYCB1;1* mRNA could be part of the compensation mechanism that helps maintain proliferation at low, non-freezing temperatures.

5.3 Global analysis of cold-responsive gene expression in roots

Gene expression changes in response to low temperature are crucial for low-temperature acclimation leading to freezing tolerance as well as for adaptation for growth and development at low temperatures (Hughes & Dunn 1996; Thomashow 1999; Thomashow 2001). I surveyed global gene-expression changes of Col-0 and Sha accessions that originate from habitates expected to differ greatly in temperature during the growing season to detect possible ecotypic differentiation related to growth at low temperatures. I also compared the effects of acute- and chronic-cold treatments of the two accessions to identify sets of genes that respond specifically to these cold treatments. About 5395 cold-responsive genes were identified (Fig. 4.1) in either Col-0 or Sha accession. Of these, 82% were specific for acute- or chronic-cold treatment and 63% were accession specific. These results show that acute- and chronic-responses involve overlapping as well as specific patterns of gene expression, and that both types of responses are influenced by the genetic background of the accession. The number of genes responsive to cold was larger for Col-0 than for Sha (Fig. 4.1). For a large dataset

of shared genes between Col-0 and Sha (Fig 4.9), the magnitude of the chronic-cold response of Sha was 5 fold lower than that of Col-0; whereas, the magnitudes of the acute-cold responses were equal. These results strongly suggest that Sha is less sensitive than Col-0 to chronic cold treatment. This might reflect some form of "cold adaptation" by Sha to growing at low temperature.

The variation in gene expression I found is consistent with recent studies showing that for any pair of seven *Arabidopsis* accessions between 1428 and 3344 genes were differentially expressed and that these accessions encompassed about 80% of the moderate- to high-frequency nucleotide polymorphisms (Kliebenstein *et al.* 2006). Natural genetic variation in *Arabidopsis* accessions has been shown to affect low-temperature acclimation and freezing tolerance (Alonso-Blanco *et al.* 2005; Hannah *et al.* 2006) as well as the effects of cold acclimation on leaf senescence (Masclaux-Daubresse *et al.* 2007).

Examination of changes in expression of individual genes in large datasets is time consuming and may not clearly reveal the biological meaning of the changes. Therefore, the major emphasis of the analysis presented here was to detect trends in gene expression by screening for significant co-regulation of genes grouped in similar functional groups or biological processes (Al-Shahrour *et al.* 2004; Al-Shahrour *et al.* 2005). The possible biological relevance of some of these trends is discussed in the following sections.

5.3.1 Abiotic and biotic stress responses

Changes in the expression of genes in the "response to phytohormones" group were mainly found in response to acute-cold treatment (Table 4.2A). This suggests that endogenous levels of ethylene, jasmonic acid, ABA, salicylic acid, and gibberellic acid or sensitivity to these signal molecules may increase in response to acute cold treatment. It has been shown that exogenous application of ethylene, ABA, salicylic acid and jasmonic acid can reduce chilling injury or increase antifreeze activity in plants; and, increased endogenous levels of these molecules are associated with low temperature tolerance (Chen *et al.* 1983; Lång *et al.* 1989; Xin & Browse 2000; Yu, Griffith & Wiseman 2001; Gonzalez-Aguilar *et al.* 2004; Yoshikawa, Honda & Kondo 2007). ABA is well-known to be involved in responses to abiotic stress such as drought and salinity (Gusta, Trischuk & Weiser 2005; Fujita *et al.* 2006). Ethylene, salicylic acid, and

jasmonic acid with known functions in pathogen defense and biotic stress signaling (Thomma *et al.* 1998; O'Donnell *et al.* 2001; Lorenzo & Solano 2005) are also involved in chilling- and freezing- tolerance (Yu *et al.* 2001; Gonzalez-Aguilar *et al.* 2004; Yoshikawa *et al.* 2007).

Genes in the group "disaccharide biosynthetic process" (Fig. 4.2) were mainly affected in response to acute cold treatment (Table 4.2A, 4.3). Accumulation of glucose, maltose, fructose, sucrose, and trehalose is induced by low temperature and associated with cold acclimation (Wanner & Junttila 1999; Xin & Browse 2000; Cook *et al.* 2004; Kaplan *et al.* 2004). I confirmed that trehalose-6-phosphate phosphatase and β-amylase known to be associated with cold acclimation (Cook *et al.* 2004; Kaplan *et al.* 2004) were induced by acute-cold treatment (Table 4.3). Cold-induction of β-amylase is associated with the accumulation of maltose. Maltose is a compatible-solute that stabilizes the photosynthetic electron transport chain as well as proteins in the stroma (Kaplan & Guy 2004; Kaplan & Guy 2005). Sucrose-phosphatase 2 (SPP2, At3g52340) and one putative sucrose-phosphatase (At1g51420) catalyze the last step in sucrose synthesis (Fig 4.2A) (Lunn *et al.* 2000). Accumulation of sucrose in response to low temperature may serve as a compatible osmolyte that helps protect proteins or membranes against damage during low temperature exposure (Crowe *et al.* 1990; Wanner & Junttila 1999; Xin & Browse 2000; Cook *et al.* 2004; Kaplan *et al.* 2004).

5.3.2 Phenylpropanoid and flavonoid pathways

The phenylpropanoid pathway (Fig. 4.3, 4.4) and the flavonoid biosynthetic pathway, which is a branch of the phenylpropanoid pathway (Fig. 4.3), produce secondary metabolites with a wide range of important functions as structural components, protectants against biotic and abiotic stresses, and signaling molecules (Dixon & Paiva 1995; Weisshaar & Jenkins 1998). Studies of maize have shown that the accumulation of anthocyanins in response to short-term cold treatment is associated with the induction of key genes in the phenylpropanoid/flavanoid pathways including those encoding PAL, chalcone synthase, 4-coumarate:CoA ligase, and chalcone isomerase (Christie, Alfenito & Walbot 1994). Numerous genes grouped under "phenylpropanoid biosynthetic process" and "flavonoid biosynthesis process" (Table 4.2A, 4.4) were induced by cold treatment of Col-0 or Sha. Seven cold-induced genes encoded key enzymes at the major steps in flavonoid biosynthesis (Fig. 4.3). MYB domain protein 12 (At2g47460)

was also induced in response to low temperature. This is a R2R3-MYB type transcription factor that activates transcription of chalcone synthase and flavonol synthase genes (Mehrtens *et al.* 2005).

Cold induction of the flavonoid pathway is interesting because of its possible role in protection against cold stress. Low temperature may increase the levels of reactive oxygen species (ROS) (Prasad *et al.* 1994) generated as by-products of photosynthesis and respiration processes (Vranová, Inzé & Van Breusegem 2002; Apel & Hirt 2004). Increased ROS levels resulting from low temperature or other abiotic stresses is toxic and can lead to cell death. It has been proposed that flavonoids induced by low temperature serve as antioxidants that scavenge ROS (Prasad 1996; Wuguo, Xingwang & Jilan 1997; Swiderski *et al.* 2004; Irena Kruk, lstrok & adna 2005). Flavonoid content is also correlated with frost resistance in *Rhododendron* (*Rhododendron* L.) leaves (Swiderski *et al.* 2004). Cold-induction of genes encoding enzymes in flavonoid biosynthesis is correlated with freezing tolerance in various *Arabidopsis* accessions (Hannah *et al.* 2006). Under conditions of cold acclimation, a phenylpropanoid deficient mutant of oilseed rape showed a greater increase in leaf weight and higher growth rates than did wild-type plants (Solecka & Kacperska 2003). In summary, these finding and the present study support the hypothesis that cold-induction at the level of mRNA of the phenylpropanoid and flavonoid pathways is part of a cold-adaptation process important for the growth and development of *Arabidopsis* at low, nonfreezing temperatures.

The branch of the phenylpropanoid pathway concerned with the biosynthesis of monolignols (Fig. 4.4) is also regulated by cold at the mRNA level. Six genes encoding enzymes in monolignol biosynthesis and the gene encoding O-methyltransferase family 2 protein (At1g33030), which is thought to be involved in lignin methylation (Bugos, Chiang & Campbell 1991; Ibrahim, Bruneau & Bantignies 1998), were up-regulated by cold-treatment of Col-0 or Sha (Table 4.4 and Fig 4.4). Lignin results from the oxidative polymerization of the monolignols coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Boerjan *et al.* 2003). Lignification is a differentiation of cell walls that occurs mainly after cell-enlargement has ceased and has a structural function in strengthening tissues, particularly vascular tissues (Fry 1988; Cooper 2000). Lignification can also be induced in response to mechanical stimulation (Cipollini Jr 1997) and as part of the response to infection by pathogens and potential pathogens (Kawasaki *et al.* 2006). S-adenosyl-L-methionine:transcaffeoyl CoA 3-O-methyltransferase, shown to be induced by cold in the present study has been implicated in the disease defense response (Zou & Taylor 1994; Busam *et al.* 1997; Martz *et al.* 1998).

5.3.3 Terpenoid biosynthetic pathways

Several key enzymes in terpenoid biosynthesis were repressed at the mRNA level by cold treatment. Included in more than 40,000 compounds produced in terpenoid pathways are the hormones GA, ABA, brassinosteroids, and cytokinins as well as plant steroids that are important membrane constituents (Croteau, Kutchan & Lewis 2000; Aharoni *et al.* 2005; Phillips *et al.* 2006). The down-regulation of key genes in GA and ABA (Fig. 4.5) biosynthesis or in the biosynthesis of the cycloartenol precursor of brassinosteroids (Fig. 4.6) documented in this study are likely to have strong effects on growth and development. Deficiency mutations of gibberellin 3-beta-hydrolase in *Arabidopsis* result in a dwarf phenotype (Talon, Koornneef & Zeevaart 1990; Hedden & Kamiya 1997). In addition to its known functions in drought-, salt- and cold stress (Nambara & Marion-Poll 2005), ABA has a role in growth regulation. Mutants deficient in ABA DEFICIENT1/zeaxanthin epoxidase (At5g67030) show reduced endogenous levels of ABA and reduced cell-size resulting in smaller leaves, inflorescences, and flowers (Barrero *et al.* 2005). ABA may also inhibit growth under stress conditions, and promote growth in the absence of stress (Cheng *et al.* 2002; Sharp & LeNoble 2002).

Functions of brassinosteroids include the positive regulation of cell enlargement, cell division and organ formation; responses to stress; and, modulation of other hormone responses (Clouse & Sasse 1998; Nemhauser & Chory 2004). *Arabidopsis* mutants deficient in the triterpenoid pathway that reduced brassinosteroid levels have been implicated in dwarfing (Schaller 2003). The natural loss-of-function allele *brx* in *Arabidopsis* Uk-1 shows reduced root growth resulting in a reduction in the size of both root meristem and mature cells (Mouchel, Briggs & Hardtke 2004). This effect is due to a root-specific deficiency in brassinosteroid bioynthesis (Mouchel, Osmont & Hardtke 2006). Because the *BRX* gene was not represented on the gene chip, I measured the abundance of *BRX* mRNA by RT-qPCR, but found no statistically significant effects of chronic cold-treatment on expression of this gene in either Col-0 or Sha.

In summary, the correlation of reduced root growth with chronic cold repression of genes involved in ABA, GA, and brassinosteroid biosynthesis suggests that reduced accumulation of one or more of these hormones may limit growth in the cold. This hypothesis is consistent with the view that plant growth and development in cold environments are likely to be modulated by the regulation of hormone homeostasis (Hannah *et al.* 2005; Lee *et al.* 2005).

5.3.4 Lipid metabolism, membrane structure and membrane function

Cold-induction of genes in the choline biosynthetic pathway (Fig. 4.7) might be an adaptive advantage for low temperature growth. Expression of phospholipase D Zeta 2 (PLDZ2) (At3g05630), and S-adenosyl-L-methionine:phosphoethanolamin N-methyltransferase (PEAMT) (AT3g18000) was induced in Sha but not in Col-0 by chronic cold treatment. This suggests that choline is likely to be accumulated as a chronic-cold specific response of Sha and, possibly, other cold habitat accessions. Choline is a precursor for the synthesis of glycine betaine in certain plants, which helps protects these plants from cold stress or other abiotic stresses (Kishitani *et al.* 1994). Although betaine does not normally accumulate in *Arabidopsis*, transgenic *Arabidopsis* plants expressing a bacterial choline oxidase are able to produce betaine. These plants show significantly improved freezing tolerance, even though the accumulation of betaine is much less than that found in species that naturally accumulate betaines (Hayashi *et al.* 1997; Sakamoto & Murata 2000; Sakamoto *et al.* 2000). Choline is also a precursor of phosphatidylcholine, which is a major membrane component. The x*ipotl1* mutant, which is deficient in PEAMT required for phosphocholine biosynthesis (Fig. 4.7), shows a short-root phenotype due to reduced cell length of the elongation zone, proximal and distal regions of the differentiation zones as well as abnormalities in root epidermis cells (Cruz-Ramirez *et al.* 2004).

Genes encoding enzymes required for synthesis of the glycolipids digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) (Fig. 4.8) present in the plastid and plasma membranes (Benning 1998; Awai *et al.* 2001) were induced by chronic-cold treatment of Sha (Table 4.9). Increased levels of SQDG in chloroplast membranes are known to compensate for decreases in phospholipid resulting from phosphate (Pi) starvation (Yu & Benning 2003). It has also been proposed that DGDG replaces membrane phospholipids, which are degraded to provide a source of Pi for normal growth (Cruz-Ramirez *et al.* 2006). *Phosphate transporter2* and *Phosphate transporter3* were strongly induced by chronic-cold treatment of Sha, and considerably less so by the same treatment of Col-0 (Table 4.9). Sha also showed a stronger induction than Col-0 of two genes required for the development of root hairs (*MRH2* and *MRH6*), which are likely to be induced in response to nutrient deficiencies. Taken together, this leads me to propose Pi availablity is a factor limiting growth of Sha at 10 °C. This deficiency directly or indirectly induces compensatory responses that

include increased glycolipid production, increased formation of root hairs, and increased biogenesis of phosphate transporters. Interestingly, chronic cold treatment specifically induces the expression of several Sha genes concerned with sulfate transport, nitrate transport and copper transport (Table 4.9). This suggests that growth at low temperatures might result in a more general deficiency in ion transport and compensatory responses.

5.3.5 Growth and development

Plants grown at nonfreezing low temperatures grow more slowly and are smaller in size than warm-grown plants (Levitt 1980; Hughes & Dunn 1990). Growth and development depend on cell division and cell enlargement. During cell division, the primary cell wall is synthesized in the cell plate and is capable of subsequent expansion. New materials including cellulose microfibrils have to be incorporated to increase surface area of the existing cell wall architecture, which is rate limiting for cell growth (Aidid & Okamoto 1993; Cosgrove 1993; Cosgrove 1997). After cells stop expanding, a secondary cell wall is deposited within the boundary of primary wall. The secondary wall is much thicker and stronger than the primary wall, accounts for the most of the carbohydrate in biomass, and provides mechanical strength (Fry 1988; Cooper 2000). Thus, in principle, the limited growth of plants in the cold could involve changes in the expression of genes concerned with regulating the cell cycle, primary cell wall formation associated with cell enlargement, or the differentiation of the secondary cell wall.

5.3.5.1 Cell cycle regulation

Most of the 89 core cell cycle genes I surveyed did not show robust cold-responsiveness at the 2-fold level. For example, although genes encoding B-type cyclins were induced by cold-treatment of Col-0 and Sha (Fig 4.10), only *CYCB1;1* was induced >2-fold by chronic cold treatment, confirming the results in Chapter 3 (Table 4.13). B-type cyclins are positive regulators that govern the G2-to-M phase progression. Ectopic expression of *CYCB1;1* under the control of the *CDC2aAt* (*CDKA;1*) promoter in *Arabidopsis* markedly accelerates root growth without altering the pattern of lateral root development suggesting that *CYCB1;1* expression is growth limiting in roots (Doerner *et al.* 1996).

Similarly, ectopic expression of *CYCB1;2* induces extra endoreduplication/cell division in trichomes (Schnittger *et al.* 2002). This suggests that *CYCB1;4*, *CYCB2;1* and *CYCB2;2* clustered in Group I, and *CYCB1;3*, *CYCB2;3*, *CYCB2;4* and *CYCB3;1* clustered in Group II may have functions similar to *CYCB1;1* in promoting G2-to-M progression in the cold.

CYCP4;3 expression was induced by chronic-cold treatment 3-fold in Col-0 and 2-fold in Sha (Table 4.13). The functional significance of this induction is not known. While there is evidence that P-type cyclins have a role in phosphate signaling, *CYCP* mRNA levels do not appear to be affected by phosphate starvation and reapplication of phosphate in plant cell cultures (Torres Acosta *et al.* 2004).

D-type cyclins are thought to regulate G1-to-S progression and some responses of the cell cycle to external stimuli (Inzé & De Veylder 2006). Ectopic expression of *Arabidopsis* D-type cyclin *CYCD2;1* in transgenic tobacco cells accelerates the rate of cell cycling and results in an overall increase in growth rate (Cockcroft *et al.* 2000). Of the D-type cyclin genes surveyed, only *CYCD1;1* was cold-responsive and was repressed 2-fold by chronic cold treatment (Table 4.13).

In contrast to these findings, Rymen et al. (2007) have reported the repression of a diverse range of cyclin and cyclin-dependent kinase transcripts in maize leaves maintained at a low (4 °C) night temperature. This discrepancy could reflect differences in the plants used, the organs tested (leaves versus roots), or the regime used for cold treatment (cold nights/warm days versus continuous cold). Cyclins and cyclin-dependent kinases are known to be regulated primarily at the posttranslational level (Dewitte & Murray 2003). My results show, at least for the accessions tested, that if cold has major effects on the cell cycle, these effects are not at the mRNA level.

5.3.5.2 Cell enlargement, secondary wall formation, and vascular differentiation

Cold responsive genes with potential functions in cell enlargement and cell wall differentiation were identified. The LTP protein family has been implicated in wall loosening (Nieuwland *et al.* 2005). Genes encoding members of this family showed high-level induction in Sha, Col-0, or both accessions mainly in response to chronic cold treatment (Table 4.9). Included among the genes induced by chronic cold treatment enriched in Sha were (Table 4.5, 4.9): proline-rich extensin-like family proteins, which are structural components of plant cell (Tierney and Varner, 1987; Cassab, 1998); α-

expansin 18 and β-expansin, which have the cell wall loosening activity (Cosgrove, 1998, 2001); pectinesterases/pectin methylesterases, with roles in cell enlargement and stem elongation (Pilling, Willmitzer & Fisahn 2000; Micheli 2001); the leucine-repeat rich extensin ATHRGP1 (At3g54590), and ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 1(At2g33460).

Particularly relevant to the reduced growth observed at low temperatures may be genes grouped under "secondary cell wall biosynthetic process" and "xylem histogenesis" that were enriched in the set of genes repressed by chronic cold treatment of Col-0 or Sha (Table 4.7). Included in this set are Irregular Xylem 1 (IRX1)/cellulose synthase 8 (At4g18780), Irregular Xylem 3 (IRX3)/cellulose synthase 7 (At5g17420), Irregular Xylem 5 (IRX5)/cellulose synthase 4 (At5g44030), Irregular Xylem 6 (IRX6)/COBRA-LIKE4 (At5g15630), Irregular Xylem 9 (IRX9) (At2g37090), and Irregular Xylem 12 (IRX12)/laccase 4 (At2g38080). Cellulose microfibrils, which are important for the tensile strength of cell walls, are synthesized by complexes of cellulose synthase catalytic subunits organized into large plasma membrane bound structures known as rosettes (Saxena & Brown 2005; Somerville 2006). *Arabidopsis* mutants deficient in cellulose synthase activity show a specific reduction or even complete loss of cellulose deposition in the secondary cell wall and exhibit slightly reduced growth rate or smaller plant size compared to the wild-type plants (Turner & Somerville 1997; Taylor *et al.* 1999; Brown *et al.* 2005). Deficiency mutants of IRX1, IRX3 and IRX5, which encode the cellulose synthase catalytic subunits AtCesA8, AtCesA7 and AtCesA4 respectively, or IRX6, result in a severe reduction of cellulose in the secondary cell wall (Taylor *et al.* 1999; Taylor, Laurie & Turner 2000; Taylor *et al.* 2003). This suggests that down-regulation of genes concerned with cellulose biosynthesis might contribute to the reduced growth of *Arabidopsis* at low temperatures. Interestingly, laccase, which is required for the polymerization of monolignols in the lignification of secondary cell walls (Boerjan *et al.* 2003) was also down-regulated by chronic cold treatment of Col-0 and Sha.

Repression of genes concerned with secondary cell formation is also relevant to vascular tissue differentiation. Tracheary elements, which transport water in mature xylem, and fibers associated with vascular tissues, have highly thickened secondary cell walls enriched in cellulose and lignin. This suggests that chronic cold treatment, which represses expression of genes required for secondary wall formation, might retard vascular tissue differentiation or weaken the structures formed. This hypothesis is consistent with the finding that transcription factors that regulate the formation of

vascular tissues were repressed by chronic cold treatment. These include the genes *Vascular Related Nac-Domain Protein 7* (*VND7*), *ATHB-8*, and *Altered Phloem Development* (*APL*) grouped under "xylem histogenesis", which were enriched in chronic cold-repressed dataset of Sha but not Col-0 (Table 4.2B, 4.7). VND6 and VND7 are plant-specific NAC-domain transcription factors that can induce transdifferentiation of many cell types into metaxylem-like and protoxylem-like vessels elements, respectively. Deficiences in VND6 and VND7 specifically inhibit the formation of the respective vascular structures in roots (Kubo *et al.* 2005). APL is a MYB coiled-coil-type transcription factor responsible for phloem identity in *Arabidopsis* that helps determine the arrangement of xylem and phloem (Bonke *et al.* 2003). Mutants deficient in APL exhibit a defect in the organization of roots in which cells with xylem characteristics form at positions where phloem normally forms. These mutants have short, determinate roots with only occasional lateral branches. ATHB-8 is a member of the homeodomain-leucine zipper family of transcription factors (Baima *et al.* 2001). It has been suggested that is part of a positive feedback in which auxin signaling induces the expression of ATHB-8, which in turn promotes differentiation of procambial cells and cambial cells to form vascular tissues. Loss-of-function alleles of ATHB-8 do not, however, display obvious abnormalities.

5.3.6 Cold-treatment and accession specific gene expression

GO-term enrichment provides a rough picture of major trends in gene expression associated with cold-responses, which can be verified by examining the expression patterns of individual genes. The results of this analysis are summarized as a simplified model in Figure 5.1. Only genes in the functional groupings concerned with flavonoid biosynthesis appear to be induced by both acute and chronic cold treatment. The largest fold induction, in both Col-0 and Sha, was flavonoid-3'-hydroxylase, which is at the branch point leading to anthocyanins (Table 4.4, Fig. 4.3). This suggests that activation of anthocyanin biosynthesis is a major, common response to acute- and chronic-cold treatment. In contrast, genes concerned with electron transport and light-reactions in photosynthesis were repressed by both cold treatments in both accession. This is consistent with the well-documented, general reduction of these functions associated with growth at low temperatures (Huner *et al.* 1993; James, Grace & Hoad 1994; Huner *et al.* 1998; Allen & Ort 2001; Schuler & Werck-Reichhart 2003). Thus, regulation at the

mRNA level appears to be an important mechanism for down-regulating energy metabolism in cold environments.

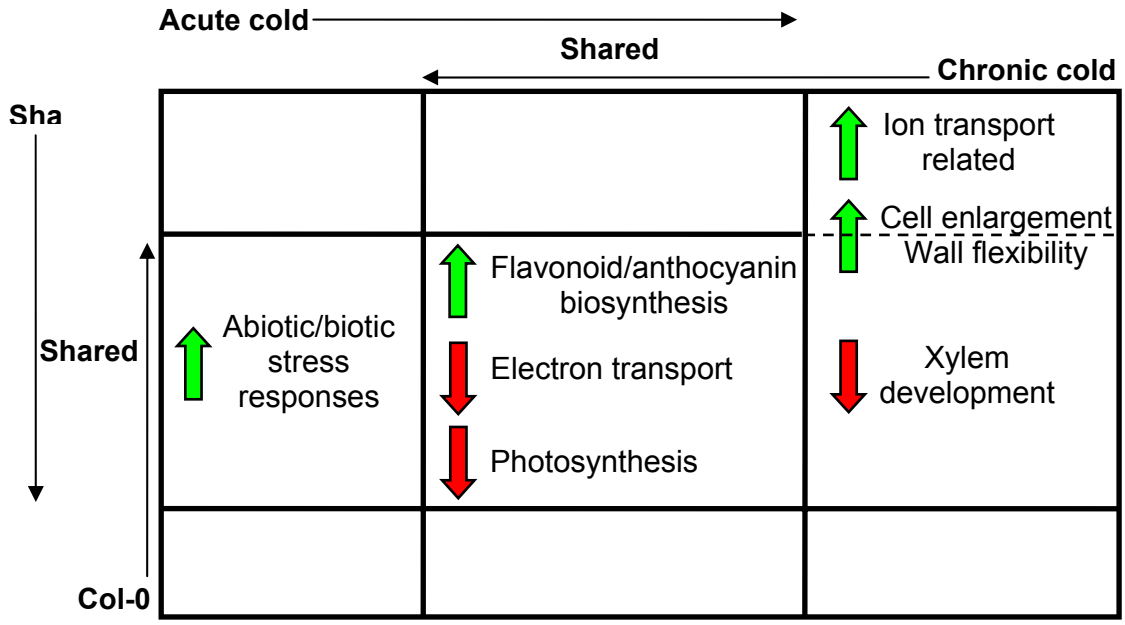


Figure 5.1. A simplifiec model of functional groupings of cold responsive genes as a result of the intereaction between cold treatments and accessions. Green arrows indicate up-regulation. Red arrows indicate down-regulation. Upper row for responses specific for Sha, and lower row for responses specific for Col-0. Left column for responses specific for acute cold treatment, and right column for responses specific for chronic cold treatment. Middle column or row indicates shared responses. Dash line indicates unique genes related to cell wall enlargement and flexibility that either specific for Sha or overlapped with Col-0 in the acute cold response.

I confirmed earlier studies showing that acute-cold treatment induces expression of a broad range of *Arabidopsis* genes concerned biotic and abiotic stress (Fowler & Thomashow 2002; Kreps *et al.* 2002; Seki *et al.* 2002; Lee *et al.* 2005). The breadth of this response emphasizes that brief exposure to cold, even at temperatures as high as 10 °C, is perceived by plants as a form of stress. Responses to cold stress depend on CBF-dependent (Gilmour *et al.* 1998; Liu *et al.* 1998; Shinwari *et al.* 1998) and CBF-independent signaling pathways (Xin & Browse 1998; Zhu *et al.* 2004; Zhu *et al.* 2005), and involve rather rapid activation of gene expression in the timeframe of as early as in

15 min to approximately two hours (Stockinger *et al.* 1997; Gilmour *et al.* 1998; Fowler & Thomashow 2002; Seki *et al.* 2002; Zarka *et al.* 2003; Oono *et al.* 2006). These rapid responses are known to result in cold acclimation and increased freezing tolerance, which are believed to contribute to the survival of *Arabidopsis* in cold environments (Thomashow 1999; Xin & Browse 2000; Van Buskirk & Thomashow 2006).

Unexpectedly, global induction of stress-related genes was strongly dependent on the nature of the cold treatment: induction was restricted primarily to the acute-cold response in both Sha and Col-0. This strongly suggests that growth at low, non-freezing temperatures is not recognized by *Arabidopsis* plants as a stress per se. Moreover, mechanisms must exist for suppressing prolonged stress responses in the cold. It would be interesting to know if this suppression is specific for cold stress or other stresses as well. Whatever the exact mechanism, these results clearly imply that general stress responses are not essential for growth of *Arabidopsis* at low temperatures.

Several other processes and pathways respond primarily to chronic-cold treatment and are likely to be cold adaptations. Included in this group are genes encoding ion transporters; genes concerned with compensation for Pi deprivation; and, genes required for formation of root hairs. Rates of root elongation at 10 °C were reduced by 18% and 14% for Col-0 and Sha, respectively (Fig. 3.2). Intriguingly, under the same conditions genes encoding primary wall constituents and enzymes concerned with cell enlargement and pectin metabolism were induced by chronic-cold treatment, while those genes important for secondary wall formation such as those encoding cellulose synthase and laccase required for lignification are repressed. These findings and the cold-repression of genes concerned with fiber and vascular tissue formation suggest as a working hypothesis that chronic cold treatment should increase the flexibility of roots and cell wall extensibility as a compensatory response to the reduced root growth in the cold. The working hypothesis is consistent with earlier reports showing that cell turgor of maize roots was reduced under osmotic stress or low water potential but elongation recovered at lower rates at later timepoints (Frensch & Hsiao 1995; Wu *et al.* 1996). Wu *et al.* (1996) found that the maize primary root tips at low water potential are more extensible than roots at high water potential, and that the increase in wall extension properties is associated with increses in expansin activity and in wall susceptibility to expansins. This result indicated that higher expansin activity and increase in cell wall susceptibility to expansins play a role in cell-wall enlargement and maintaining root elongation at root tips under osmotic stress or low water potential.

Differences in the cold-responsive gene expression of Col-0 and Sha could reflect natural genetic variation, ecotypic differentiation to the advantage of Sha or the disadvantage of Col-0 for growth in cold environments. No over-represented functional groupings were specific for Col-0 or Sha in acute up-regualtion response (Table 4.10A), suggesting there is no ecotypic differentiation of Col-0 for the cold-responsive traits studied. Although some genes concerned with cell expansion were Sha specific, other genes in this grouping as well as genes concerned with cell-wall flexibility and vascular differentiation were shared (Table 4.7, 4.9). The only good candidate for ecotypic differentiation of Sha was chronic-cold induction of pathways concerned with ion transport, Pi starvation, and root hair development. Of the 12 genes in this category, 9 were specifically induced in Sha (Table 4.9). Cold-enriched ion transport could, therefore, be of adaptive significance for the growth of Sha in cold environments.

5.4 Concluding remarks and outlook

Low temperature not only affects the survival of plants, but also their phenological and developmental plasticity. Responses of gene expression to chronic cold may function in cold adaptation to initiate or fine-tune physiological processes and developmental programs. Transcript profiling identified major trends in cold-related gene expression. One especially interesting lead is that cold-treatment rapidly induces stress-related genes, which are then suppressed or down regulated in prolonged cold exposure. This raises the possibility that suppression of stress responses is of advantage for growth in cold environments. The physiological significance of general stress responses and their down-regulation could be explored using informative mutants in combination with stress-treatments to detect cross-talk between the pathways. For example, studies of panels of mutants deficient or enhanced in cold-responsive could be used to establish whether the general stress response depends on known cold-perception pathways concerned with cold acclimation.

Other promising leads are the chronic-cold responsive pathways concerned with ion-transport related processes and cell wall expansion and differentiation. Technologies are available examine how the observed changes in gene expression are related to their proposed effects at the biochemical and physiological level. It would be interesting to compare the nutritional requirement and uptake by roots of Pi, sulfate, copper and nitrate

in Sha and Col-0. Physical measurements could be used to detect changes in cell-wall extensibility and root flexibility (Cosgrove 1993). Fingerprinting technologies could be used to characterize changes in cell wall polysaccharides (Lerouxel *et al.* 2002) and membrane lipids (Welti *et al.* 2002).

The approaches I developed could be applied to *Arabidopsis thaliana* accessions identified in large-scale screens that are capable of growing at high relative growth rates at low, non-freezing temperatures. These approaches could also be extended to *Thellungiella salsuginea*, an *Arabidopsis*-related species found in subarctic regions. *Thellungiella salsuginea* is emerging as a model species for molecular elucidation of abiotic stress tolerance (Inan *et al.* 2004; Griffith *et al.* 2007). Based on comparisons of the *Arabidopsis* genome sequence and *Thellungiella* ESTs, 60-95% of the nucleotides are identical in the two species.

6.0 References

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7.0 Appendix

Due to the redundancy of enriched genes in each GO term between Table 4.2, 4.8, and 4.10, I only showed a complete set of genes enriched in Table 4.10 to ease the presentation and discussion. Tables below represent genes in Table 4.2 or 4.8. Other gene enrichment in particular GO terms were presented in the Tables in Chapter 4.

Table I. Enrichment of genes in various GO terms in sets of acute cold up-regulated genes shared between Col-0 and Sha accessions.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
response to ethylene stimulus											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor (MYB74)	At4g05100	35.95	144.15	18.56	4.01	0.52	27.50	96.45	19.28	3.51	0.70
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11
ethylene-responsive protein, putative/PDX1L4	At2g38210	168.30	656.66	100.13	3.90	0.59	139.26	641.79	85.84	4.61	0.62
ethylene-responsive factor, putative	At4g18450	8.11	17.64	7.82	2.17	0.96	9.51	21.08	14.19	2.22	1.49
ethylene-responsive element-binding protein, putative	At1g72360	193.70	445.39	236.26	2.30	1.22	136.63	300.17	163.22	2.20	1.19
response to salt stress											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70

myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor (MYB74)	At4g05100	35.95	144.15	18.56	4.01	0.52	27.50	96.45	19.28	3.51	0.70
myb family transcription factor	At1g01520	5.15	46.96	6.91	9.12	1.34	4.65	38.28	5.13	8.24	1.10
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
zinc finger (C2H2 type) family protein (ZAT12)	At5g59820	291.40	840.73	409.01	2.89	1.40	386.45	1094.20	594.22	2.83	1.54
sodium-inducible calcium-binding protein (ACP1)	At5g49480	1286.75	3023.07	2115.08	2.35	1.64	924.50	3090.30	1144.51	3.34	1.24
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11
SNF1-related protein kinase 2-7	At4g40010	138.12	362.41	417.66	2.62	3.02	204.00	516.22	650.96	2.53	3.19
response to jasmonic acid stimulus											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor (MYB74)	At4g05100	35.95	144.15	18.56	4.01	0.52	27.50	96.45	19.28	3.51	0.70
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11
FAD-binding domain-containing protein	At2g34810	20.98	159.89	165.20	7.62	7.87	174.90	410.54	417.07	2.35	2.38
response to cadmium ion											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11

response to salicylic acid stimulus											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb-related protein CAPRICE (CPC)	At2g46410	64.60	321.70	69.45	4.98	1.08	60.59	176.57	51.49	2.91	0.85
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11
phytoalexin-deficient 4 protein (PAD4)	At3g52430	89.19	216.47	97.09	2.43	1.09	69.84	207.04	70.92	2.96	1.02
response to gibberellic acid stimulus											
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB13)	At1g06180	34.61	179.61	161.26	5.19	4.66	32.68	110.47	88.21	3.38	2.70
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11
gibberellin-regulated family protein/structural constituent of cell wall	At5g14920	215.86	798.11	367.39	3.70	1.70	150.58	309.36	171.44	2.05	1.14
response to UV-B											
bZIP protein / elongated hypocotyl 5 (HY5)	At5g11260	249.31	517.71	294.22	2.08	1.18	249.33	607.57	286.05	2.44	1.15
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
flavanone 3-hydroxylase (F3H)	At3g51240	2217.12	4898.76	5133.61	2.21	2.32	1699.21	5392.74	3693.49	3.17	2.17
UDP-glycosyltransferase/ sinapate 1-glucosyltransferase (UGT84A1)	At4g15480	15.23	187.42	118.43	12.30	7.77	10.84	225.56	38.76	20.81	3.58
transducin family protein / WD-40 repeat family protein	At5g52250	149.97	628.64	224.55	4.19	1.50	196.16	519.30	304.23	2.65	1.55
phenylpropanoid metabolic process											
cinnamoyl-CoA reductase-related	At2g23910	61.43	514.47	879.34	8.38	14.32	164.73	766.72	572.23	4.65	3.47

cinnamoyl-CoA reductase-related	At4g30470	394.03	1390.03	979.00	3.53	2.48	435.33	1976.10	810.41	4.54	1.86
cinnamoyl-CoA reductase-related	At5g14700	18.85	152.14	25.56	8.07	1.36	14.64	121.36	24.45	8.29	1.67
4-coumarate:CoA ligase 3 (4CL3)	At1g65060	191.79	399.16	993.43	2.08	5.18	181.28	843.71	782.65	4.65	4.32
flavanone 3-hydroxylase (F3H)	At3g51240	2217.12	4898.76	5133.61	2.21	2.32	1699.21	5392.74	3693.49	3.17	2.17
flavonoid 3'-hydroxylase (F3'H)	At5g07990	51.17	354.30	880.49	6.92	17.21	31.91	371.41	420.83	11.64	13.19
myb family transcription factor (MYB12)	At2g47460	108.07	1071.67	407.78	9.92	3.77	194.82	1164.41	297.06	5.98	1.52
O-methyltransferase family 2 protein	At1g33030	6.35	18.31	17.13	2.88	2.70	7.39	18.73	8.36	2.54	1.13
chorismate mutase 1, chloroplast (CM1)	At3g29200	362.45	1014.62	575.54	2.80	1.59	452.82	1174.68	565.80	2.59	1.25
response to auxin stimulus											
auxin-responsive family protein	At5g53590	350.57	846.17	501.69	2.41	1.43	358.39	829.10	370.81	2.31	1.03
auxin-responsive AUX/IAA family protein/SOLITARY ROOT(SLR)	At4g14550	73.10	296.18	114.31	4.05	1.56	62.81	222.30	70.05	3.54	1.12
auxin-responsive GH3 family protein	At5g51470	10.59	25.12	9.82	2.37	0.93	15.23	41.70	36.89	2.74	2.42
auxin-responsive protein, putative	At4g36110	8.55	41.54	7.44	4.86	0.87	7.71	21.63	10.07	2.80	1.31
auxin-responsive protein / indoleacetic acid-induced protein 28 (IAA28)	At5g25890	722.86	1674.30	667.62	2.32	0.92	832.16	1725.25	628.59	2.07	0.76
myb family transcription factor (MYB4)	At4g38620	277.14	884.45	482.38	3.19	1.74	185.70	714.22	227.24	3.85	1.22
myb family transcription factor (MYB44)	At5g67300	929.98	2427.08	1327.56	2.61	1.43	782.33	1580.57	819.30	2.02	1.05
myb-related transcription factor/circadian clock associated 1 (CCA1)	At2g46830	275.16	3209.59	341.23	11.66	1.24	42.44	2984.46	334.72	70.32	7.89
myb family transcription factor	At3g09600	35.36	207.01	17.43	5.85	0.49	11.86	229.36	18.08	19.33	1.52
myb family transcription factor	At5g37260	146.84	1184.93	609.22	8.07	4.15	146.11	741.61	400.63	5.08	2.74
myb family transcription factor	At3g09610	11.37	45.45	7.82	4.00	0.69	5.22	48.47	8.78	9.28	1.68
late elongated hypocotyl (LHY)	At1g01060	547.90	2775.58	212.86	5.07	0.39	64.22	2631.69	263.89	40.98	4.11

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table II. Enrichment of circadian rhythm-related genes in set of chronic cold up-regulated genes specific to Col-0 accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
circadian rhythm											
early flowering 4 (ELF4)	At2g40080	128.33	72.36	1297.24	0.56	10.11	1329.62	151.88	1067.44	0.11	0.80
phytoclock 1 (PCL1)	At3g46640	121.84	135.59	372.39	1.11	3.06	430.52	87.54	260.78	0.20	0.61
zinc finger protein CONSTANS (CO)	At5g15840	5.28	23.82	11.95	4.51	2.27	5.02	11.43	5.36	2.28	1.07
timing of CAB1 1/ABI3-interacting protein 1 (AIP1)	At5g61380	334.77	324.15	746.57	0.97	2.23	878.01	348.93	681.11	0.40	0.78
response regulator 3 (ARR3)	At1g59940	102.88	196.95	230.63	1.91	2.24	41.01	54.13	78.73	1.32	1.92
pseudo-response regulator 5 (APRR5)	At5g24470	153.91	96.40	523.50	0.63	3.40	423.61	306.59	325.01	0.72	0.77

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table III. Enrichment of genes in various GO terms in sets of chronic cold up-regulated genes specific to Sha accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
cell wall organization and biogenesis											
proline-rich extensin-like family protein	At5g49080	243.21	216.00	430.94	0.89	1.77	82.64	96.55	298.21	1.17	3.61
proline-rich extensin-like family protein	At4g08410	68.42	84.13	98.33	1.23	1.44	37.86	44.30	144.01	1.17	3.80
proline-rich extensin-like family protein	At5g06640	205.91	180.78	276.26	0.88	1.34	191.42	117.52	612.42	0.61	3.20
proline-rich extensin-like family protein	At3g54580	153.30	97.97	180.05	0.64	1.17	111.56	57.15	340.91	0.51	3.06
proline-rich extensin-like family protein	At2g24980	263.10	176.88	332.65	0.67	1.26	217.98	103.78	575.83	0.48	2.64
proline-rich extensin-like family protein	At5g06630	214.70	145.39	328.24	0.68	1.53	224.46	119.29	882.00	0.53	3.93
proline-rich extensin-like family protein	At5g35190	331.36	293.47	557.15	0.89	1.68	322.60	247.03	831.40	0.77	2.58
α-expansin 18	At1g62980	389.45	514.60	573.38	1.32	1.47	376.59	397.66	854.90	1.06	2.27
ATHRGP1, structural constituent of cell wall	At3g54590	339.07	420.24	409.91	1.24	1.21	260.93	251.39	746.73	0.96	2.86

glycolipid biosynthetic process[b]											
phospholipase D Zeta 2 (PLDZ2)	At3g05630	75.50	47.47	99.72	0.63	1.32	26.59	16.03	129.73	0.60	4.88
monogalactosyldiacylglycerol synthase type C (MGD3)	At2g11810	196.05	122.58	263.81	0.63	1.35	29.71	43.46	298.16	1.46	10.04
sulfolipid synthase (SQD2)	At5g01220	1448.87	777.29	1388.65	0.54	0.96	592.03	424.10	1744.82	0.72	2.95
choline biosynthetic process											
phosphoethanolamine N-methyltransferase 1	At3g18000	376.28	554.34	580.12	1.47	1.54	487.17	462.72	1058.12	0.95	2.17
sulfate transport											
sulfate transporter	At4g08620	21.54	26.73	22.41	1.24	1.04	56.03	59.75	276.50	1.07	4.93
sulfate transporter (Sultr1;3)	At1g22150	5.49	7.46	6.81	1.36	1.24	6.65	11.23	77.41	1.69	11.63
sulfate transporter, putative	At1g23090	19.49	71.25	41.36	3.65	2.12	52.96	122.21	118.14	2.31	2.23
root development											
phospholipase D Zeta 2 (PLDZ2)	At3g05630	75.50	47.47	99.72	0.63	1.32	26.59	16.03	129.73	0.60	4.88
Morphogenesis of Root Hair 2 (MRH2)	At3g54870	58.60	43.38	78.74	0.74	1.34	60.93	43.64	158.80	0.72	2.61
Morphogenesis of Root Hair 6 (MRH6)	At2g03720	317.02	365.43	479.66	1.15	1.51	541.41	463.48	1258.57	0.86	2.32
leucine-rich repeat/extensin 1 (LRX1)	At1g12040	23.40	34.93	34.19	1.49	1.46	21.12	26.75	92.43	1.27	4.38
Nitrate Transporter 2.1	At1g08090	12.01	7.57	19.21	0.63	1.60	10.18	8.01	43.10	0.79	4.23
copper transporter 1 (COPT1)	At5g59030	68.82	80.47	78.51	1.17	1.14	51.07	69.06	111.31	1.35	2.18

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table IV. Enrichment of flavonoid biosynthetic-related genes in sets of chronic cold up-regulated genes shared between Col-0 and Sha accessions.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
flavonoid biosynthetic process											
flavonol synthase 1 (FLS1)	At5g08640	776.60	1542.79	2904.80	1.99	3.74	1025.38	2529.11	2568.04	2.47	2.50
flavanone 3-hydroxylase (F3H)	At3g51240	2217.12	4898.76	5133.61	2.21	2.32	1699.21	5392.74	3693.49	3.17	2.17
flavonoid 3'-hydroxylase (F3'H)	At5g07990	51.17	354.30	880.49	6.92	17.21	31.91	371.41	420.83	11.64	13.19
chalcone isomerase (CHI)	At3g55120	613.36	1085.09	2576.37	1.77	4.20	963.96	2473.53	2529.41	2.57	2.62
UDP-glucoronosyl/UDP-glucosyl transferase family protein	At1g30530	127.06	288.53	508.55	2.27	4.00	63.53	499.22	331.48	7.86	5.22

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table V. Enrichment of photosynthesis-related genes in sets of acute cold down-regulated genes specific to Col-0 accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
photosynthesis											
PSII 43 KDa protein/PSBC	AtCg00280	1208.00	352.23	154.99	0.29	0.13	452.32	427.11	173.05	0.94	0.38
PSII D2 protein	AtCg00270	473.56	156.20	98.39	0.33	0.21	209.09	208.41	110.13	1.00	0.53
PSII T protein/PSBT	AtCg00690	17.30	7.14	7.20	0.41	0.42	9.56	7.21	5.23	0.75	0.55
photosystem II subunit P-2	At2g30790	16.66	7.57	6.40	0.45	0.38	6.50	6.33	4.70	0.97	0.72
chlorophyll A-B binding protein / LHCII type I (LHB1B2)	At2g34430	6015.34	1844.22	1460.13	0.31	0.24	3592.31	1266.69	884.16	0.35	0.25
chlorophyll A-B binding protein / LHCII type II (LHCB2.2)	At2g05070	2434.82	1141.66	334.43	0.47	0.14	830.49	884.30	409.17	1.06	0.49
chlorophyll A-B binding protein / LHCII type III (LHCB3)	At5g54270	2055.87	766.94	489.22	0.37	0.24	1035.82	575.81	338.80	0.56	0.33
chlorophyll A-B binding protein 4/ LHCI type III CAB-4 (CAB4)	At3g47470	1693.70	509.93	450.12	0.30	0.27	826.40	396.33	303.28	0.48	0.37
chlorophyll A-B binding protein, chloroplast (LHCB6)	At1g15820	825.65	281.31	267.83	0.34	0.32	554.84	286.67	259.67	0.52	0.47
cytochrome f/PETA	AtCg00540	1392.84	679.52	1227.07	0.49	0.88	1243.33	698.36	1028.61	0.56	0.83
hypothetical protein/YCF3	AtCg00360	169.66	76.64	74.42	0.45	0.44	117.39	84.93	83.44	0.72	0.71

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table VI. Enrichment of circadian rhythm-related genes in sets of acute cold down-regulated genes specific to Sha accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
circadian rhythm											
early flowering 4	At2g40080	128.33	72.36	1297.24	0.56	10.11	1329.62	151.88	1067.44	0.11	0.80
ABI3-interacting protein 1 (AIP1)	At5g61380	334.77	324.15	746.57	0.97	2.23	878.01	348.93	681.11	0.40	0.78
myb family transcription factor, myb family transcription factor	At3g46640	121.84	135.59	372.39	1.11	3.06	430.52	87.54	260.78	0.20	0.61
glycine-rich RNA-binding protein (GRP7)	At2g21660	4223.90	4431.95	7844.11	1.05	1.86	9700.33	4770.14	7795.86	0.49	0.80
flavin-binding kelch domain F box protein (FKF1) / adagio 3 (ADO3)	At1g68050	596.47	326.81	584.45	0.55	0.98	623.50	84.92	308.31	0.14	0.49
MAP kinase 7	At2g18170	216.96	150.37	241.30	0.69	1.11	271.12	99.53	139.51	0.37	0.51
with no lysine(K) 1 (WNK1)	At3g04910	543.20	285.83	483.19	0.53	0.89	784.28	385.55	637.58	0.49	0.81

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table VII. Enrichment of genes in various GO terms in sets of acute cold down-regulated genes shared between Col-0 and Sha accessions.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
photosynthesis											
photosystem I subunit D-2 (PSAD-2)	At1g03130	182.48	15.40	21.81	0.08	0.12	73.92	12.90	18.22	0.17	0.25
photosystem I light harvesting complex gene 3 / LHCI type III (LHCA3.1)	At1g61520	2484.69	691.92	799.06	0.28	0.32	1401.57	463.91	459.72	0.33	0.33
photosystem I reaction center subunit VI, chloroplast, putative / PSI-H, putative (PSAH2)	At1g52230	403.88	104.46	166.95	0.26	0.41	266.93	71.35	75.25	0.27	0.28
photosystem I subunit D-1 (PSAD-1)	At4g02770	684.03	141.73	280.90	0.21	0.41	526.10	153.71	189.61	0.29	0.36
photosystem I subunit O (PSAO)	At1g08380	1035.40	333.31	288.90	0.32	0.28	715.08	243.74	255.41	0.34	0.36
photosystem I subunit L (PSAL)	At4g12800	624.50	133.49	259.95	0.21	0.42	330.09	53.72	116.65	0.16	0.35
photosystem I subunit K (psaK)	At1g30380	1804.08	894.54	933.66	0.50	0.52	1283.63	588.98	653.35	0.46	0.51
photosystem II core complex proteins (PSBY)	At1g67740	392.87	146.19	161.67	0.37	0.41	286.05	118.22	146.05	0.41	0.51
light harvesting complex PSII (LHCB4.2)	At3g08940	517.54	43.68	83.99	0.08	0.16	158.55	16.81	22.31	0.11	0.14
photosystem II reaction center PsbP family protein	At3g55330	37.70	15.89	27.11	0.42	0.72	40.06	15.04	24.03	0.38	0.60
chlorophyll A-B binding protein / LHCI type I (CAB)	At3g54890	1248.38	333.82	399.95	0.27	0.32	1247.47	463.98	421.81	0.37	0.34
chlorophyll A-B binding protein CP29 (LHCB4)	At5g01530	1096.24	483.22	524.15	0.44	0.48	949.80	418.60	376.71	0.44	0.40
light-harvesting complex II protein 5 / LHCIIc (LHCB5)	At4g10340	2020.11	877.63	870.72	0.43	0.43	1369.10	581.00	576.00	0.42	0.42
CP12 domain-containing protein	At3g62410	69.99	9.75	18.90	0.14	0.27	131.34	11.12	22.26	0.08	0.17
cellular polysaccharide catabolic process											
4-alpha-glucanotransferase, putative / disproportionating enzyme, putative	At5g64860	142.74	69.70	114.38	0.49	0.80	194.34	64.22	126.81	0.33	0.65
pectinesterase family protein	At5g55590	40.58	19.87	16.05	0.49	0.40	29.49	14.27	19.02	0.48	0.64
α-amylase-like 3	At1g69830	354.09	64.67	324.82	0.18	0.92	468.08	142.05	264.25	0.30	0.56
isoamylase 3 / starch debranching enzyme, putative	At4g09020	183.61	66.85	259.21	0.36	1.41	200.31	50.50	151.36	0.25	0.76
electron transport											

cytochrome P450, family 71, subfamily A, polypeptide 16 (CYP71A16)	At5g42590	1083.41	412.58	174.38	0.38	0.16	919.94	336.88	432.80	0.37	0.47
cytochrome P450, family 71, subfamily A, polypeptide 19 (CYP71A19)	At4g13290	1080.85	334.93	231.75	0.31	0.21	745.07	310.47	397.98	0.42	0.53
cytochrome P450, family 71, subfamily B, polypeptide 28 (CYP71B28)	At1g13090	196.56	63.49	152.28	0.32	0.77	168.48	80.15	69.82	0.48	0.41
cytochrome P450, family 78, subfamily A, polypeptide 8 (CYP78A8)	At1g01190	780.22	348.09	314.31	0.45	0.40	1303.11	519.69	472.99	0.40	0.36
cytochrome P450, family 87, subfamily A, polypeptide 9 (member of CYP89A9)	At3g03470	137.03	24.49	26.58	0.18	0.19	64.01	19.65	17.04	0.31	0.27
cytochrome P450, family 705, subfamily A, polypeptide 4 (CYP705A4)	At4g15380	109.08	37.91	64.15	0.35	0.59	66.54	22.81	26.11	0.34	0.39
cytochrome P450, family 705, subfamily A, polypeptide 12 (CYP705A12)	At5g42580	313.38	92.16	30.73	0.29	0.10	453.45	138.68	164.78	0.31	0.36
cytochrome P450, family 708, subfamily A, polypeptide 3 (CYP708A3)	At1g78490	193.59	74.64	33.81	0.39	0.17	57.93	8.92	12.10	0.15	0.21
cytochrome P450, family 710, subfamily A, polypeptide 1 (CYP710A1)	At2g34500	574.43	203.26	21.42	0.35	0.04	550.55	238.22	89.66	0.43	0.16
cytochrome P450, family 735, subfamily A, polypeptide 2 (CYP735A2)	At1g67110	195.00	62.19	7.56	0.32	0.04	395.78	88.40	14.16	0.22	0.04
cytochrome c oxidase subunit II, putative	At2g07695	104.68	48.61	92.63	0.46	0.88	104.29	42.56	97.83	0.41	0.94
ubiquinol-cytochrome C reductase complex 14 kDa protein, putative	At5g25450	45.53	13.43	14.53	0.29	0.32	39.51	13.31	22.62	0.34	0.57
FAD-binding domain-containing protein	At4g20840	146.28	68.24	94.03	0.47	0.64	261.01	46.30	137.83	0.18	0.53
FAD-binding domain-containing protein	At5g11540	109.66	48.47	18.21	0.44	0.17	252.52	115.25	68.12	0.46	0.27
FAD-dependent oxidoreductase family protein	At5g67290	157.06	71.44	111.09	0.45	0.71	130.29	58.05	120.96	0.45	0.93
flavin-containing monooxygenase family protein / FMO family protein	At5g61290	13.84	5.99	6.76	0.43	0.49	20.35	6.68	6.43	0.33	0.32
photosystem I subunit E-2	At2g20260	211.37	80.60	96.12	0.38	0.45	174.38	45.07	61.76	0.26	0.35
NADH dehydrogenase subunit 9/nad9	AtMg00070	44.31	18.11	11.62	0.41	0.26	57.02	18.25	39.22	0.32	0.69
ferredoxin--NADP(+) reductase, putative / LEAF FNR 2	At1g20020	66.02	17.85	26.15	0.27	0.40	73.44	20.20	17.29	0.28	0.24
ferredoxin--NADP(+) reductase, putative /LEAF FNR 1	At5g66190	364.94	166.62	137.63	0.46	0.38	344.07	143.98	107.65	0.42	0.31
ferredoxin-related/electron carrier	At4g32590	56.96	28.06	52.73	0.49	0.93	56.97	26.15	52.26	0.46	0.92
ascorbate peroxidase 6 (APX6)	At4g32320	30.66	12.78	19.70	0.42	0.64	32.49	15.27	28.06	0.47	0.86
monooxygenase, putative (MO3)	At5g05320	150.81	48.11	73.90	0.32	0.49	92.12	39.12	46.90	0.42	0.51
glutaredoxin family protein	At1g03850	1681.70	707.43	2170.85	0.42	1.29	1705.64	751.67	1470.06	0.44	0.86
ferric reduction oxidase 3 (FRO3)	At1g23020	266.87	112.76	172.65	0.42	0.65	309.46	139.49	235.97	0.45	0.76

glutaredoxin family protein	At5g58530	137.71	59.98	154.65	0.44	1.12	151.34	64.60	171.64	0.43	1.13
choline monooxygenase, putative (CMO-like)	At4g29890	144.28	36.71	57.42	0.25	0.40	122.28	39.54	49.11	0.32	0.40
plastocyanin	At1g76100	116.28	10.66	10.63	0.09	0.09	91.74	10.01	10.55	0.11	0.12

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table VIII. Enrichment of genes in various GO terms in sets of chronic cold down-regulated genes specific to Col-0 accession.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
electron transport											
cytochrome P450, family 71, subfamily A, polypeptide 19 (CYP71A19)	At4g13290	1080.85	334.93	231.75	0.31	0.21	745.07	310.47	397.98	0.42	0.53
cytochrome P450, family 71, subfamily B, polypeptide 13 (CYP71B13)	At5g25140	49.12	65.42	19.10	1.33	0.39	18.67	36.48	10.91	1.95	0.58
cytochrome P450, family 71, subfamily B, polypeptide 37 (CYP71B37)	At3g26330	299.35	237.15	102.16	0.79	0.34	16.75	17.05	9.62	1.02	0.57
cytochrome P450, family 705, subfamily A, polypeptide 13 (CYP705A13)	At2g14100	273.13	207.29	126.66	0.76	0.46	261.82	242.75	149.97	0.93	0.57
cytochrome P450, family 705, subfamily A, polypeptide 18 (CYP705A18)	At3g20090	31.18	13.95	14.32	0.45	0.46	26.49	13.78	11.94	0.52	0.45
cytochrome P450, family 706, subfamily A, polypeptide 7 (CYP706A7)	At4g12330	331.49	59.29	76.74	0.18	0.23	4.35	7.19	4.70	1.65	1.08
cytochrome P450, family 710, subfamily A, polypeptide 2 (CYP710A2)	At2g34490	834.35	758.51	246.31	0.91	0.30	802.56	832.12	417.72	1.04	0.52
cytochrome P450, family 735, subfamily A, polypeptide 1 (CYP735A1)	At5g38450	39.11	21.73	7.20	0.56	0.18	10.76	12.25	6.93	1.14	0.64
cytochrome b (MTCYB) (COB) (CYTB)	At2g07727	92.11	54.97	35.93	0.60	0.39	73.26	40.53	49.00	0.55	0.67
ubiquinol-cytochrome C reductase complex 14 kDa protein, putative	At5g25450	45.53	13.43	14.53	0.29	0.32	39.51	13.31	22.62	0.34	0.57
FAD-binding domain-containing protein	At4g20820	151.55	74.05	35.68	0.49	0.24	50.62	57.21	28.08	1.13	0.55
FAD-binding domain-containing protein	At5g44410	80.46	18.19	8.43	0.23	0.10	20.03	15.51	9.37	0.77	0.47
flavin-containing monooxygenase family protein / FMO family protein	At1g62540	136.73	30.93	10.17	0.23	0.07	11.34	17.65	12.70	1.56	1.12
flavin-containing monooxygenase family protein / FMO family protein	At5g07800	26.21	28.23	11.37	1.08	0.43	31.38	28.79	18.01	0.92	0.57
flavin-containing monooxygenase family protein / FMO family protein	At1g62560	388.52	387.13	173.81	1.00	0.45	102.33	225.30	170.23	2.20	1.66
monooxygenase, putative (MO3)	At5g05320	150.81	48.11	73.90	0.32	0.49	92.12	39.12	46.90	0.42	0.51
ferredoxin family protein	At4g14890	97.45	46.29	33.33	0.47	0.34	92.21	64.45	64.73	0.70	0.70
PSII D2 protein/psbD	AtCg00270	473.56	156.20	98.39	0.33	0.21	209.09	208.41	110.13	1.00	0.53
similar to NADH dehydrogenase subunit 5/nad5.1	At2g07711, AtMg00513	9.42	12.33	4.65	1.31	0.49	7.15	11.15	5.34	1.56	0.75

photosystem I reaction center subunit PSI-N	At5g64040	665.91	419.96	219.87	0.63	0.33	387.80	208.87	150.80	0.54	0.39
oxidoreductase N-terminal domain-containing protein	At1g66130	22.61	35.29	8.46	1.56	0.37	9.91	41.30	7.79	4.17	0.79
glucose-methanol-choline (GMC) oxidoreductase family protein	At1g14190	155.07	103.44	67.18	0.67	0.43	160.80	102.35	112.85	0.64	0.70
(S)-2-hydroxy-acid oxidase, peroxisomal, putative	At3g14415	332.34	480.96	88.89	1.45	0.27	468.08	547.09	260.36	1.17	0.56
polyamine oxidase 5	At4g29720	560.49	349.12	247.99	0.62	0.44	602.66	364.67	309.97	0.61	0.51
photosynthesis, light harvesting in photosystem II											
PSII I protein (psbI)	AtCg00080	608.03	311.10	198.78	0.51	0.33	292.70	292.67	196.93	1.00	0.67
PSII D2 protein (psbD)	AtCg00270	473.56	156.20	98.39	0.33	0.21	209.09	208.41	110.13	1.00	0.53
PSII 10KDa phosphoprotein (psbH)	AtCg00710	532.44	301.31	174.76	0.57	0.33	335.46	341.10	168.91	1.02	0.50
monoterpenoid metabolic process											
terpene synthase-like sequence-1,8-cineole	At3g25820	1227.68	834.73	324.20	0.68	0.26	835.82	719.80	623.39	0.86	0.75
terpene synthase-like sequence-1,8-cineole	At3g25830	5387.07	3545.21	2537.32	0.66	0.47	4021.34	2595.68	2697.97	0.65	0.67
oligopeptide transport											
proton-dependent oligopeptide transport (POT) family protein	At1g22550	498.13	355.07	209.27	0.71	0.42	629.96	443.63	353.94	0.70	0.56
proton-dependent oligopeptide transport (POT) family protein	At3g01350	173.90	66.20	85.48	0.38	0.49	194.34	164.22	102.03	0.84	0.52
proton-dependent oligopeptide transport (POT) family protein	At3g53960	25.92	11.90	7.12	0.46	0.27	14.90	8.72	15.38	0.59	1.03
proton-dependent oligopeptide transport (POT) family protein	At1g33440	13.23	7.88	6.34	0.60	0.48	22.67	9.21	13.63	0.41	0.60
proton-dependent oligopeptide transport (POT) family protein	At2g37900	30.70	10.81	4.49	0.35	0.15	8.25	18.44	6.17	2.24	0.75
nitrate transporter (NTP3)	At3g21670	68.84	105.79	32.96	1.54	0.48	78.39	117.02	101.28	1.49	1.29

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

Table IX. Enrichment of genes in various GO terms in sets of chronic cold down-regulated genes shared between Col-0 and Sha accessions.

		Col-0					Sha				
		Median expression value			Ratio*		Median expression value			Ratio*	
Annotation	AGI No.	warm	acute	chronic	acute	chronic	warm	acute	chronic	acute	chronic
secondary cell wall biosynthetic process (sensu Magnoliophyta)											
cellulose synthase subunit A8 (CESA8)/IRX1	At4g18780	154.11	158.95	27.16	1.03	0.18	176.55	180.86	35.03	1.02	0.20
cellulose synthase subunit A7 (CESA7)/IRX3	At5g17420	241.67	247.85	49.40	1.03	0.20	273.99	226.41	69.82	0.83	0.25
cellulose synthase subunit A4 (CESA4)/IRX5	At5g44030	238.31	124.70	48.98	0.52	0.21	161.63	55.24	40.88	0.34	0.25
COBRA-LIKE4 (COBL4)/IRX6	At5g15630	76.29	58.14	16.68	0.76	0.22	102.43	68.94	20.74	0.67	0.20
glycosyl transferase family 43 protein/IRX9	At2g37090	182.60	143.08	51.80	0.78	0.28	176.69	130.54	77.35	0.74	0.44
laccase 4/IRX12	At2g38080	193.42	214.51	37.01	1.11	0.19	241.56	263.47	60.49	1.09	0.25
electron transport											
cytochrome P450, family 71, subfamily A, polypeptide 16 (CYP71A16)	At5g42590	1083.41	412.58	174.38	0.38	0.16	919.94	336.88	432.80	0.37	0.47
cytochrome P450, family 71, subfamily B, polypeptide 26 (CYP71B26)	At3g26290	514.19	154.14	190.02	0.30	0.37	92.78	52.80	8.77	0.57	0.09
cytochrome P450, family 78, subfamily A, polypeptide 5 (CYP78A5)	At1g13710	154.90	159.59	62.90	1.03	0.41	287.67	311.09	142.60	1.08	0.50
cytochrome P450, family 78, subfamily A, polypeptide 8 (CYP78A8)	At1g01190	780.22	348.09	314.31	0.45	0.40	1303.11	519.69	472.99	0.40	0.36
cytochrome P450, family 87, subfamily A, polypeptide 9 (member of CYP89A9)	At3g03470	137.03	24.49	26.58	0.18	0.19	64.01	19.65	17.04	0.31	0.27
cytochrome P450, family 96, subfamily A, polypeptide 4 (CYP96A4)	At5g52320	15.42	20.37	5.69	1.32	0.37	78.03	102.94	26.84	1.32	0.34
cytochrome P450, family 705, subfamily A, polypeptide 1 (CYP705A1)	At4g15330	33.80	25.59	11.11	0.76	0.33	28.35	21.60	6.66	0.76	0.24
cytochrome P450, family 705, subfamily A, polypeptide 12 (CYP705A12)	At5g42580	313.38	92.16	30.73	0.29	0.10	453.45	138.68	164.78	0.31	0.36
cytochrome P450, family 706, subfamily A, polypeptide 2 (CYP706A2)	At4g22710	544.21	742.42	114.45	1.36	0.21	726.52	787.93	69.82	1.08	0.10
cytochrome P450, family 708, subfamily A, polypeptide 3 (CYP708A3)	At1g78490	193.59	74.64	33.81	0.39	0.17	57.93	8.92	12.10	0.15	0.21
cytochrome P450, family 710, subfamily A, polypeptide 1 (CYP710A1)	At2g34500	574.43	203.26	21.42	0.35	0.04	550.55	238.22	89.66	0.43	0.16

cytochrome P450, family 712, subfamily A, polypeptide 1 (CYP712A1)	At2g42250	427.66	525.81	22.83	1.23	0.05	1192.30	596.44	319.57	0.50	0.27
cytochrome P450, family 735, subfamily A, polypeptide 2 (CYP735A2)	At1g67110	195.00	62.19	7.56	0.32	0.04	395.78	88.40	14.16	0.22	0.04
cytochrome P450, putative	At4g20240	19.44	13.93	7.80	0.72	0.40	48.02	29.25	9.09	0.61	0.19
FAD-binding domain-containing protein	At5g11540	109.66	48.47	18.21	0.44	0.17	252.52	115.25	68.12	0.46	0.27
FAD-binding domain-containing protein	At1g30760	457.69	435.45	93.36	0.95	0.20	1025.71	897.09	205.45	0.87	0.20
FAD-binding domain-containing protein	At2g46760	26.35	19.01	5.55	0.72	0.21	37.67	22.60	9.47	0.60	0.25
flavin-containing monooxygenase, putative / FMO, putative	At5g25620	23.36	22.80	6.82	0.98	0.29	27.67	23.03	9.31	0.83	0.34
plastocyanin	At1g76100	116.28	10.66	10.63	0.09	0.09	91.74	10.01	10.55	0.11	0.12
plastocyanin-like domain-containing protein	At3g27200	516.39	479.19	171.22	0.93	0.33	567.46	426.11	274.87	0.75	0.48
rubredoxin family protein	At5g17170	120.22	116.65	31.94	0.97	0.27	163.39	120.44	41.13	0.74	0.25
ferredoxin--NADP(+) reductase, putative / LEAF FNR 1	At5g66190	364.94	166.62	137.63	0.46	0.38	344.07	143.98	107.65	0.42	0.31
photosystem I reaction center subunit IV (PSAE1)/PSA E1 KNOCKOUT	At4g28750	702.58	396.98	269.32	0.57	0.38	594.86	223.85	193.67	0.38	0.33
lipoxygenase, putative	At3g22400	190.63	206.34	94.54	1.08	0.50	169.09	120.33	67.75	0.71	0.40
choline monooxygenase, putative (CMO-like)	At4g29890	144.28	36.71	57.42	0.25	0.40	122.28	39.54	49.11	0.32	0.40
glutaredoxin family protein	At5g14070	13.58	7.64	4.07	0.56	0.30	22.44	10.88	4.67	0.48	0.21
photosynthesis, light harvesting in photosystem I											
chlorophyll A-B binding protein (LHCA2)	At3g61470	1726.68	913.30	571.49	0.53	0.33	1525.40	503.35	528.10	0.33	0.35
chlorophyll A-B binding protein / LHCI type I / LHCA1	At3g54890	1248.38	333.82	399.95	0.27	0.32	1247.47	463.98	421.81	0.37	0.34
photosystem I subunit O (PSAO)	At1g08380	1035.40	333.31	288.90	0.32	0.28	715.08	243.74	255.41	0.34	0.36

* Ratios of the median expression values of cold-treated relative to warm controls. Median value is based on three biological replicates.

8.0 List of abbreviations and trivial names

$A_{260,\ 280}$	absorbance at 260 nm or at 280 nm
ABA	abscisic acid
ADA2	transcriptional adapator protein 2
ATP	Adenosine 5'-triphosphate
AU	arbitrary unit
CBF	C-Repeat Binding Factor
CoA	Co-enzyme A
COR	Cold-Regulated gene
COR15a	Cold-regulated 15a
cRNA	Complementary RNA
CRT	C-Repeat
C_T	threshold cycle of quantitative real-time PCR
CTP	cytidine-5'-triphosphate
dCTP	deoxy-cytidine-5'-triphosphate
DEPC	diethylpyrocarbonate
df	degrees of freedom
dNTP	Deoxynucleoside triphosphates
DREB	Dehydration Responsive Element Binding Protein
EDTA	ethylenediamine tetraacetic acid
EF1	Elongation factor 1
ESK1	Eskimo 1
GA	gibberellic acid
GC/MS	gas chromatography/mass spectrometry
HOS	High expression of OSmotically responsive gene
ICE1	Inducer of CBF Expression 1
KIN	Cold-induced
LTI	Low-Temperature Induced
MOPS	3-(N-morpholino)propanesulfonic acid
PCR	polymerase chain reaction
RD29A	Response to drought 29A
R_n	threshold value for quantitative realtime PCR fluorescence signal
ROS	reactive oxygen species

RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SSC	Sodium chloride-Sodium Citrate
TBE	Tris-Boric acid-EDTA
T-DNA	Transferred DNA
UDP	Uridine 5'-diphosphate
ZAT12	Zinc-finger protein ZAT12

9.0 Curriculum vitae

Personal particulars

Last Name:	Lee
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Educational background

2008 Doctoral dissertation at Friedrich Miescher Institute and Institute of Botany under supervision of Prof. Dr. Frederick Meins Jr (FMI) and Prof. Dr. Christian Körner (Institute of Botany, University of Basel). Members of the committee: Prof. Dr. Thomas Boller (Institute of Botany), and Dr. Yoshikuni Nagamine (FMI).

Dissertation title: Effects of chronic cold treatment on root elongation and gene expression in *Arabidopsis thaliana*.

2003 M. Sc. in Genetic engineering and molecular biology, Universiti Putra Malaysia, Serdang, Malaysia.

Dissertation title: Characterization of expressed genes isolated from oil palm vegetative, normal and abnormal inflorescence meristems using a EST approach.

2000 B. Sc. (Honours), in Plant biotechnology, Universiti Putra Malaysia, Serdang, Malaysia.

Dissertation title: Construction of a Transformation Vector Containing The No Apical Meristem (*NAM*) Gene and Development Of a Floral-Dip Transformation Procedure for Microtomato and *Arabidopsis thaliana.*

Honours and awards

Zürich-Basel Plant Science Doctoral Studentship funded by Syngenta, AG (2004-present).

SNSF postdoctoral fellowship for prospective researchers (2008 - 2009, provisionally awarded with conditions).

Poster presentations

Yang Ping Lee, Christian Körner and Fred Meins (2005) Cold Adaptation of Plant Growth and Development. Poster presentation at FMI Annual Meeting, Prontesina, Switzerland.

Yang Ping Lee, Christian Körner and Fred Meins (2006) Global Effects of Chronic Cold Treatment on Gene Expression. Poster presentation at FMI Annual Meeting, Murten, Switzerland.

Lee, Y.P., Fleming, A.J., Meins, F., and Körner Ch. (2007) Global effects of chronic cold treatment on gene expression. Zurich-Basel Plant Science Center Symposium. Plant Reproductive Systems. ETH, Zurich, Switzerland

Seminars

Yang Ping Lee (2005) Cold adaptation in plant growth and development. Oral presentation in Research Seminar in Plant Biology, Basel, Switzerland.

Yang Ping Lee (2006) Transcriptional programs of cold adaptation in Arabidopsis thaliana ecotypes. Oral presentation in Research Seminar in Plant Biology, Basel, Switzerland.

Yang Ping Lee (2007) Molecular responses of chronic cold treated *Arabidopsis*. Invited talk on Plant stress tolerance monthly seminar. Max-Planck-Institute of Molecular Plant Physiology, Potsdam, Germany.

Publications

Chai-Ling Ho, Yen-Yen Kwan, Mei-Chooi Choi, Sue-Sean Tee, Wai-Har Ng, Kok-Ang Lim, Yang-Ping Lee, Siew-Eng Ooi, Weng-Wah Lee, Jin-Ming Tee, Siang-Hee Tan, Harikrishna Kulaveerasingam, Sharifah Shahrul Rabiah Syed Alwee and Meilina Ong Abdullah. (2007). Analysis and functional annotation of expressed sequence tags (ESTs) from multiple tissues of oil palm (*Elaeis guineensis* Jacq.). ***BMC Genomics 8:381, doi:10.1186/1471-2164-8-381***.

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Yang Ping Lee, Andrew Fleming, Frederick Meins Jr, Christian Körner (2008) Global effects of chronic cold treatment on gene expression of two *Arabidopsis thaliana* accessions.