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Gene Expression Comparison by Microarray Analysis between Two *Triticum aestivum* Cultivars that Differ in Freezing Tolerance.

Simon Drouin

A Thesis

in

The Department

Of

Biology

Presented in Partial Fulfillment of the Requirements

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ABSTRACT

Gene Expression Comparison by Microarray Analysis between Two *Triticum aestivum* Cultivars that Differ in Freezing Tolerance.

Simon Drouin, Concordia University, 2004

Cold tolerance in plants is a complex trait that occurs in many plants during growth at low temperature, a process known as cold acclimation. This process is a multigenic system and there is broad variation in the range of freezing tolerance that can be achieved by different wheat cultivars. To identify genes that are regulated by cold treatment and to characterize their expression profiles a microarray was constructed with PCR amplified cDNA inserts from 1187 wheat Expressed Sequence Tags (ESTs) that represent 948 genes. Two *Triticum aestivum* L. cultivars, Norstar and Glenlea that differ in their capacity to develop freezing tolerance were used to compare the changes in gene expression during cold acclimation.

Microarray analysis is a powerful and rapid approach to gene discovery and for studying global gene expression. In species in which large scale mutant screening and transgenic studies are not currently practical, genotype comparison is an especially good approach to identify candidate genes for important agronomic traits.

The analysis reveals that the transcript levels of over 300 genes were altered by cold treatment. Among these, 65 genes were differently regulated in the freezing-tolerant and the less-freezing-tolerant cultivars on at least one time point. These genes encode for protein kinases, putative transcription factors, Ca^{2+} binding proteins, Golgi localized

protein, inorganic pyrophosphatase, cell wall associated hydrolase, 2-oxoglutarate/malate translocator, and aspartate-tRNA ligase.

DEDICATION AND ACKNOWLEDGEMENTS

I dedicate this thesis to my other (much better) half, Geneviève Lefebvre, to my mother, Johanne, my father, Pierre, my kid brother, Andrek, and my big brother Mirco.

I would like to acknowledge the scientific, moral and financial support provided by my supervisor, Dr. Patrick Gulick, with whom I had several years of fruitful research. I would also thank the members of my lab, past and present, most importantly my friends Fred Piot and Damiano Ferraro, which helped me retain my sanity during my masters' degree, and the "Hole-in-the-Wall" gang.

I cannot write an acknowledgement page without mentioning the invaluable moral support provided by the McKibbins Pub, more specifically by the barmaid of the "Hole-in-the-Wall" gang for the last three years, Jennifer.

TABLE OF CONTENTS

1.0 LIST OF FIGURES

2.0 LIST OF TABLES

LIST OF ABBREVIATIONS USED

Abbreviations for Units of Measure

BLAST	Basic Local Alignment Search Tool
Cy-3	cyanine-3
Cy-5	cyanine-5
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ELIP	early light-induced protein
FT	freezing tolerance
LT	low temperature tolerance
LT_{50}	lethal temperature for 50% of the plants
MIP	membrane intrinsic protein
NIP	NOD26-like membrane intrinsic protein
PIP	plasma membrane intrinsic protein
PS	photosynthesis
PSI	photosystem I
PSII	photosystem II
RT	reverse transcription/transcriptase
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
TIGR	The Institute for Genomic Research
TIP	tonoplast intrinsic protein

3.0 INTRODUCTION

Low temperature (LT) is a major cause of crop damage, and the ability of plants to develop freezing tolerance (FT) during cold acclimation is a determining factor in their survival in temperate environments. Exposure of plants to LT produces morphological, biochemical, and physiological changes that are often highly correlated with plant FT and winter survival. There is a great deal of genetic variation both between and within species for the process of cold acclimation and in the ultimate degree of FT that can be achieved. Previous molecular and genetic analyses have demonstrated differential gene expression and the increased accumulation of several proteins during cold acclimation in several species. These proteins could be classified into three groups based on the presumed function: structural proteins that might be involved in protecting the cell during LT stress, enzymes involved in the biosynthesis of different osmoprotectants, in the desaturation of lipids and in the antioxidative response, and regulatory proteins that control the mechanisms of LT response (Breton et al. 2000 and references therein). The phenotypic expression of cold tolerance is controlled by a large number of genes with complex interactions. The complexity of the LT response creates a challenge to differentiate genes responsible for LT acclimation and cold hardiness from those associated with metabolic adjustment to LT. Progress in understanding the genetic regulation of this multigenic trait has been slow. Identification of the genes contributing to FT and understanding how they are regulated is important in establishing the appropriate strategy to improve FT in crop species. To achieve this goal, global genome wide approaches to identify the genetic components involved in this multigenic trait are essential.

3.1 Microarrays in Plant Stress Response Biology

Microarray technology has become one of the important tools in biological research. Northern blots, which measure gene expression, rely on the specific hybridization of a labeled nucleic acid molecule (the probe) to its complementary sequence (the target) out of a membrane-bound complex mixture. However, this method is best suited to the study of a few genes, since specific probes and individual blots have to be prepared for every few genes that are analyzed. The reverse strategy is used in microarray-based experimental design. The probes, which are usually oligonucleotides or PCR amplified cDNA clones, are fixed to a solid support and the target cDNA mixture derived from a mRNA population that is isolated from tissues of interest is labeled and hybridized to the probes on the solid support. The probes can be produced by high-throughput methods and printed on the microarray at high density. This allows the experimenter to measure relative abundance for the mRNAs of a multitude of genes in a single experiment. The ability to label different target samples with fluorophores possessing different emission and excitation characteristics allows for simultaneous hybridization with two target populations and thus direct comparison of the relative levels of gene expression in the two samples. A typical experiment is to compare a control sample to a treatment sample to detect gene induction or repression in response to the treatment. The expression levels for each probe are usually expressed as the ratio of the experimental sample intensity over the control sample intensity. The representation of gene expression in terms or ratios rather than absolute values alleviates the problems associated with variability in the DNA concentration or the GC content of the individual probe spots. Dyes sometimes affect the hybridization efficiency of some targets. To control for these effects, replicate hybridizations are done as reverse-labeling

experiments, in which the dyes used for labeling the control and experimental samples are swapped. If the dyes have identical effects on the hybridization efficiencies of both samples, the ratios of intensity of treatment compared to the control will be the same as that observed in the "normal" experiment.

There are two widely used methods to microarray fabrication. The first, which we used in our experiments, is the spotting and cross-linking of specific PCR products to a glass support, usually referred to as cDNA microarrays. The second method, mostly used by Affymetrix, is the *in-situ* synthesis of oligonucleotides by photolithography. Both methods have their advantages and disadvantages. PCR-based microarrays are more flexible, cheaper to produce and more widely accessible to the average researcher than commercially produced oligonucloetide microarrays. Production of oligonucleotide-based arrays also requires the prior knowledge of the sequences to be spotted, whereas PCR-based arrays do not, though it is customary to use cDNA clones for which EST sequences are available. Oligonucleotide-based arrays are therefore more ideally suited to model organisms where the genomic sequence is known for which a large EST database is available.

The large size DNA amplicon, normally several hundred bp to a few kb in length, which is normally used for printing cDNA arrays, may hybridize to several closely related members of a gene family. Also, cDNA arrays are not ideally suited to study splice variants, as these kinds of studies require the generation of exon-specific sequences. These two problems are reduced in oligonucleotide-based arrays, where it is possible to produce oligonucleotides specific to individual gene family members, or specific to different intron splice junctions when there is sufficient sequence information available to identify all members of a gene family and where mRNA splice variants are

known. The relatively high cost of commercial oligonucleotide microarrays is compensated for by the large number of genes that are represented on recent versions of these arrays, which substantially lowers the cost of analysis on a per gene basis.

Plant biologists are now using microarrays extensively to study gene expression in plants subjected to different abiotic stresses. Of particular interest are studies investigating plant expression profiles in response to drought, salt and cold stresses. Seki et al. (2001) used a 1300 gene cDNA *Arabidopsis thaliana* microarray to characterize changes in gene expression in response to cold and drought, and found that the majority of genes with changed expression are affected by both stresses. However, in another comparison of drought, cold and salt stress, this time using Affymetrix *A.thaliana* oligonucleotide microarrays, Kreps et al. (2002) report that approximately 30% of the genome is regulated by at least one of these stress conditions, but that the majority of the gene expression changes are specific to individual stress conditions. They found that only about 5% of the gene expression changes are common to the three stress conditions at the acute phase (3h), and less than 0.5% by 27h exposure. Seki et al. (2002) confirmed the Kreps et al. results using a 7000 gene *A.thaliana* cDNA when they found only 22 genes common to cold, drought and salt stress conditions, out of 524 genes which had their expression changed in at least one of these stress conditions. Other microarray studies of environmental stress in plants include the characterization of salt shock over a time course in maize roots (Wang et al., 2003), salt shock in rice (Kawasaki et al., 2001), drought and salt stress in barley (Ozturk et al., 2002), high light conditions (Rossel et al., 2002), oxidative stress (Desikan et al., 2001), recovery from dehydration (Oono et al., 2003), and response to UV radiation in maize (Casati and Wolbot, 2003).

3.2 Photosynthesis, Photoinhibition, and Cold Stress

Upon exposure to low temperatures, plants show an immediate partial inhibition of photosynthesis and of the carbon fixation metabolic pathway. This inhibition leads to the accumulation of phosphorylated intermediates, depletion of the phosphate pool, and the depletion of the complex carbohydrate pool (Hurry et al., 2000; Leonardos et al., 2003). In cold-hardy species, new leaves grown under cold temperatures seemingly overcome this inhibition using three mechanisms. First, the activity of the Calvin cycle enzymes is increased to compensate for the lower rate of synthesis. Second, the cell readjusts the pool of phosphates available by maintaining the cellular phosphate concentration constant, and drawing phosphate from the vacuoles if needed. Last, there is an upregulation of the genes encoding members of the sucrose synthesis pathway, and a downregulation of the starch anabolism genes (Hurry et al., 2000; Strand et al., 1997; Strand et al., 2003). Sucrose is an important cryoprotectant, protecting the cell against freezing. Sucrose is also an important energy source, and is a precursor of other cryoprotective compounds such as proline and glycine betaine (Hurry et al., 2000).

The sustained decrease of photosynthetic efficiency observed when plants are subjected to excessive light pressure conditions is described as photoinhibition. This phenomenon can be the result of either the irreversible inhibition of the PSII complex due to damage to its D1 reaction center, or to the reversible inhibition of the PSII complex caused by non-photochemical energy dissipation (Gray et al., 2003).

Warm-grown plants shifted to cold temperature conditions (5°C) exhibit photoinhibition. The light harvesting and O_2 generation mechanisms are temperature independent, while low temperature conditions slow down the carbon fixation pathway kinetics (Hurry et al., 2000). The excess level of light excitation can lead to oxidative

damage due to higher level of unutilized electrons. These excess electrons can damage photosystem subunits, particularly the PSII D1 reaction center, leading to an increased production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxyde (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$). ROS can severely damage DNA, proteins, and lipids in a cell, and can lead to premature cell death.

Plants have several physiological responses to excess light pressure. First, a fraction of the PSII complexes switch from their dimeric form to a monomeric form. The monomeric form decreases the absorption cross-section of PSII, lowering the amount of gathered light energy. Photoinactivated PSII complexes might also be involved by dissipating light energy as heat (Ivanov et al., 2003). The ROS load in the chloroplast is reduced through the xanthophyll epoxidation pathway. Xanthophylls are carotenoids that can quench triplet chlorophyll and singlet oxygen species through reversible deepoxidations. They are known to be associated with the light harvesting complexes of both photosystem I and photosystem II (Król et al., 1999). The diepoxide violaxanthin is converted to antheraxanthin, which is then further deepoxidated to zeaxanthin. These reversible reactions are catalyzed by the enzyme violaxanthin depoxidase. It is also suggested that zeaxanthin might protect the chloroplast lipid membrane against peroxidation by directly scavenging ROS. Cereals show increased xanthophyll pools when overwintering which further supports the concept of xanthophylls as a means to dissipate excess energy. The early light-inducible proteins (ELIP) are known to be induced in response to cold treatment (Adamska and Kloppstech, 1994). It has been suggested that ELIPs may be zeaxanthin-binding proteins that protect the photosynthetic apparatus from over-excitation induced by low temperature.

Finally, plants can reduce the light pressure by blocking photons before they get to the chlorophyll antennae. The accumulation of anthocyanins in vegetative tissues is known to correlate with low-temperature conditions and high light pressure conditions. Anthocyanins are red pigments that preferentially absorb the green and ultraviolet wavelengths of light, while still absorbing the blue wavelength at a lesser level. The key enzyme in anthocyanin biosynthesis is chalcone synthase. This enzyme controls a metabolic bottleneck for the formation of various flavonoid precursors, including those for anthocyanins. Chalcone synthase is known to be upregulated by increased concentrations of sucrose present in the plant cell during cold acclimation.

3.3 Aquaporins and Cold Acclimation

Water transport across the lipid bilayer was thought to be uniquely driven by diffusion through the lipid-bilayer of the plasma membrane until the discovery that mercuric compounds inhibit water transport in human erythrocytes. Further studies in the late 1980s have shown the existence of membrane intrinsic proteins (MIPs) that form transmembrane pores. These proteins facilitate the passive movement of water across lipid membranes, and are thus dubbed aquaporins (Baiges et al., 2002). About 10 aquaporins have been identified in mammals, whereas *Arabidopsis thaliana* expresses about 35 different aquaporins, and maize 31 (Quigley et al., 2001).

Aquaporins fall under different categories corresponding to their amino acid composition, length of the N- and C-terminus tails and the presence of tracts of specific conserved amino acids. There are four subgroups of MIPs: plasma membrane intrinsic proteins (PIPs), tonoplast instrinsic proteins (TIPs), NOD26-like MIPs (NIPs) and Small and basic intrinsic proteins (SIPs). MIP proteins represent a large part of the protein

content of lipid membranes. The PIP1 subfamily, composed of 5 genes in *A. thaliana*, represents at least 1% of the total protein content in *A. thaliana* membranes. PIP2 family members were found to represent up to 15% of the total membrane protein content in spinach leaves (Baiges et al., 2002; Li et al., 2000; Quigley et al., 2001; Terashima and Ono, 2002). Experiments have shown that some MIPs can carry urea, glycerol, and carbon dioxide across membranes in addition to water. Some MIPs even exclude water and are specific to other compounds (Aharon et al., 2003; Quigley et al., 2001).

The importance of MIPs in cold acclimation and freezing tolerance is still unclear. It has been shown that the overexpression of the PIP1b protein in *Nicotiana tabacum* grown under normal conditions is beneficial to the plant. However, no growth advantage was observed in salt stressed plants, and a deleterious effect was observed for drought stressed plants (Aharon et al., 2003). Other salt stress experiments conducted in barley have shown both induction and repression for different PIP1 and PIP2 family members (Katsuhara et al., 2002). Javot et al. (2002) have reported that osmotic stress inhibits expression of the PIP1 gene subfamily

3.4 Objectives

Wheat cultivars have a remarkable range of FT and are thus an excellent model to study this trait. Several LT responsive genes have been identified in wheat by screening for genes with differential expression. (Houde et al. 1992; Chauvin et al. 1993; Danyluk et al. 1994; Danyluk et al. 1996; Danyluk 1997; N'Dong et al 2002; Breton et al. 2003). However, these methods are limited, time consuming and do not give the global view of coordinated expression and the interactions among the expressed genes. To accelerate the pace of discovery, 1200 EST sequences were generated from clones identified from a

cDNA library of cold acclimated winter wheat and used to construct a cDNA amplicon based microarray. The arrays were used to monitor gene expression in two cultivars that have different degrees of FT, the winter wheat Norstar with an LT_{50} (lethal temperature that kills 50% of cold acclimated seedlings) of -19°C and the spring wheat Glenlea with an LT_{50} of -8°C. This genotypic comparison helps to differentiate between genes associated with the development of FT from those associated with LT stress. Significant changes in the expression pattern of 327 genes were detected over the course of 36 days of cold acclimation. Comparisons between the two cultivars showed that 65 of these were differently regulated in the tolerant and the less tolerant cultivar. Of these, 36 encode proteins with predicted function and 17 genes encode proteins of unknown function that had not previously been identified as cold regulated genes. Novel cold regulated genes include those encoding protein kinases, putative transcription factors, Ca^{2+} binding proteins, inorganic pyrophosphatase, cell wall associated hydrolase, and a 2-oxoglutarate/malate translocator.

3.5 Contribution of the Author

This project was done in collaboration with Dr. Fathey Sahran's laboratory at UQAM, Guylaine Poisson, also from UQAM, and Dr. Patrick Gulick from Concordia University. Dr. Sahran's laboratory's contributions are the following: plant growth, RNA extraction, cDNA library construction, virtual subtraction. Guylaine Poisson's contribution was the statistical analysis of the data using the GeneSpring software package. Dr. Gulick's contribution was in the analysis of the results and the writing of the manuscript. The author's contribution to the project was such: EST functional assignments, microarray design and construction, microarray hybridizations, analysis of

the results, clustering. The author also contributed significantly to the writing of the manuscript. This project has been submitted for publication.

4.0 MATERIALS AND METHODS

N.B. All work was performed by Simon Drouin, unless otherwise noted.

4.1 Plant Material and Growth Conditions

This part was done by Dr. Fathey Sahran's laboratory at UQAM.

The spring wheat *Triticum aestivum* L. cv Glenlea, LT_{50} -8°C, and the winter wheat *T. aestivum* L. cv Norstar, LT_{50} - 19°C, were used in this study. Seeds were germinated in moist sterilized vermiculite for five days in the dark and two days under artificial light. Cool-white fluorescent and incandescent lighting was combined to provide an irradiance of 250μmol $m^{-2}s^{-1}$. The temperature was maintained at 25 ± 1°C (15h photoperiod) during the day and 20 ± 1°C during the night. The relative humidity was 70 ± 5%. Seedlings were watered daily with a nutrient solution (0.5g/L of 20:20:20; N:P:K). At the end of this period, control plants were maintained under the same conditions of light and temperature for and additional one and six days. Cold acclimation was performed by subjecting germinated seedlings to a temperature of 6 ± 1°C during the day (10h photoperiod and 175μmol $m^{-2}s^{-1}$) and 2 ± 1°C during the night for one, six and 36 days. Based on seedling dry weight, the control seedlings of eight and 13 days (one and six days beyond the seven days of germination) have the same physiological age as seedlings that were cold acclimated for six and 36 days (beyond the seven days of germination), respectively. Gene induction in plants cold acclimated for one and six days was measured by comparison to eight day old control plants (referred to as Control one day) and plants cold treated for 36 days were compared to 13 day old control plants

(referred to as Control six days). Plants were harvested three hours before the end of the day cycle.

4.2 cDNA Library Construction

This part was performed in Dr. Fathey Sahran's laboratory at UQAM.

In brief, poly $(A)^+$ RNA from one day cold acclimated wheat cultivar Norstar was purified using an oligo dT-cellulose batch procedure (Danyluk and Sarhan, 1990). Virtual subtraction was carried out to enrich for cDNAs from low abundance mRNAs. Colonies containing plasmids with inserts were picked and replicated onto nylon membranes (HybondN, Amersham Pharmacia).

First strand cDNA from one day cold acclimated Norstar was labeled with digoxigenin, and hybridization and detection were done. Approximately 30% of colonies showing the lowest hybridization signal were chosen for sequencing.

4.3 EST Sequencing and Annotation

EST sequences were obtained from plasmids containing inserts from randomly selected and virtual subtraction colonies. The ESTs were generated by single-pass sequencing of the cDNA inserts on a Beckman CEQ2000 automated sequencer (Center for Structural and Functional Genomics, Concordia University). The quality of EST sequences was assessed using PHRED/PHRAP, and vector and low sequences and low quality regions were trimmed using LUCY (TIGR). The EST sequences were queried against the TIGR gene indices using Wheat Version 6.0, Barley Version 6.0, Rice Version 12.0, and Arabidopsis Version 10.0 indices and annotated using the TIGR descriptions. Some additional annotation of differentially expressed genes was taken

from GenBank "nr" data set. Independent members of gene families were scored as individual genes, and thus some independent clones on the microarray may have similar annotations.

4.4 cDNA Microarray

The cDNA insert for each EST was amplified from the plasmids in two 50μL reactions using a protocol of Hegde et al. (2000) with some modifications. The 50μL reaction mix contained 75mM Tris-HCl (pH 8.8), 20mM $(NH_4)_2SO_4$, 0.01% Tween-20, 2.5mM $MgCl_2$, 0.2μM Forward-New primer, 0.2μM Reverse-New primer, 2mM each of dATP, dTTP, dCTP, dGTP, and 1.5U of Taq DNA polymerase (MBI Fermentas). Nanopure water was used in all preparations. The vector specific primer sequences were: Forward-New, CGACTCACTATAGGGCGAATTGGGTA, and Reverse-New, CCTCACTAAAGGGAACAAAAGCTGGA. The cycling conditions started with two minutes of denaturation at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 52°C, three minutes at 72°C. The cycling was concluded by a 7:30 minutes at 72°C. To avoid clone identification errors, PCR amplification products were maintained in the same order as was used in the 96 well plates used for EST sequencing.

Quantification and quality of the PCR product was assessed by electrophoresis of a 6μL aliquot in a 1.5% agarose gel at 4V/cm until the dye front had migrated ¾ of the length of the gel. The MBI-Fermentas 1kb DNA ladder (#SM0313) was used as a standard for molecular weight and quantity. Visualization and quantification were done semi-automatically with a Syngene GeneGnome imager. Samples with multiple bands were flagged and were not used in further analyses. Insert sizes, previously determined

by plasmid digestion, were confirmed. PCR products that did not correspond to their expected sizes were also flagged and not used in further analyses.

The duplicate PCR products of two 50µL reactions were combined and purified using the Millipore 96-well multiscreen filter plates following the protocol detailed in Hegde P. *et al.* (2000). The PCR products were lyophilized in a Speed-Vac and resuspended in 10µl Nanopure water. Aliquots of 5µL were transferred to 384-well plates and mixed with an equal volume of a 90% (v/v) DMSO, 100mM KCl, and 40mM Tris-Cl (pH 6.5) solution.

4.5 Microarray Spotting

Microarrays were produced by spotting the PCR products on Corning CMT-GAPS 2 or UltraGAPS glass slides using a Virtek arrayer robot equipped with Telechem Stealth II quill pins in a 24-pins configuration. The arrayer used default parameters, except that "dwell time" was 0.3 second and "vertical speed" was 0.5cm/sec. The relative humidity inside the robot enclosure was kept at 45% during the spotting process. The spots were spaced on a 0.3mm grid; each product was spotted four times, two spots side by side, in two locations. The array included several previously characterized cold regulated genes from *T. aestivum*. After spotting, slides were baked at 80°C for two hours and UV cross-linked at 120mJ using a Stratalinker (Strategene). Processed slides were stored in a light-tight microscope slide storage box until they were used.

4.6 Target Preparation

Two independent reactions were done for each labeled target sample. For reverse transcription, 4µg of poly-T primer (18-20mer) were added to 10µg of total RNA in a

total volume of 14.5μL, heated at 70°C for 10 minutes, centrifuged briefly, and cooled at room temperature for 10 minutes. A 14.5μL aliquot of master mix was added to achieve final concentrations of 1x Superscript II RT buffer (Life Technologies), 0.01M dithiothreitol (DTT), 1.7mM dATP, dCTP, dGTP, 1mM dTTP, 0.69mM aminoallyl-dUTP (Sigma), and 400U SuperScript II RT. The reaction mixture was incubated at 42°C for four to 16 hours. The product was treated with 10μg of RNAse A and 5U of RNAse H at 37°C for 30 minutes.

To remove unincorporated aa-dUTP and free amines, the cDNA reaction was mixed with five volumes of PB buffer and purified on a QIAquick column (Qiagen) according to the manufacturer's specifications, excepted that 750μl of phosphate wash buffer (5mM KPO_4 (pH 8.5), 80% ethanol, in Nanopure water) was used in place of PE buffer. The wash was done twice. Also, 4mM KPO_4 (pH 8.5) was used as elution buffer in place of EB buffer. The elution was done twice, each with 30μl of KPO_4, and the sample was lyophilized.

For the target labeling reactions, one fresh tube each of Cy-3 and Cy-5 monoreactive ester dye (Amersham Pharmacia #PA23001 (Cy-3), #PA25001 (Cy-5)) was dissolved in 73μL DMSO. The lyophilized cDNA was dissolved in 4.5μl of fresh 0.1M Na_2CO_3 (pH 9.0), 4.5μL of the appropriate Cy-dye solution was added and the reaction was incubated for one hour in the dark. The reaction was quenched with 4.5μL of 4M hydroxylamine, incubated 15 minutes in the dark, and then 35μl of 100mM sodium acetate (pH 5.2) were added. Samples were purified on QIAquick columns (Qiagen) according to the manufacturer's protocol and using Qiagen PE and EB buffers. At the final elution step, pairs of columns containing the same cDNA sample, each originating from 10μg of total RNA, labeled with the same Cy-dye were eluted into the same tube,

yielding a final volume of approximately 120µl. The eluates were lyophilized and stored in the dark at -20°C.

4.7 Microarray Pre-Hybridization

Microarrays were pre-hybridized with 40µL aliquot of a solution composed of DIG Easy Hybridization buffer (Roche), 5µg/µL salmon sperm DNA, 0.5µg/µL yeast tRNA, and 11µg/µL bovine serum albumin (BSA) at 42°C under Hybrislip coverslips (Grace Biolabs) for approximately 45 minutes. The slides were washed by total immersion seven times in Nanopure water, twice in isopropanol, and dried under a flow of filtered compressed air. The washed slides were hybridized within the hour.

4.8 Microarray Hybridization

All incubations were done in the dark and exposure to light was minimized during manipulations. The Cy-3 and Cy-5 labeled lyophilized cDNA were dissolved in 35µL of DIG Easy Hybridization buffer, (Roche), 0.5µg/µL salmon sperm DNA, and 0.5µg/µL yeast tRNA, heated at 95°C for three minutes, centrifuged at 10,000 x g for two minutes, and kept in a 42°C water bath until use. A 30µL aliquot of hybridization solution was pipetted to the centre of a Hybrislip coverslip (Grace Biolabs) and the micorarray slide was gently placed on the drop, array face down, taking care not to produce air bubbles. The slides were immediately placed in an air- and light-tight hybridization chamber containing four distilled water-saturated pieces of Whatman paper, and incubated at 42°C overnight (approximately 16 hours).

4.9 Post-Hybridization Processing

The slide was dipped in low-stringency buffer (1x SSC, 0.2% SDS) preheated to 42°C. The slide was slightly shaken in solution to remove the coverslip. All washing steps were done in a glass microscope slide staining dish made light-tight with aluminum foil wrapping. The slides were washed with gentle agitation in 42°C 1x SSC, 0.2% SDS for 10 minutes, twice in 42°C 0.1x SSC, 0.2% SDS for 10 minutes, once in room temperature 1x SSC for 8 minutes and once in room temperature Nanopure water for five minutes. For each pair of comparisons three to six replicate hybridizations were carried out. To dry them, the slides were held with the spotted side facing up, and dried using filtered compressed air with the air flow towards the bar-code sticker. The slides were visually inspected and the wash procedure was repeated from the 1x SSC wash step if they were deemed unclean. The slides were stored in a light-tight microscope storage slide until they were scanned.

For each pair of comparisons three to six replicate hybridizations were carried out. For each experiment, at least one dye-swap slide was done in which the labeling of control and treatment target cDNA with Cy-3 and Cy-5 were reversed.

4.10 Microarray Scanning

The slides were scanned on an Axon 4000b scanner at a 10μm resolution. Each line was scanned twice and averaged. The laser power was set at 100% for both excitation wavelengths (532nm and 635nm). The photomultiplier tube voltages were tuned to balance the signal from both Cy-3 and Cy-5 dyes as much as possible. The images were saved as multi-channel TIFF files and the images were analyzed using the GenePix 3.0 software (Axon Instruments). Low quality spots including misshapen spots

and those obscured by dust motes, scratches, buffer spots were flagged and subsequently disregarded in the analysis.

4.11 Statistical Analysis

This part was done by Guylaine Poisson, UQAM.

The statistical analysis was done using the GeneSpring software package version 5.0.3 (Silicon Genetics, Redwood City, CA, USA). The four replicate spots on each slide were averaged. The dye-swap normalization method, on a per spot basis, was first applied to applicable experiments, and all slides were normalized, on a per chip basis, using the Lowess regression method. Variances were calculated using cross-gene error model and on-chip replicates. The multiple testing correction model used the Benjamini and Hochberg False Discovery Rate model. A gene was judged to have a significant induction or repression of expression for a treatment when: (1) it had P-value < 0.05 when tested against the hypothesis of no treatment effect (Treatment / Control = 1), and (2) a 50% or greater increase or decrease in expression (i.e. T/C above 1.5 or below 0.66). Genes differentially expressed in the winter wheat, Norstar, and the spring wheat, Glenlea, cultivars were detected in two ways: (1) with the hypothesis that there was no difference in gene induction in the two cultivars for a particular cold treatment (i.e. the expression ratio for cold treated winter wheat/control winter wheat plants = the expression ratio for cold treated spring wheat/control spring wheat plants (WT / WC = ST / SC)), and (2) by direct comparison of target cDNAs from the two cultivars with the same cold treatments tested with the hypothesis that there were no differences in expression in the two cultivars (WT/ST = 1). The relative level of gene expression in the control plants was not measured directly, but it was calculated as follows:

Norstar Control d1 /Glenlea Control d1 =

(Norstar Expression d 6 / Glenlea Expression d 6) x (Glenlea Induction d 6 / Norstar Induction d 6) =

(Norstar Expression d 6 / Glenlea Expression d 6) x ((Glenlea Expression d 6 / Glenlea Control d 1) / (Norstar Expression d 6 / Norstar Control d 1))

Comparison of gene expression of the six day control plants was calculated similarly, using the combination of direct comparison of plants cold acclimated for 36 days and the measure of gene induction in each cultivar at 36 days.

4.12 Clustering Analysis

Clustering analysis was performed using the Genesis software (Sturn, et al., 2002). The analysis included genes which had altered expression in at least one time point and that had valid data for all time points in at least one cultivar (e.g. Norstar cultivar, Control 1d vs Treatment 1d, C1d vs T6d, C6d vs T36d all had to be present and valid). The analysis included 215, and 136 genes from the winter and spring data sets, respectively; 128 of the genes were common to both data sets. Expression values for the same gene measured in the two cultivars were entered into the analysis in order to detect pattern similarity and differences between the two cultivars. Average-linkage hierarchical clustering was used, using Euclidian distances.

5.0 RESULTS AND DISCUSSION

Cold acclimation and development of FT are multigenic traits that allow plants to tolerate extreme winter conditions. These mechanisms are genetically controlled and induced upon exposure to LT. It has been shown in *Arabidopsis* that about 30% of the genome may be regulated at the level of transcript abundance by abiotic stresses and approximately 25% of the genome has changes in mRNA levels of two-fold or more when plants are just cold treated (Kreps et al. 2002). In a large genome such as that of hexaploid wheat, whose diploid compliment of genes likely similar to the estimated 32,000 to 52,000 genes of rice (Goff et al., 2002, Yu et al., 2002), the identification and function analysis of this large number of genes is a challenging task and requires the development of high throughput analysis. Microarray analysis is a powerful tool for global analysis of gene expression through the measurement of changes in specific transcript levels. Though significant regulation can occur at other levels, including translation and protein activation, transcription profiling offers a key insight into a plant's response to LT treatment that is amenable to global analysis. Comparative gene expression analysis between tolerant and less tolerant cultivars is a means to identify and prioritize key candidate genes that may be investigated in depth by other means. The expression profiling of large number of LT-regulated genes in plants makes it possible to identify classes of genes with similar or related biochemical function, patterns of expression mediated by common regulatory pathways, and most importantly to distinguish patterns of expression that are specific to cultivars that have superior FT.

5.1 EST Annotation and Microarray Design

EST sequences were generated by single-pass sequencing of cDNA clones from a cDNA library made from crown and shoots of LT treated wheat seedlings. Annotation for the wheat ESTs was derived from BLAST queries against the TIGR gene indices for wheat, barley, rice and *Arabidopsis* or from the GenBank "nr" database. Out of 1187 high quality ESTs, 783 were assigned a functional description derived from BLAST queries against TIGR gene indices, and 386 ESTs with high scoring blast hits (E-value below $1x10^{-10}$) in the TIGR databases were classified as either unknown, hypothetical, putative, ESTs or ORFs, or without functional descriptions. Only 18 ESTs had no hit with an E-value below $1x10^{-10}$. Contig assembly within our EST set and with wheat tentative contigs (TCs) in the TIGR database that had identity above 96% with our sequences indicated our EST collection contains 801 singletons, while 383 ESTs were clustered into 147 contigs, giving a unigene set of 948 genes. We constructed a wheat microarray with PCR amplified cDNA clone inserts from 1187 ESTs and conducted large-scale comparison of gene expression in two wheat cultivars that differ in their capacity to develop FT. This approach facilitates differentiation between genes associated with general LT response from those related to the development of superior FT.

5.2 Gene induction and repression in response to cold treatment

Gene expression was measured over a time course of cold acclimation in two *T. aestivum* cultivars that have a marked difference in their degree of FT. The winter wheat Norstar achieves an LT_{50} of -19°C after 36 days of growth at 4°C whereas the spring wheat Glenlea has an LT_{50} of only -8°C under the same growth conditions. Microarray analysis was used to compare changes in gene expression profiles during cold

acclimation in the two cultivars by monitoring transcript levels after one, six, and 36 days of growth at LT. Two approaches were used: (1) the measurement of gene induction and repression as detected by microarrays hybridized to pairs of first-strand target cDNAs derived from cold acclimated and non-acclimated control plant total RNAs, and (2) direct comparison of pairs of labeled target cDNAs derived from Norstar and Glenlea that had been cold acclimated for six or 36 days. The experimental design, shown in Figure 1, allowed a thorough comparison of the gene expression profiles of the two cultivars. Direct comparison of mRNA populations from one cultivar taken at two time points or samples from cultivars taken at the same time point of the treatment was used to derive most expression data, but the design allowed additional data to be deduced by calculation. For example, ratio of expression levels in control plants of the two cultivars was calculated from ratio of gene induction between one and six days in the two cultivars multiplied by the ratio of expression between the two cultivars measured at six days of cold acclimation.

Genes were classified as having increased or decreased in expression in response to LT if the ratio of treatment and control (T/C) signals were significantly different from one, with $P < 0.05$. There were 337 genes that had induction or repression levels that were statistically significant and with changes of 50% or more on at least one time point. Detailed data for these genes are summarized in Table 1 and listed in Table 2. The use or choice of threshold values is somewhat debatable since even small changes in gene expression may be biologically important, especially for regulatory genes. The choice of higher thresholds used elsewhere is influenced by higher levels of statistical confidence associated with larger changes in RNA signals; however there are exceptions to the trend

to have lower P-values associated with larger changes in gene expression. The 1.5 and 0.66 threshold values for data presented in Table 1 were chosen

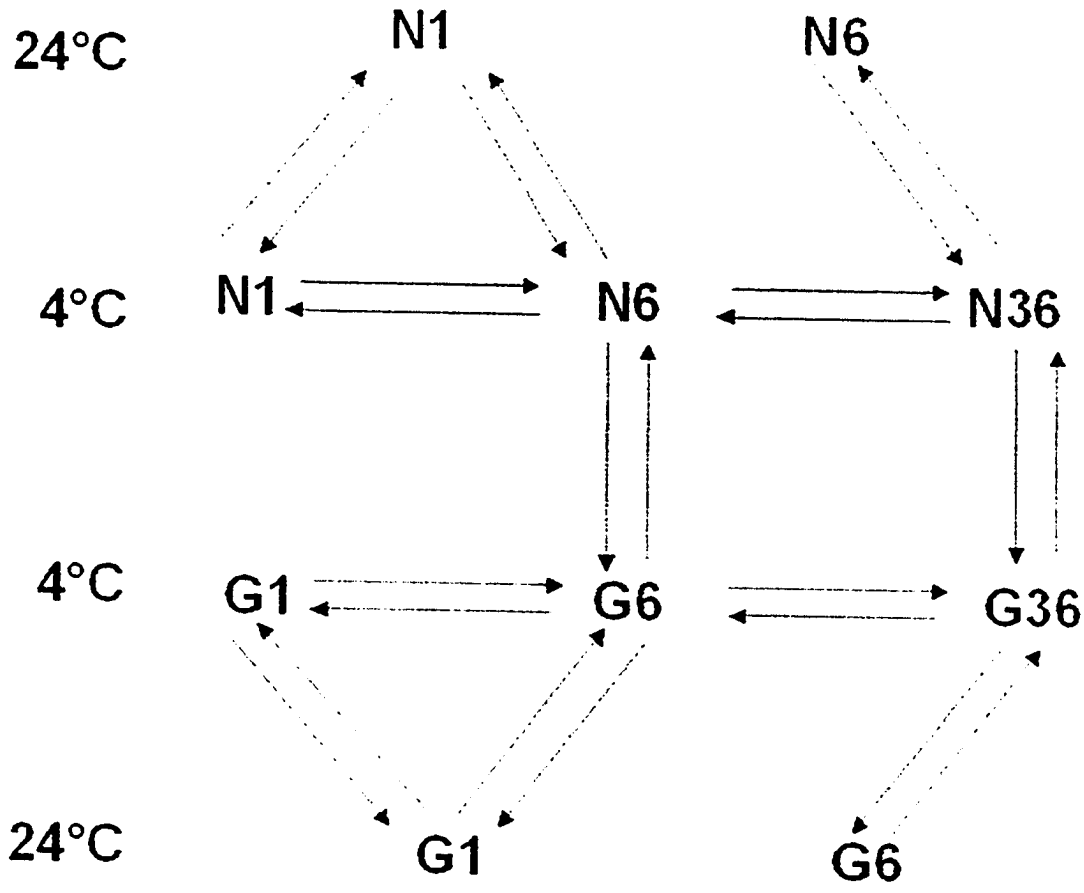


Figure 1. Experimental design. The experimental design is based on the series of pairwise comparisons. Notation: N – winter wheat cultivar, Norstar; G - the spring wheat cultivar, Glenlea; numbers indicate the number of days plants were grown at the indicated experimental temperatures. Before day one of the experiment, all plants had been germinated and grown for seven days at 24°C.

Days of Treatment	*Upregulated Genes*		*Downregulated Genes*	
	Norstar	Glenlea	Norstar	Glenlea
1	74	44	78	32
6	90	66	115	60
36	57	45	77	27

Table 1. Number of Genes with Changes in Expression 1.5-fold or more and P-Value < 0.05 in Winter and Spring Wheat after One, Six, and 36 Days of Cold Acclimation.

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
A02_F	0.45	0.025541	1.95	0.0011	1.47	0.187836	0.64	0.109664	0.75	0.017634	2.83	0.028914	0.88	0.71574	0.74	0.585992	TC70414	cold regulated protein
A04_F	0.91	0.522746	0.82	0.18082	0.97	0.860685			0.68	0.002505	0.34	7.63E-05	0.95	0.73118	0.33	0.000277	TC64810	ribuso small subunit
A17_F			0.99	0.96924	1.14	0.580027	0.64	0.031255	0.60	0.000902							TC69081	ribosomal protein L3
A75_F			1.62	1.7E-05	1.07	0.687256	1.18	0.330565	2.09	8.8E-09	1.19	0.246175	1.29	0.14132			TC85062	cyclophilin A-3
B17_F			1.01	0.97196	1.05	0.827366	0.72	0.583461	0.62	0.00072	0.97	0.780617					TC63170	catalase 2
B20_F			0.70	0.07001	1.26	0.458041	0.82	0.270161	0.67	0.005149			1.14	0.63857			TC88655	hypothetical protein
B24_F	2.36	0.000388	3.50	0.00012	5.75	0.001774	2.32	6.98E-05	2.88	6.18E-08	0.77	0.156585	0.84	0.24528			TC86051	chlorophyll a/b-binding protein (WCAB)
B29_F			1.20	0.31012	1.06	0.825405			0.64	0.000341							TC66944	hypothetical protein
B34_F	1.05	0.795955	1.06	0.64391	2.26	0.018435	1.03	0.866275	1.21	0.219309	0.57	0.000169	0.92	0.494			TC83306	chlorophyll a/b binding protein (CP29)
B59_F			2.05	0.00015	1.24	0.558062	3.09	0.115785	2.36	6.34E-06			0.79	0.0872			TC88664	Mg-chelatase subunit (XANTHA-F)
B60_F			1.16	0.24581	0.97	0.807531	2.26	0.00571	2.09	2.95E-06	1.16	0.125529	1.32	0.5217			TC67796	cold acclimation protein (WCOR825)
C02	0.57	0.008195					0.62	0.031685			0.78	0.184398	0.95	0.84121			TC65757	ascorbate peroxidase
C03			1.12	0.40494	0.95	0.820067			0.63	0.000178							TC65467	ribosomal protein S16
C09	0.63	0.041918	0.77	0.05623	0.87	0.448627	0.53	0.013456	0.51	7.97E-07	0.87	0.256487	0.84	0.13706	0.64	0.033639	BU979312	expressed protein
C103	3.44	0.001549	1.73	0.00835	1.26	0.129513	3.20	0.006777	2.35	6.01E-07	2.89	0.027985	1.47	0.02361			BQ608834	hypothetical protein
C105	3.59	1.43E-05	3.91	2.4E-05	2.37	0.000046	3.40	3.16E-06	3.13	5.56E-12	2.41	2.12E-06	1.12	0.41996	1.28	0.411464	TC88565	cold acclimation protein (WCOR615)
C108	3.89	5.07E-07	2.12	0.00112	1.33	0.133923	3.50	0.000113	3.29	6.17E-15	2.44	0.00376	1.81	0.02635			TC93229	F15K9.6
C119	0.69	0.42398	2.25	6.7E-05	1.64	0.001561	1.38	0.138307	2.62	7.31E-07	1.97	0.000275	1.02	0.87925	1.27	0.363033	TC85497	nonspecific lipid-transfer protein
C14	0.87	0.36977	0.43	0.00095	0.50	0.011295	0.69	0.104747	0.36	1.25E-07	0.40	2.9E-06	0.68	0.00348	0.55	0.022894	TC63405	ribuso activase
C150			1.13	0.5983	1.25	0.481617	1.03	0.881772	1.81	0.000228	0.90	0.585403	0.69	0.65062			TC72920	unknown protein
C152									0.66	0.002351							TC87024	heat shock protein 70
C161	9.26	0.000352	11.75	8.4E-05	14.80	8.86E-07	11.70	0.004261	19.92	7.71E-07	47.56	0.001279	2.15	0.00216	5.46	0.051505	TC96342	low-temperature induced protein
C162	0.84	0.260669	0.62	0.01583	0.95	0.768192	1.10	0.491071	1.21	0.132895	1.22	0.114214	1.18	0.27452			TC72163	glyoxalase I
C170	0.65	0.044628	1.57	0.01344	1.23	0.599772	0.62	0.074015	0.81	0.038651	0.60	0.000234	0.72	0.13973	1.01	0.951375	TC63101	orf107a
C172			1.21	0.22479	2.46	0.00026	1.04	0.826058	1.38	0.036412	2.55	0.000521	0.99	0.95233	1.07	0.81594	TC85585	Ribonuclease 2 precursor
C173	1.11	0.642358	1.15	0.21639	0.92	0.683578	1.34	0.044424	1.70	0.00244	0.85	0.07166	1.00	0.98931			TC86207	photosystem II subunit (22kDa)
C176							0.63	0.034386	0.63	0.041613							BAB09809	phosphatase 2C-like
C177	1.90	4.83E-05	2.03	2.6E-06	2.08	0.001973	1.07	0.632965	1.53	1.84E-05	0.46	0.00027	0.91	0.45737	0.43	0.005486	TC63105	chlorophyll a/b-binding protein
C179	2.16	0.002806	2.36	0.00052			1.83	0.018549	3.10	0.000823	4.46	8.29E-07	0.86	0.29801	3.34	0.072302	TC86189	sucrose:fructan 6-fructosyltransferase
C180			0.92	0.60237	0.88	0.547316	0.61	0.018824	0.97	0.753978	1.04	0.829269	0.81	0.22455			TC84932	ribosomal protein S29
C184			1.34	0.18166	1.45	0.062652	2.45	0.000453	2.36	2.49E-06	1.69	0.000168	1.25	0.2081			TC85879	monodehydroascorbate reductase
C191	1.79	0.000455	2.16	4.7E-05	0.86	0.372749	1.71	0.006536	2.43	3.66E-09	0.88	0.340141	0.73	0.07307			TC87050	chalcone synthase 1
C193							1.50	0.020508	1.28	0.420885	1.12	0.766122					TC70160	hypothetical protein
C196	1.51	0.006828	1.18	0.16194	0.89	0.437954	1.94	0.006617	1.62	0.002286	0.90	0.244555	1.56	0.01683	1.14	0.701633	TC67656	remorin 2
C197	1.28	0.112845	1.05	0.75397	1.43	0.500097	1.85	0.003176	1.62	3.45E-07	1.00	0.990395	1.55	0.0197	1.14	0.622711	TC87822	unknown protein
C200			1.67	0.00019	1.69	0.070751	1.07	0.71836	1.55	1.65E-06	1.07	0.511317	1.06	0.65228	1.21	0.544019	TC66034	cyclophilin A-2
C205	1.18	0.202893	1.09	0.41271	1.24	0.135721	1.43	0.019818	1.67	8.05E-06	1.55	0.000348	1.12	0.37591	2.12	0.124046	TC64307	polyubiquitin 4
C208			0.97	0.85692	0.88	0.454281	1.11	0.423734	1.17	0.10396	1.74	0.002879	1.19	0.39989			TC67262	beta 5 subunit of 20S proteasome
C209					1.14	0.808938	1.38	0.068871	2.15	0.018497							TC90307	At2g24280/F27D4.19
C21			0.78	0.11986	0.90	0.57154	0.49	0.013785	0.46	0.000002	0.95	0.894219	0.85	0.26969			TC97268	unknown protein
C213			1.92	0.00344			3.58	6.13E-06	3.64	4.12E-08	2.33	1.78E-06	1.52	0.39995			TC73110	expressed protein
C215	0.87	0.404781	0.84	0.20492	0.54	0.022941	0.82	0.182229	0.90	0.151523	0.86	0.110572	0.99	0.96698			TC84730	elongation factor 1-gamma (EF-1-gamma)
C22	0.57	0.027446	0.71	0.04417	1.48	0.106078	0.50	0.013612	0.49	3.02E-05	0.86	0.242937	0.90	0.48498	0.78	0.281403	TC67631	unknown protein
C24	0.54	0.024014	0.69	0.02941	0.92	0.575898	0.45	0.002215	0.48	4.61E-05	0.80	0.035253	0.84	0.40132	0.72	0.165127	TC82928	pyruvate dehydrogenase E1 beta subunit
C25			0.65	0.08973	0.76	0.397841	0.63	0.030951	0.60	0.000272			0.81	0.32828			BJ215021	expressed protein
C26	0.94	0.712248	0.75	0.03779	1.17	0.393392	0.65	0.019366	0.61	0.000339	0.38	0.000491	0.90	0.39171	0.52	0.042929	TC83130	LHCI-680 photosystem I antenna protein
C27			0.57	0.06201	0.63	0.052811	0.91	0.528623	0.66	0.004981	0.81	0.398056	0.73	0.18608			TC89306	gb protein
C270	1.22	0.214206	1.33	0.07767	0.91	0.611007	1.11	0.556683	1.52	8.73E-05	1.32	0.005657	0.80	0.28228			TC66378	expressed protein
C272			1.03	0.84736	0.75	0.153636	2.07	0.000264	1.98	3.92E-07	1.26	0.093689	1.37	0.21561			TC65072	gamma-thionin
C275	0.55	0.017152	0.75	0.04274	0.58	0.033441	0.62	0.036845	0.48	2.56E-06	0.65	0.034685	0.77	0.11335			TC63692	metallothionein-like protein
C28	8.42	0.015491	21.93	1.6E-06	10.11	0.047902	19.74	0.044044	30.70	0.000304	12.88	0.000263	2.43	0.00444	3.45	0.076178	TC92066	ice recrystallisation inhibition protein
C287			1.12	0.36165	0.90	0.61468	0.63	0.030025	0.89	0.234353	1.63	0.082998	0.70	0.04762			TC63530	ribosomal protein S15
C288					1.26	0.715031	2.80	0.001823					0.90	0.61147			TC86939	protein kinase
C29	1.67	0.009139	3.17	0.00074	5.24	0.01143	2.74	0.079932	3.60	1.78E-07	20.29	0.00068	1.95	0.01062	3.80	0.067314	AL821437	cold acclimation protein
C290	1.29	0.043567	1.50	0.01111	1.75	0.421018	2.17	0.000915	2.42	7.34E-10	4.62	6.74E-08	1.51	0.05044			TC141164	biotin carboxyl carrier protein
C296	0.78	0.135947	0.79	0.07281	0.80	0.180889	0.69	0.066816	0.73	0.002761	0.66	0.000149	0.81	0.10808	0.72	0.165254	TC84210	histone H4

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
C298			2.09	0.0004	1.47	0.055141	0.93	0.683984	1.44	0.004912	2.05	0.007262	1.12	0.44555			TC63131	photosystem I reaction centre subunit N
C300	0.89	0.542482	1.54	0.1003	1.16	0.529922	0.59	0.048788	0.80	0.050284	1.17	0.115692	0.85	0.35225			TC67024	expressed protein
C301	0.69	0.070485	0.52	0.00137	0.72	0.118221	0.76	0.154679	0.52	1.09E-05	0.32	0.000116	0.72	0.00654	0.41	0.022819	TC68028	PE-PGRS homolog (MAG24-1)
C302			1.17	0.21202	1.30	0.164234	1.96	0.041311	1.05	0.609784			1.60	0.0308			CA630452	hypothetical protein
C306			0.63	0.05007	0.74	0.344924	0.68	0.095535	0.63	0.003206			0.78	0.45528			TC74850	cysteinyl-tRNA synthetase
C307			0.95	0.8059	2.27	0.217369	0.49	0.016959	0.48	2.52E-05			0.85	0.72179			TC64789	glyceraldehyde 3-phosphate dehydrogenase A
C308	0.63	0.015974	0.89	0.41853	1.26	0.176347	0.55	0.01172	0.69	0.002042			0.87	0.28533			TC92222	At1g54320/F20D21_50
C31	0.67	0.050741	0.63	0.0045	0.70	0.139874	0.73	0.051074	0.81	0.068564	0.60	0.000938	1.02	0.90493	1.27	0.407022	TC84092	elongation factor 1-alpha (EF-1-alpha)
C313	1.17	0.232231	1.20	0.22629	0.77	0.120077	1.35	0.056966	1.88	0.001797	1.03	0.723158	1.25	0.17277	1.57	0.218419	TC82491	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
C314			1.56	0.00217	1.04	0.831917	1.13	0.355995	1.70	0.000952	1.47	0.015824	1.27	0.19251			TC72446	AT3g19170/MVI11_8
C319			0.80	0.10793	0.93	0.689265	0.81	0.045629	0.53	0.000018			0.68	0.47193			TC82748	Poly-A Binding Protein
C320			0.90	0.66056	1.08	0.840759	0.56	0.020731	0.42	9.6E-06	0.64	0.019729	1.76	0.06804			TC92606	Y15E3A.3
C325			0.88	0.38787	0.58	0.042877	0.96	0.789332	0.89	0.13045	1.00	0.99055	0.93	0.80382			TC87788	ubiquitin-specific protease 7
C328							2.00	0.015024									TC78218	cold acclimation protein (WCOR413)
C33							0.53	0.012883									TC71662	expressed protein
C334	0.64	0.050798	0.51	0.0032	0.80	0.254833	0.71	0.073722	0.55	0.000104	0.32	0.000767	0.67	0.06025			CA634358	expressed protein
C338			0.88	0.37074	0.98	0.882059	0.87	0.338652	0.63	0.000189	0.96	0.787916	1.25	0.18213			TC67545	calcium-dependent protein kinase
C339	0.77	0.168743	0.59	0.00474	0.87	0.434204	0.72	0.046075	0.69	0.005136	0.38	0.000188	0.89	0.38494	0.49	0.152217	TC99520	OSJNBa0088K19.5
C34			0.76	0.05683	0.92	0.627317	0.51	0.013208	0.55	6.88E-05	1.05	0.617258	0.66	0.11617			TC82469	RNA binding protein
C340			0.84	0.51378			0.54	0.039558									TC71941	CTP synthase
C342	0.66	0.047468	0.49	0.00232	0.84	0.461863	0.81	0.244733	0.66	0.012293	0.39	0.001606	0.70	0.02813			TC86554	expressed protein
C35			0.66	0.02467	0.86	0.460317	0.61	0.035635	0.51	0.000048	1.05	0.792925	0.74	0.27442			TC70223	unknown protein
C36							4.54	9.98E-08	4.74	2.33E-09	8.22	7.31E-08	3.58	0.00287			TC72617	cold acclimation protein (WCOR80)
C38	0.67	0.051439	0.64	0.01987	0.68	0.061229	0.65	0.025232	0.66	0.000354	0.96	0.670514	1.14	0.35743	1.41	0.286753	CA729384	aquaporin PIP1
C40			1.09	0.79352			0.79	0.386646	0.64	0.019751							BE426494	hypothetical protein
C41			0.61	0.00787	0.76	0.35074	0.73	0.122488	0.71	0.082879			0.79	0.25577			TC71943	unknown protein
C44	0.75	0.098791	0.70	0.03301	0.58	0.028925	0.78	0.197814	0.73	0.0034	0.69	0.010977	0.81	0.16791	1.10	0.743959	TC86727	ATP citrate lyase
C465	0.60	0.0258	0.77	0.06382	0.44	0.011859	0.72	0.090786	0.73	0.001011	0.33	7.37E-05	0.49	0.0078	0.79	0.299733	TC66723	unknown protein
C468			0.80	0.13643	0.72	0.168497	0.62	0.035472	0.56	0.000341	1.35	0.452154	0.97	0.87153			TC76042	expressed protein
C469					0.74	0.47169	0.97	0.910187	0.63	0.017585			0.81	0.50606			TC91916	expressed protein
C47									0.65	0.011769							TC68854	unknown protein
C472	1.23	0.248069			1.27	0.245181			1.77	3.67E-05	1.81	9.07E-07	1.25	0.25306	1.48	0.273575	TC84126	ribuso large subunit
C473	0.59	0.04923	0.56	0.00629	0.65	0.047069	0.75	0.203976	0.48	1.97E-05	1.02	0.896363	0.83	0.32454			BG262503	proline-rich protein
C51	0.79	0.146145	0.70	0.01458	0.69	0.094162	0.82	0.197449	0.86	0.088184	0.60	1.02E-05	1.04	0.78158	1.47	0.25189	TC83994	elongation factor 1-alpha (EF-1-alpha)
C514			0.67	0.02055	0.57	0.029995	0.54	0.019981	0.59	0.000268	0.52	0.001239	0.88	0.50313			TC88885	hypothetical protein
C515					0.43	0.030248	0.80	0.398128									TC69805	isovaleryl-CoA dehydrogenase
C517	0.79	0.148817	0.62	0.00255	0.72	0.073527	0.70	0.062979	0.74	0.007626	0.47	5.2E-07	0.87	0.40374	0.87	0.598205	TC65213	histone H2B.2
C520	0.75	0.100443	0.49	0.00053	0.52	0.015241	0.73	0.068155	0.71	0.002866	0.44	3.77E-05	0.98	0.90274	0.96	0.860368	TC64704	alpha-tubulin
C55			1.55	0.09146	0.96	0.824812	3.35	1.88E-05	2.65	1.24E-06			2.26	0.00149			TC67115	cold acclimation protein
C582					2.09	0.006793	0.70	0.202544					1.08	0.58901	0.94	0.866379	TC65831	metallothionein-like protein
C583			1.98	0.00023	1.85	0.000159	1.51	0.123415	2.62	8.57E-08	1.67	0.000167	0.85	0.19504	1.13	0.647374	TC65818	nonspecific lipid-transfer protein
C60	0.63	0.035966	0.52	0.01767			0.78	0.163593	0.70	0.001519	0.53	0.010614	0.80	0.10462			TC63285	fructose-bisphosphate aldolase
C63	1.52	0.030724	1.29	0.09305	1.22	0.311886	1.51	0.023433	1.72	0.000289	1.93	7.99E-05	1.06	0.67993	1.86	0.153227	TC64384	polyubiquitin
C65			1.26	0.36636	0.87	0.676359	1.94	0.004959	1.20	0.206678							TC68453	cold acclimation protein (WCOR413)
C652							0.59	0.020894									TC82509	delta-COP
C653	0.78	0.221986	0.37	0.00067	0.80	0.186803	0.83	0.47039	0.32	1.5E-06	0.56	0.023867	0.64	0.01253			TC63643	carbonic anhydrase
C658			0.83	0.48928			0.66	0.035851					0.97	0.92575			TC87202	hypothetical protein
C663			0.59	0.00884	0.59	0.241918	0.77	0.143557	0.79	0.047441			0.84	0.42054			TC85804	Ps16
C665			1.13	0.58257			0.60	0.039111	0.57	2.58E-05			0.78	0.52462			B85295	DNA mismatch repair protein
C666							0.83	0.677931	0.57	0.002564			1.02	0.95433			TC87994	phenylalanine ammonia-lyase
C67							2.51	0.000447	1.64	0.003288			1.21	0.39359			BJ216287	polyprotein
C68	1.22	0.224934	2.33	5.1E-05	2.84	4.45E-06	1.65	0.255321	2.76	2.99E-05	3.80	1.18E-06	1.03	0.87942	2.00	0.075885	TC86203	lipid transfer protein precursor
C73	1.02	0.939982					0.84	0.277726	0.77	0.015147	0.40	0.00428	0.73	0.15871			TC64946	actin 2
C75	0.62	0.090466	2.03	0.00229	1.37	0.187733	0.51	0.028622	0.98	0.820423	2.69	0.007102	0.68	0.18407	1.09	0.833671	BQ170273	peptidase-like protein
C79	2.69	9.04E-08	2.67	3.1E-05	3.71	2.89E-05	1.90	0.042128	2.55	9.37E-06	0.86	0.07462	0.92	0.53236	0.45	0.001662	TC63281	chlorophyll a/b-binding protein

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
C83	1 07	0 630099	1 22	0 12394	0 97	0 856822	1 41	0 067015	1.80	1.38E-06	0 97	0 666601	1 31	0 08796	1 01	0 960934	TC65868	phosphoethanolamine methyltransferase
C86	5.32	4.65E-07	12.79	9.6E-08	4.32	0.01143	3.37	0.002602	8.27	6.9E-10	5.08	0.001774	1 24	0 18041	1.38	0 38156	BE213332	glycine rich protein
C94	0 76	0 153368	0 75	0 06171	0 72	0 098424	0 84	0.285662	0.91	0 389321	0.43	0.000329	1 20	0 25167	0 79	0 464526	TC63233	expressed protein
C95	24.16	1.26E-05	3.76	1.6E-06	1 69	0 190707	11.03	0.000687	12.14	0.000199	3.40	8.12E-06	3.40	0.00221	3 94	0 066807	TC87317	actin depolymerization factor-like protein
E08_F	0 71	0 597674	1 08	0 6744	0 95	0 823914	0 91	0 920843	0.61	0.000112							TC63639	oxygen-evolving enhancer protein 3-1
E15_F			1.37	0 14445	1 06	0 823262			0.63	0.00083							TC71453	hypothetical protein
E25_F			0 91	0 46719	2.30	0.004489	1 36	0 122465	1.55	0.000215	2.06	0.002065					TC83987	expressed protein
E29_F			0.51	0.00292	0 76	0.14759	0 66	0.05495	0.58	0.000022	0.40	0.002853	0.79	0 47338			TC63664	photosystem I reaction center subunit VI
E31_F	6.63	0.00045	6.52	1.4E-05	2.43	0 074737	6.85	0.00117	20.50	3.6E-06	6.84	0.000943	3.75	0.00207			TC92493	COR39
F04_F			1.45	0.1341	1.01	0 97317			0.64	0.001815							CA716838	ribuso oxygenase
F07_F	0.54	0.040827	0.57	0.00618	0 90	0 58331	0.46	0.001552	0.47	9.49E-06	0.52	0.000847					CA677710	oxygen-evolving enhancer protein 2
F26_F	0 89	0.423594	0.79	0 12445	0 69	0 062574	0 90	0 466524	0 94	0 383904	0.63	0.000169	1.04	0.73149	1.62	0 212132	TC84022	elongation factor 1-alpha (EF-1-alpha)
F29_R	0.60	0.048209	2.17	0.00057	1.23	0 22292	0.48	0.022192	1.12	0 38505	3.23	0.002212	0.66	0 10691	1.12	0.742082	TC93925	LRK10 receptor-like kinase
F31_F							1.95	5.51E-05	1.85	0 056626	3.10	0.191194					TC67765	OSR8
F32_F	0.70	0 069353	0.58	0.00542	0.84	0.420618	0 68	0 057031	0.60	0.000803	0.34	0.003306	0.92	0 51322			TC65552	unknown protein
F33_F	1.70	0 064108	1.81	4.2E-06	2.93	0.013705	1.44	0.015577	1.74	1.34E-06	0.71	0.029389	0.99	0 97414			TC86043	chlorophyll a/b-binding protein (WCAB)
F34_F			1.60	0.00146	1.74	0.127823	1 30	0 150807	1.99	0.000166							TC85257	cyclophilin A-1
F46_R	1.57	0.03889	2.21	2.2E-06	2.00	0.002438	1.58	0.15131	2.59	4.59E-07	1.94	4.03E-05	0.93	0.55538	1.48	0.271645	TC65820	nonspecific lipid-transfer protein precursor
F56_F	1 16	0 383421	0.86	0 29353	0 92	0 576236			1.00	0 963014	0.49	8.68E-06	0.89	0 48288	0.47	0.012656	TC64829	ribuso small subunit
F66_R			0.72	0 07627	1 68	0 270301	1 00	0.978559	0.63	0.000757	0 88	0.263557	0.97	0 82219			TC98686	oxidoreductase short-chain dehydrogenase
F70_R					3 94	0 183198			1 39	0 607631	0.13	0.000517					TC90187	hypothetical protein
F71_F	0 81	0 209274	0 95	0 66138	0.59	0.02045	0 77	0 124736	0.81	0.042631	0 87	0.333221	0.82	0 47404			TC65683	ribosomal protein L10A
F79_F	0 88	0.427155	0.76	0 07411	0.60	0.031218	0.65	0.036372	0.72	0.006319	0.61	0.000694	1.07	0 6261			TC65549	HSP80-2
J260	0.58	0.024593	0 62	0 10118			0.63	0.034593	0.58	0.000816			0 85	0 29187	0 73	0 230115	TC83332	expressed protein
J262			0.63	0.005	0 75	0 408369	0.67	0.030396	0 77	0 479973	0.36	0.000912	0.74	0.20306			CA696957	ribuso small subunit
J264	0 81	0.199332	0 86	0 22291	0 77	0 227262	0 81	0 186806	1 15	0 062816	0.65	0.003674	1.03	0.82582	1 05	0.830874	TC68502	AT3g61860/F21F14_30
J265	0 67	0.057263	0.57	0.01376	0 68	0 070291	0 86	0 452537	0.59	0.000203	0.77	0.007524	0 87	0.3018	0 69	0.124327	CA678069	ribuso large subunit
J275	9.93	2.61E-06	4.99	4E-08	2.14	0.000269	5.08	2.76E-05	4.02	4.51E-14	3.58	1.45E-05	1.87	0.0236	2.76	0.080003	TC87867	LT101.1 LT induced protein
J276	0 85	0 359841	0.65	0.00426	0.74	0 114678	0 73	0 0814	0.68	0.003032	0.74	0.0278	0.88	0 46874	1.28	0.397562	TC65938	profilin
J277			0 71	0 11228	0 71	0 082324	0.61	0.039914	0 96	0 841493	1.16	0.666539	0.78	0.65802			TC71462	expressed protein
J278			0.58	0.00728	0.61	0.031025	0 82	0 410515	0.49	4.14E-05	0.83	0.029865	0.84	0 21273			TC84129	ribuso large subunit
J279	0.54	0.029292	1.98	0.00041	1.35	0 106371	0.48	0.026154	0 90	0 434807	2.84	0.060782	0.69	0.01276	0.95	0.868534	TC72165	expressed protein
J286							2.53	0.005614					1.57	0 06424			TC88089	expressed protein
J293	0 59	0 10405					1 23	0 781078			0.57	0.042631					TC66914	IAA1
J294			1 23	0 46025	1 59	0 406701			0.65	0.000546							TC73151	ISW2-like ATPase
J296	1 04	0.767946	2.37	0.001	2.07	0.000237	1.77	0.042762	2.69	1.07E-05	1.48	0 051779	0.79	0 2952	0 96	0 889145	TC65819	nonspecific lipid-transfer protein precursor
J302	8.50	3.93E-06	5.87	1.8E-12	2.59	0.000151	4.48	0.000222	4.42	9.64E-09	2.96	1.66E-07	2.14	0.00565	3.15	0 08028	TC87869	LI101.1 LT induced protein
J303			0 70	0 10128	0.48	0.011523	0 73	0 06386	0.71	0.002602	0.29	0.00232					TC82851	beta-glucosidase
J325	1 00	0 977894	0.73	0.02932	0.74	0 146836	0 82	0 216291	0.81	0.046597	0.48	2.37E-06	0.87	0 25403	0.90	0.603153	TC84524	histone H2B 2
J326							0 99	0 970351	0.64	0.000409							TC88170	glutamate permease
J327			0.57	0.0081	0 68	0 115989	0.68	0.034515	0 81	0 075208	0.62	0.058132	0.99	0.9556			TC82473	expressed protein
J330	14.59	5.91E-10	15.86	1.5E-10	5.82	1.5E-06	6.85	0.001032	9.82	1.2E-08	3.50	1.24E-08	1.55	0.028	2 04	0.123317	TC87536	LEA/RAB-related COR protein
J333	0 83	0 269314	0.47	0.00219	0 68	0 089546	0 78	0 343353	0.35	0.000005	0.25	3.09E-05	0.69	0.04938	0 51	0.178212	TC63231	ribuso activase
J339	14.49	0.031279	6.04	2.4E-05	2.15	0.033313	21.23	0.000469	11.32	4.22E-07	11.63	6.27E-05	4.59	0.0023	9.62	0.049081	TC93659	WCS120
J342	0.48	0.016542	1.66	0.04599	1.45	0 328321	0.42	0.005441	0.43	4.41E-06	1.37	0 214085	0.61	0 16223			TC68241	hypothetical protein
J348	0 82	0 199299	0.64	0.00914	0 66	0 069667	0.70	0.037487	0.74	0.002739	0.56	5.73E-05	0.80	0.14996	0.70	0.168475	TC84206	histone H4
J349	0 72	0 082159	0.57	0.00619	0.54	0.019712	0 68	0 069829	0.38	6.27E-07	0.59	0.000282	0.73	0.01314	0.50	0 292304	TC67768	OSR8
J353							0.33	0.004709									TC87761	dehydration-responsive protein (RD22)
J354	0.68	0 053875	0.67	0.01644	0.89	0 438902	0 80	0 159892	0.66	7.15E-05	0 84	0.192902	1.01	0.90943	0 68	0.107361	TC63306	apospory-associated protein C-like
J357			0 95	0.90772	1 08	0 920141			0.52	0.000893							TC67028	ultraviolet-B-repressible protein
J360			1.13	0.56285	1.11	0 670993			0.54	0.003145							TC63930	senescence-associated protein
J362	0.58	0.025289	0.51	0.00368	0 89	0 550904	0.66	0.021834	0.67	0.002815	0.48	1.26E-05	1.21	0 27271	0.78	0.409274	BE418083	photosystem II subunit (10 kDa)
J366			0.63	0.01331	0 62	0 267073	0.72	0.049481	0.68	0.00195	0.36	0.000883	0.83	0 25008			TC65233	histone H3
J374	9.88	8.61E-08	7.85	2.5E-05	2.41	4.75E-06	11.84	0.000119	22.81	1.21E-10	7.20	1.87E-07	4.74	0.00201	10.20	0.049147	TC87737	dehydrin (WZY1-1)
J377	0.67	0.048638	0.69	0.02083	0 77	0 117134	0.51	0.015444	0.44	6.4E-08	0.50	0.000118	0.68	0 4112	0.40	0.037099	TC69422	plastid ribosomal protein CL9
J386			0.70	0.04048	0 83	0 307543	0 73	0 071422	0.72	0.004843	0.34	0.000153	1.17	0 32643			TC85771	beta-tubulin 3

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
J392	0 78	0 373782	1 04	0 82327	1 01	0 950916	0 89	0 52054	1 02	0 79134	0.57	0.000324	1 19	0 32186	0 92	0 767634	TC66688	proline-rich protein
J393	0 99	0 961028	0 88	0 31667	0 99	0 956547			1 00	0 994194	0.36	0.000106	0 93	0 54789	0.44	0.009057	TC64830	ribuso small subunit
J407	0 87	0 345647	0.70	0.01487	0 73	0 103757	0 68	0 056602	0.66	0.000377	0.62	0.0001	0 81	0 1084	0.87	0 556271	TC65168	histone H2B 2
J412	0 91	0 591826	1 15	0 30758	1 02	0 919189			1.23	0.026594	1.54	2.62E-05	1 27	0 1789	1.20	0 482202	TC100480	dehydrin (LEA D-11)
J440			2.08	0.00203	1 87	0 259063	1 24	0 573292	2 16	0 104399			0 84	0 189			TC89661	ribuso small subunit
J441	0 80	0 200906	0 79	0 12261	1 09	0 665189	0 74	0 064372	0.80	0.016984	0.39	0.000238	0 98	0 88767	0 40	0.136738	TC85963	chlorophyll a/b-binding protein
J442	0.47	0.020493	1.73	0.0382	1 29	0 418178	0.50	0.010575	0.62	0.000981	1 63	0 12468	0 85	0 39619	0.79	0 614119	TC102138	ubiquitin ligase E2
J444	2.97	2.68E-08	3.25	1.8E-05	4.64	0.000228	2.81	2.28E-07	3.05	7.82E-06	0 97	0 772972	0 80	0 21847	0.38	0.004362	TC63552	chlorophyll a/b-binding protein (91R)
J445	0.77	0 255329	0.52	0.00334	0 82	0 465482	0 72	0 10528	0.59	0.000284			1 00	0 97955			TC64324	plastocyanin precursor
J455	0 99	0 919344			0 89	0 471224			1.29	0.003294	1.51	0.000496	0.95	0 71788	1.12	0.72208	No Hit	expressed protein
J458	1 00	0 978175	0.54	0.00494	0.54	0.016337			0.60	5.46E-05	0.21	2.15E-05	0.66	0.01707	0.28	0.002954	TC65326	ribuso small subunit
J459	4.50	0.001696	7.49	5.2E-06	11.93	4.01E-05	8.22	0.0082	14.65	4.97E-08	34.35	3.91E-07	2.43	0.00596	6.06	0 055491	TC85842	cold acclimation protein
J462							2.90	0.026459	2.67	1.01E-06							TC86237	glutamate decarboxylase
J463	0 90	0 498704	1 18	0 18855	0 88	0 381736	1.39	0 151764	1.76	4.62E-05	0 93	0 502396	1 21	0 2015			TC85495	ferredoxin
J466	0.71	0 061581	0.73	0.0276	0 68	0 067687	0 76	0 09701	0.79	0.02119	0.55	0.000616	1 06	0 62601	1.11	0.679205	TC65161	histone H2B 2
J468	0 98	0 866633	0 80	0.08977	0 75	0 1813	0 77	0 144995	0 93	0.481691	0.65	0.000785	0 96	0 75127	1.35	0.348689	TC64334	ribosomal protein S4 (S7)
J470	4.56	4.04E-06					11.55	7.07E-06	13.46	4.82E-09	5.77	7.98E-06	6.67	0.00146	9.09	0.051272	TC85079	LEA14-A
J478	1 01	0 976327	0 90	0 34157	0.65	0.034702	0 76	0 123964	0.72	0.001029	0.76	0.028578	0 82	0 14282	1.11	0 704044	TC85216	OSJNBa0029H02 11
J481	1.60	0.014739	1.59	0.00938	2.08	0.000145	1 31	0 081212	1.91	0.009128	1 00	0 964677	0.71	0.05299			TC63174	chlorophyll a/b-binding protein
J482	0 79	0 153325	0 91	0 55764	0 97	0 917664	0.39	0.00391	0 84	0 056713	0 91	0 332774	0.69	0.03556	1.22	0.480726	TC86035	actin depolymerization-like protein
J486	2.66	0.000204	2.71	6.9E-05	3.90	0.000541	2.39	0.000189	2.28	1.49E-07	1 17	0 075578	0.69	0.04976	0.41	0.12203	TC86049	chlorophyll a/b-binding protein
J487							2.07	0.003755	2.02	0.000117			1 10	0 66202			TC76653	thioredoxin homolog
J516	0.59	0.023799	1.12	0 43629	0 72	0 288011	0.35	0.005939	0.61	0.001458	0.44	0.000102	0.68	0.02046	0 93	0 835255	TC63682	orf107a
J517	7.72	1.59E-08	4.81	5.1E-11	2.28	0.000495	4.50	3.87E-05	3.87	1.91E-12	3.19	6.92E-08	1.96	0.01569	2.66	0.09354	TC91471	low-temperature induced protein lt101 2
J532	0 67	0 068513	0.32	0.00043	0.56	0.020556	0.52	0.00248	0.46	5.9E-07	0.30	2.21E-05	0 80	0 66921			TC65980	33kDa PSII oxygen evolving protein
J536	0 80	0 210483	0 87	0 28046	0 77	0.099856			0 91	0.197897	1.68	0.000255	0.74	0 12327			TC84127	ribuso large subunit
J537			0.54	0.00495	0.62	0.041888	0 86	0 371763	0.56	0.000166							TC82817	hypothetical protein
J547			1 01	0 95389	0 99	0 946666			0.61	0.000453							TC87284	cytochrome B5 reductase
J551									0.59	0.000103							TC63842	long-chain acyl-CoA synthetase
J554			1 09	0 62735	1 06	0 838059			0.60	0.000291							TC96510	fasciclin-like arabinogalactan-protein 7
J557			0 86	0 57717	1 06	0 8737			0.63	0.000163							TC66728	transketolase
J573			1 08	0 60812	1 07	0 781445			0.62	0.000232							TC65243	histone H3
J595	0.83	0 242349	0.56	0.00206	0.56	0.028521	0 87	0.401962	0.53	1.56E-05	0.51	0.000961	0 87	0 45136	0.65	0.122123	TC66956	3-phosphoglycerate kinase
J597	0.68	0.047264	0.32	0.00034	0.43	0.00964	0 67	0 078239	0.31	5.53E-06	0.23	9.29E-05	0.74	0 07389	0.25	0.002966	TC65328	ribuso small subunit
J598	0.99	0 922873	0.77	0.04183	0 78	0 146379	1.02	0 893014	0.83	0.040548	0.36	0.00017	0 93	0 51797	0.45	0.018192	TC64793	ribuso small subunit
J602	1.50	0.005741	1.75	0.00026	1.78	0.000721	1 28	0 087441	1.51	0.000271	0.63	0 28756	0 85	0 27865			TC64630	chlorophyll a/b-binding protein
J603	2.66	0.000324	1 25	0 26748	1.92	0 137974	2.76	0.011273	1.57	0.000081			1 08	0 57583			TC86935	amine oxidase
J615							3.93	0.001346									TC85075	LEA14-A
J617			2.01	0.00601			4.31	2.33E-05									TC76348	dehydrin/LEA group 2-like protein
J619	10.91	2.65E-09	2.91	0.00169	1.34	0.112488	8.60	0.000157	7.49	4.63E-06	3.99	3.29E-07	2.14	0.0079			TC87319	actin depolymerization-like protein
J621	0.69	0.043072	0.58	0.00837	0.72	0.075384	0 62	0.054477	0.41	1.55E-06	0.71	0.045861	1 15	0 39214	1.63	0.229562	TC84388	translation initiation factor 5A
J622	0.68	0 082435	0.55	0.00592	0.63	0.039288	0.54	0.01688	0.42	2.73E-05	0.39	7.99E-05	0 84	0 31223	0.65	0.07869	TC72840	OSJNBb0066J23 1
J624			0 82	0 20688	0.77	0 184661			0.57	0.000105							TC84173	ribuso small subunit
J625	0.57	0.025894	0.65	0.01593	0 81	0 251526	0.55	0.007831	0.53	9.13E-05	0.77	0 105038	0.73	0 20605	0.53	0.021996	BJ276977	expressed protein
J626	0.58	0.033183	1.73	0.01984	1.52	0 38559	0.32	0.005826	0.41	0.000104	1.53	0 185696	0.73	0 15969	1.25	0.419692	TC63138	hypothetical protein
J627	0 62	0 053764	0.74	0.04954	0 89	0 489444	0.51	0.004498	0.52	8.11E-06	1 07	0 672415	0 83	0 3958	0.78	0.343562	TC89624	thioredoxin homolog
J636	1.11	0 40918	0.58	0.00562	0 80	0 185384	0 96	0 823011	0.64	5.74E-05	0.72	0.004994	1 15	0 31565	2.51	0.99831	TC64862	ribuso small subunit
J637	1.35	0 057701	0.58	0.00284	0 81	0 251733	1 02	0 903006	0.68	0.000532	0.69	0.002609	1 24	0 131	1.05	0.847381	TC66793	heat shock protein
J638	0.62	0.035376	0 92	0 65397	0 83	0 365131	0.55	0.015694	0.54	8.75E-05	1 06	0 555956	0 82	0 13154	0.86	0.540194	TC85766	latex-abundant protein
J642							4.70	0.003998	6.85	0.000389			2.87	0.00253			TC79747	unknown protein
J645	0.70	0 068577	0.63	0.01333	0 73	0 087557	0.70	0.041951	0.63	0.000236	0.66	0.002612	1 13	0 35561	0 85	0.488863	TC63534	photosystem II subunit (10 kDa)
J648			0 76	0 22061	0 91	0 623932	0.56	0.025025	0.55	0.00011			0 80	0 30853			TC82445	histone H2A 2.1
J649	0.92	0 556618	0.72	0.02313	0 86	0 324497	0 96	0 789869	0.77	0.009472	0.36	2.29E-05	0 90	0 4241	0.39	0.000395	TC90819	expressed protein
J653			1 14	0 69962			1 34	0 259881	1.67	0.002112							TC85490	ferredoxin
J654	0.55	0.006938	0 72	0 06156	0 83	0 300134	0.58	0.023985	0.53	0.000429			0 93	0 67439			TC98392	CSLA7
J656	2.17	0.020972	1.41	0.03335	1 15	0 289027	2.23	0.001252	1.30	0.013078	1.29	0.03435	1 35	0 07639	1 44	0 275991	TC90737	acyl-CoA oxidase homolog

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
J657							4.63	0.00222	2.86	1.58E-05			2.35	0.00339			TC65078	gamma-thionin
J660			0.79	0.11328	4.05	0.250275	0.59	0.012853	0.56	0.000298			0.88	0.51046			TC67748	protease subunit ClpP-like protein
J661	0.90	0.467731	0.71	0.04183	0.65	0.052142	0.73	0.090022	0.84	0.124678	0.48	0.000272	0.77	0.09022	0.80	0.291472	TC90186	hypothetical protein
J662	0.95	0.747672	0.69	0.021	0.71	0.090712	0.73	0.08604	0.78	0.007345	0.55	6.69E-05	0.81	0.11067	0.68	0.138782	TC84225	hypothetical protein
J664	0.63	0.038343	0.83	0.16881	0.87	0.458566	0.51	0.003724	0.50	3.24E-05	1.03	0.802267	0.80	0.10815	0.74	0.194435	TC72186	At5g12410
J665			0.73	0.19171			0.74	0.514751	0.62	0.002673							TC65863	phosphoethanolamine methyltransferase
J666	14.44	2.78E-10	18.85	0.00001	6.08	0.003748	5.55	1.14E-05	8.41	2.72E-06	4.30	0.000256	1.64	0.00552	1.67	0.13367	TC69676	cold acclimation protein (WCOR14a)
J670			0.65	0.00825	0.86	0.56555	0.75	0.103969	0.76	0.005647	1.10	0.765545	0.88	0.48362			TC96648	expressed protein
J676	2.37	0.000112	2.47	1.2E-06	3.86	0.005273	2.09	0.002202	1.92	9.03E-08	0.80	0.065484	0.94	0.64368	0.42	0.05209	TC83195	latex-abundant protein
J677	0.64	0.063417	0.70	0.03734	0.98	0.944311	0.62	0.022766	0.68	0.000572	0.74	0.008304	0.95	0.72395	0.83	0.432869	TC83148	ubiquitin-conjugating enzyme (UBC2)
J678			0.81	0.19768	0.94	0.692082	0.52	0.010864	0.48	4.21E-05	1.02	0.926388	0.72	0.2254			TC72410	peptidyl-prolyl cis-trans isomerase
J680	4.86	0.004372	5.19	5.1E-07	4.62	1.55E-10	4.17	0.001409	8.17	3.59E-07	8.91	4.12E-09	2.10	0.0085	2.36	0.103208	TC153501	ice recrystallisation inhibition protein
J697			2.02	0.09282	1.86	0.352858	0.34	0.00954	0.35	3.09E-05	1.78	0.306747	0.70	0.31363			TC87170	unknown protein
J713			1.04	0.84352	1.00	0.984833	1.17	0.342219	1.65	7.54E-05							CA605532	transcription factor (HUA2)
J715							4.10	5.61E-06	2.82	0.000157							CA605532	expressed protein
J717			0.84	0.22207	0.69	0.558331	0.60	0.016109	0.92	0.348199	0.89	0.281077	0.90	0.44512			TC65169	histone H2B
J733	12.82	0.048491					7.05	0.003525					2.04	0.00998			TC94499	golgi-localized protein (GRIP)
J737			0.88	0.30558	0.70	0.080574	0.65	0.044253	0.76	0.005715	1.03	0.929959					TC87407	ribosomal protein L34
J757	0.87	0.423056	0.71	0.01925	0.75	0.122948	1.23	0.343059	0.62	0.000361	0.96	0.603556	0.92	0.48708	0.72	0.191282	TC83643	pherophorin-dz1 protein
J762			0.52	0.0042	1.06	0.890016	0.73	0.061912	0.66	0.007896	0.83	0.455425	0.80	0.21893			TC94824	dihydrofolate reductase-thymidylate synthase
J765	16.53	6.32E-10	3.74	1.8E-06	1.89	0.088522	10.77	0.000214	6.88	9.49E-07	2.94	0.000206	2.70	0.00407	3.73	0.068617	TC69449	chitinase 2
J770			0.67	0.072	0.78	0.432588	0.77	0.159921	0.64	0.001056	0.58	0.023525					TC69014	zinc finger transcription factor
J772			0.82	0.37342	1.11	0.880444	0.84	0.254754	1.04	0.843489	0.52	0.000467					TC88326	CAAT-box DNA binding subunit B (NF-YB)
J773	0.78	0.190081	1.11	0.32296	1.23	0.22636	0.89	0.476388	1.17	0.099055	0.62	0.000153	0.78	0.1841	0.72	0.416723	TC65728	HMG1/2-like protein
J782			1.03	0.87447	0.75	0.162568	0.55	0.027946	0.76	0.022434	0.71	0.0131	1.23	0.20751			TC85327	precursor protein (AA -25 to 143)
J783							0.43	0.013784									TC84258	histone H4
J786			2.43	4.2E-05	1.17	0.521054	1.78	0.008549	1.81	2.29E-05			1.17	0.32164			TC98570	serine-rich protein
J798	0.81	0.203484	0.63	0.0074	0.60	0.035995	0.69	0.055835	0.78	0.012528	0.45	4.64E-05	0.89	0.34105	0.90	0.672034	TC105282	dehydration induced protein (ERD15)
J799	1.91	0.003296	3.13	0.00064	1.97	0.01263	2.07	0.002612	2.11	9.74E-11	1.37	0.014713	1.17	0.2688	1.03	0.923386	TC85816	expressed protein
J802			2.34	0.03535	0.65	0.11368	4.41	0.007211			0.99	0.952983	1.29	0.25628			TC73876	unknown protein
J806	1.49	0.014291	5.99	0.00032	3.60	0.095019	12.31	0.033364	14.40	2.64E-05	8.45	0.00834	1.79	0.01302	1.62	0.200502	TC68452	cold acclimation protein (WCOR413)
J811	0.61	0.03419	0.77	0.08126	0.72	0.081377	0.73	0.130566	0.74	0.002304	0.92	0.419744	0.97	0.79008	0.87	0.543763	TC71673	unknown protein
J814			0.58	0.01012	0.66	0.077573	0.70	0.043154	0.80	0.048754	0.55	0.000438	0.75	0.40338			TC68723	unknown protein
J818			0.62	0.01428			0.78	0.153434	0.79	0.033685	0.52	0.000785	0.68	0.02424			TC85304	elongation factor 1-beta (EF-1-beta)
J821			1.14	0.32062	1.17	0.270614	1.20	0.228067	1.49	1.09E-05	1.75	0.002284	1.07	0.71935			CA665766	expressed protein
J822	9.76	7.08E-06					11.84	0.000144	7.02	4.66E-05	2.46	0.022081	2.49	0.00737			TC89914	guanine nucleotide regulatory protein
J824							1.51	0.069091	1.59	0.046385			1.41	0.09218			TC86919	chaperonin 21 precursor
J832			0.64	0.01258	0.88	0.667767	0.86	0.389255	0.73	0.007412			0.91	0.78715			TC86367	EREBP-type transcription factor
J835			0.73	0.25189	0.82	0.490602	0.95	0.826545	0.74	0.011609	0.47	0.002956					TC65466	ribosomal protein S16
J840	0.72	0.103037	0.49	0.00247	0.38	0.006939	0.53	0.012667	0.47	1.92E-05	0.48	8.49E-05	0.86	0.39953	1.07	0.782751	TC82594	nuclear transport factor 2 (NTF-2)
J851			1.30	0.14783	1.18	0.478014			0.63	0.000672							TC64697	C13 endopeptidase NP1 precursor
J854	13.19	2.28E-08	12.55	6.9E-08	5.06	3.38E-05	6.74	0.001333	2.64	0.000542	2.90	6.24E-05	1.51	0.04499	1.57	0.234167	TC87536	LEA/RAB-related COR protein
J862			1.38	0.03358	1.68	0.016507	1.20	0.159225	2.34	1.34E-05	1.32	0.004413	1.18	0.32939	1.42	0.28484	TC88307	apolipoprotein D
J864			0.62	0.36807			1.45	0.029339	1.68	0.011968	3.06	0.026951					TC75884	abscisic acid-induced protein (HVA22)
J878	0.77	0.182456	0.52	0.00172	0.44	0.007226	0.62	0.034448	0.48	2.64E-06	0.69	0.002226	0.85	0.29044	1.19	0.513221	TC84434	tumor protein homolog (TCTP)
J880	0.78	0.20417	0.34	0.00056	0.80	0.214805	0.62	0.056374			0.75	0.038156	0.68	0.03113			TC64301	ribosomal protein L21
J882	1.19	0.303128	1.57	0.00059	1.60	0.032128	2.02	0.007475	2.63	1.4E-08	2.01	0.001071	1.15	0.4277	1.32	0.342636	TC63551	inorganic pyrophosphatase
J883	0.90	0.554704	0.58	0.00357	0.94	0.684164			0.39	2.84E-07	0.42	4.23E-05	0.66	0.02775	0.22	0.006394	TC64211	carbonic anhydrase
J884	1.64	0.023093	1.50	0.03405	2.09	0.004837	0.91	0.565952	1.50	0.001356	0.86	0.218345	0.76	0.13526	0.46	0.243105	TC70334	peroxidase
J885	10.24	4.15E-08	13.91	2.3E-07	22.30	0.000067	13.16	0.004151	18.70	6.87E-15	62.31	3.99E-05	2.59	0.0063	6.37	0.056311	TC92901	cold acclimation protein
J887			0.85	0.23742	0.71	0.187686	0.58	0.01187	1.03	0.759473	0.93	0.790492					TC65378	cellulose synthase-1
J893			1.28	0.1195	1.06	0.760363			0.63	0.000806							CA621935	expressed protein
J900			1.86	0.00184	2.91	0.028557	6.31	0.250682	1.36	0.020429	8.98	0.000344			1.56	0.193271	BQ161796	calcium binding EF-hand protein
J904	0.73	0.062569	0.73	0.02933	0.68	0.191962	0.79	0.15875	0.55	4.64E-06	0.69	0.026167	0.90	0.47281	1.02	0.946427	TC66809	phosphatase 2A regulatory A subunit
J908			1.18	0.17095	1.30	0.273689	0.89	0.514429	1.07	0.382344	2.10	0.008134	1.06	0.66588			TC83412	unknown protein
J921			3.10	0.00094			1.93	0.046088	2.27	2.18E-06			1.35	0.10111			No Hit	expressed protein

Clone	S1/SC Ratio	S1/SC P-value	S6/SC Ratio	S6/SC P-value	S36/SC Ratio	S36/SC P-value	W1/WC Ratio	W1/WC P-value	W6/WC Ratio	W6/WC P-value	W36/WC Ratio	W36/WC P-value	W6/S6 Ratio	W6/S6 P-value	W36/S36 Ratio	W36/S36 P-value	Accession	Annotation
J923	0 69	0 056017					0 92	0 559929			0.46	0.027496	0 90	0 57393			TC65510	sorbitol dehydrogenase-like protein
J925	0 75	0 19734	0 82	0 12897	0 75	0 115863	0 82	0 222886	1 01	0 968749	0.64	0.002824	1 21	0 20043	1 25	0 486781	TC65895	small Ras-related GTP-binding protein
J942			1 06	0 72684	1 04	0 861823	1.56	0.046511	1.44	0.001279			1.46	0 03458			TC93930	expressed protein
J946							0 78	0 259337	0.57	0.000966			0 89	0 84633			TC63597	ribosomal protein L6
J947			1 63	0 14091	1 17	0 478337	1.58	0.010466	1.63	0.00648	1 71	0 115929	1 38	0 1342			TC84816	DNA-directed RNA polymerase
J950							4.23	0.003044									TC86963	vacuolar invertase-1
J955	0 71	0 057308	0.66	0.01721	0 73	0 097579	0 86	0 34357	0.56	0.000144	0 88	0 08217	0 91	0 40813	0 81	0 429386	TC84774	ribosomal protein L36
J957							2.87	0.000542									BQ579196	HYP1
J958							6.86	3.13E-05									TC79747	unknown protein
J962			1.12	0 42229	0 99	0 985299	1.34	0.039898	1 03	0 805871	1.57	0.007412	1 26	0 31368			TC92767	AT3g24190/MUJ8_17
J982	1 02	0 878023	1 08	0 51158	1 14	0 422195	1.31	0 202529	1.57	0.000193	0.81	0 110227	0 99	0 95761	0 69	0 170393	BQ904637	expressed protein
J993			0.53	0.04091			0 79	0 215576	0.54	0.000168							TC70389	unknown protein
JB001							0.60	0.025237									TC96624	T23G18 11
JB002			0 86	0 49807	0 98	0 938919	0.49	0.013729	0.54	7.61E-05	0 91	0 778123					TC69979	Asp1
JB005	0 70	0 063062	0 87	0 41701	1 06	0 82554	0.49	0.011889	0.51	6.97E-05	1.10	0 384004	0 86	0 22323			TC92898	hypothetical protein
JB018	0 72	0 079247	0.44	0.00088	0 68	0 08568	0 68	0 098123	0.33	4.02E-06	0.21	1.69E-06	0.59	0.00172	0.28	0.002218	TC65343	ribuso small subunit
JB019	0.60	0.038436	0 74	0.0548	1 00	0 996297	0.55	0.006038	0.48	3.24E-05	0 87	0.394381	0 73	0 05053	0.60	0.084139	TC75507	unknown protein
JB020			0.64	0.017	1 03	0 953153	0.53	0.019308	0.56	0.000138			0 74	0 13518			No Hit	expressed protein
JB024	0.54	0.019452	0 88	0 51464	0 88	0 468644	0.50	0.006143	0.45	2.91E-05			0.75	0 26483			CA735542	unknown protein
JB025	1.02	0 868011	0.90	0 39009	1.08	0 672759			1 04	0 617382	0.37	0.000129	0 94	0 60294	0.46	0.001382	TC84155	heat shock protein
JB027	1 17	0 235103	2.08	2.4E-06	1.77	0.000537	1 24	0 383358	1.76	7.39E-06	1.34	0.004378	0.83	0 12607	1 30	0.364599	CA632848	lipid transfer protein precursor
JB033			0.74	0.03876	0.89	0 509474	0 82	0 253408	0.62	6.85E-05							CA593102	hypothetical protein
JB034			0 79	0.2563	0.98	0 942681	0.72	0 157344	0.59	0.001293			0.76	0 35735			CA654868	hypothetical protein
JB035	1 26	0 166294	2.63	1.1E-06	2.06	3.07E-05	1.66	0.024744	3.20	5.15E-09	2.09	2.44E-06	0.84	0 21039	1.34	0.319479	CA635093	lipid transfer protein precursor
JB036	1.10	0.524653	0.95	0 64922	1.05	0.754306			1.08	0 306053	0.47	1.6E-07	0.93	0.58691	0.49	0.026345	TC64840	ribuso small subunit
JB041			0.76	0 23287			0.62	0.034461	0 80	0.057182							TC84231	histone H4
JB048	0.61	0.043885	0.56	0.00662	0.62	0.033935	0.81	0 272249	0.51	4.24E-05	0.79	0.008197	0.91	0 42564	0 63	0 078557	CA722580	ribuso small subunit
JB049	0 73	0.097477	0.65	0.01127	0.62	0.045236	0.64	0.027591	0.60	0.020547	0.72	0.002677	0 83	0 11549	0.86	0 58593	TC84273	hypothetical protein
JB053	0.45	0.002802	0.57	0.00537	0 71	0 111083	0 77	0 187804	0.55	0.000266	0 91	0.540146	0.91	0 61561			BQ160974	unknown protein
JB057			0 77	0 07574	2.25	0.00307	0 97	0 819517	1.34	0.001606	1.93	8.32E-06	1 23	0 14132	1.02	0.942096	BQ901524	expressed protein
JB060	0 86	0 298688	0 95	0 66986	0 87	0 365884	0 81	0 205297	0.72	0.001116	0.65	0.000224	0 83	0.0945	1.17	0.542817	TC83672	malate dehydrogenase (cytoplasmic)
JB063			1 39	0 16081	1 90	0 17623	1.57	0.007409	2.35	5.62E-05	2.24	0.090886	1.13	0 4401			CA699449	temperature stress-induced lipocalin
JB074	2.29	0.000294	4.45	3.5E-06	1.56	0.002457	4.74	0.01463	8.40	7.28E-08	1.85	0.00106	2.26	0.00086	3.08	0.062803	TC65862	phosphoethanolamine methyltransferase
JB076	0.62	0 051242	0 78	0 16366	1 00	0 990163	0.51	0.020362	0 81	0 356325	1.54	0.003739	0 76	0 13594	0 94	0 794512	TC85140	thiol protease aleurain precursor
JB080	0 67	0 06933	0.73	0.03943	0 77	0 142968	0.60	0.02131	0.63	0.000344	0.98	0 811534	0.54	0.01223			TC85188	unknown protein
JB082			0.54	0.03285			0.48	0.006834									BJ230002	cold-inducible protein (CIC)
JB100			0 84	0 36729	1 09	0 639991	0.58	0.026796	0.57	0.000319			0.90	0 53556			TC64317	plastocyanin precursor
S01_F	0 68	0 072348	0 74	0 06798	0 86	0 432222	0.50	0.013599	0.53	0.000093	0 92	0 481609	0 81	0 09032			TC106933	nuclear cap-binding protein
S02_F			0 71	0 14697			0.59	0.01797	0.60	0.021578							TC87173	expressed protein
S06_F			0.71	0.0327	0 93	0 686399	0 65	0.028657	0.69	0.001161	0 96	0 931681	0.75	0.03005	0.47	0.020259	TC91686	lipoxygenase
S09_F	1 24	0 093069	1 05	0 66983	1.31	0.046564	0 75	0 147624	0.75	0.009031	0.62	3.91E-05	0 85	0 20881	0.47	0.013492	TC82882	LHCI-680 photosystem I antenna protein
S10_F	0 88	0.436224	0 98	0 88681	1 63	0.022564	0 87	0 374085	0 84	0 170787	0.54	0.000115	0 98	0 90532	0.50	0.038874	TC82882	LHCI-680 photosystem I antenna protein
S15_F			1 26	0 178	1 01	0 982223	0 71	0 49621	0 65	0.001941							CA698312	aspartate-tRNA ligase
S28_F			0 99	0 95833	1 09	0 677492	0.52	0.013749	0.55	0.000182			0 87	0 43051			TC82582	prophytepsin
S31_F	0 90	0 504483	0 90	0 44341	1 02	0 896662	0 76	0 083668	0.79	0.012773	0.64	8.73E-05	0 91	0 53294	1.05	0 837798	CA726652	14-3-3 protein homologue
S32_F	0.52	0.015202	0 69	0 17979	0 95	0 809226	0 80	0 276356	0.52	6.82E-05			0 93	0 54922			CA636920	cp31BHv
S41_F	2.89	2.87E-05	3.30	7.7E-06	4.16	1.08E-05	2.60	0.000408	2.67	1.66E-08	1.09	0.380659	0 82	0 21144	0.41	0.016274	TC86043	chlorophyll a/b-binding protein (WCAB)
S42_F			0 71	0 06952	0 87	0 519073	0.49	0.00613	0 61	0.002222	1.07	0 794283	0 77	0 09132	0 79	0 333865	TC85400	co-repressor protein

Table 2. Genes Significantly Regulated during Cold Acclimation in Winter and Spring Wheat Cultivars, Norstar and Glenlea. Abbreviations in the column headings: S – spring wheat (Glenlea); W-winter wheat (Norstar); C – control; numbers indicate days of cold treatment. Alternate columns have expression ratios and their corresponding P-values. Values in bold are significantly different from one ($P < 0.05$). Empty cells did not have data that passed quality standards. Accession numbers are for the highest BLAST hit in the TIGR Wheat Gene Index version 6.0 with the EST sequence for each gene.

to give an ample presentation of the data; P-values for each expression ratio are also given in the table.

In the winter wheat cultivar Norstar, 309 genes were induced or repressed during the cold acclimation period tested. Among these genes, 170 were significantly changed at a single time point, 96 were changed at two time points, and 43 had altered expression at all three time points. The analysis in the spring wheat cultivar Glenlea showed that 166 genes were found to be induced or repressed during the cold acclimation period tested. Among these genes, 88 were significantly changed at a single time point, 48 were changed at two time points, and 30 had altered expression at all three time points. The high proportion of genes whose change of expression were detected at only one time point indicate that the number of genes that are classified as being regulated by cold treatment is highly dependant on the degree of sampling during the course of cold treatment. The higher number of genes that were shown to have altered expression in winter wheat is partially due to the greater degree of experimental replication carried out with Norstar, and thus more genes with relatively small changes in expression could be classified as having statistically significant changes.

5.3 Differences in Gene Induction/Repression and Steady State Levels of mRNAs in Spring and Winter Wheat

To compare gene expression between the winter and spring cultivars, two methods were used. First, gene expression was evaluated by comparing gene induction and repression at one, six and 36 days acclimation versus appropriate controls in the two cultivars for all genes detected in both cultivars. Secondly, direct comparison of steady state mRNA levels in Norstar and Glenlea plants that had the same cold acclimation

regime were made. The first approach can detect genes that have different levels of induction or repression in the two cultivars that are not necessarily classified as induced or repressed in the individual cultivar analysis. For example, genes that are slightly repressed in one cultivar and slightly induced in the second cultivar may not be scored as significantly altered in individual comparisons but may be significantly different in comparison of the two cultivars. Data from these comparisons is shown in Tables 3, 4, and 5.

Plants with one day of LT treatment showed five genes that were differentially regulated in both cultivars, one of these was more strongly induced in winter wheat and four were more strongly induced in spring wheat (Table 3, column Induction W/S). Plants which were LT-treated for six days showed 37 genes that had significant differences in gene induction between the two cultivars (Table 4, column Induction W/S). Among these, 22 genes were more strongly induced in winter wheat, five of which were nearly unchanged (less than 1.3-fold increase) in the spring wheat. Fifteen genes were more strongly induced in the spring cultivar, eleven of which were unchanged or repressed in the winter wheat.

Comparison between Norstar (winter) and Glenlea (spring) after 36 days of cold acclimation showed 37 genes that were differentially induced or repressed between the two cultivars (Table 5, column Induction W/S). Among these, 13 were more strongly induced in winter wheat, than in spring wheat. Twenty four genes had a Winter Induction/Spring Induction ratio less than 0.66. The most uniform feature of this class of genes is the near absence of gene in induction in the winter cultivar. One of these genes is induced in winter wheat, and the others are repressed at 1.5-fold or lower or have

expression that is not significantly different from the control plants. In spring wheat, genes in this class include

Clone	TIGR Accession #	Annotation	Control W/S	Spring Induction	Winter Induction	Induction W/S
J470	TC85079	LEA14-A	5.69	4.56 *	11.55 *	2.53 *
J517	TC91471	LT 101 putative membrane protein	4.90	7.72 *	4.50 *	0.58 *
J275	TC87867	LT 101/ESI3 LT and salt induced	4.92	9.93 *	5.08 *	0.51 *
J330	TC87536	LEA/RAB/COR cold-responsive protein	2.60	14.59 *	6.85 *	0.47 *
J666	TC69676	WCOR14a - cold regulated protein	2.60	14.44 *	5.55 *	0.38 *

Table 3. Genes with Different Induction or Repression in Winter and Spring Wheat at One Day of Cold Acclimation. TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6.0. Control W/S was calculated as the ratio of (Expression W/S) / (Induction W/S) for plants cold treated for six days. Note the control plants for six days cold acclimation plants were the same as controls for one day cold acclimated plants. Statistical significance (*) criteria is P-value <= 0.05).

Clone	TIGR Accession	Annotation	Control W/S	Spring Induction	Winter Induction	Induction W/S	Expression W/S
C95	TC87317	Actin depolymerization factor	1.05	3.76 *	12.14 *	3.23 *	3.40 *
E31_F	TC92493	COR39 protein	1.19	6.52 *	20.50 *	3.14 *	3.75 *
J374	TC87737	Dehydrin WZY1-1	1.63	7.85 *	22.81 *	2.91 *	4.74 *
J619	TC87319	WCOR719 cold	0.83	2.91 *	7.49 *	2.57 *	2.14 *
J806	TC68452	Lipid transfer protein	0.75	5.99 *	14.40 *	2.41 *	1.79 *
J470	TC85079	LEA14-A	ND	ND	13.46 *	ND	6.67 *
C36	TC72617	WCOR80 cold acclimation	ND	ND	4.74 *	ND	3.58 *
J642	TC79747	Unknown protein	ND	ND	6.85 *	ND	2.87 *
J822	TC89914	Guanine regulatory protein	ND	ND	7.02 *	ND	2.49 *
J657	TC65078	Gamma-thionin precursor	ND	ND	2.86 *	ND	2.35 *
J733	TC94499	Golgi-localized protein	ND	ND	ND	ND	2.04 *
J459	TC85842	Probable acclimation protein	1.24	7.49	14.65	1.96	2.43 *
C272	TC65072	Gamma-thionin precursor	0.71	1.03	1.98 *	1.93 *	1.37
C213	TC73110	Unknown protein	0.80	1.92 *	3.64 *	1.90 *	1.52
JB074	TC65862	Phosphoethanolamine methyltransferase	1.20	4.45 *	8.40 *	1.89 *	2.26 *
J339	TC93659	WCS120 protein	2.45	6.04 *	11.32 *	1.87 *	4.59 *
J765	TC69449	Chitinase 2	1.47	3.74 *	6.88 *	1.84 *	2.70 *
B60_F	TC67796	WCOR825 cold acclimation protein	0.73	1.16	2.09 *	1.81 *	1.32
C197	TC87822	Unknown protein	0.89	1.05	1.82 *	1.74	1.55 *
C55	TC67115	Cold acclimation protein	1.33	1.55	2.65 *	1.70	2.26 *
E25_F	TC83987	Unknown Protein	ND	0.91	1.55 *	1.70 *	ND
J862	TC88307	Apolipoprotein D	0.69	1.38 *	2.34 *	1.69 *	1.18
C161	TC96342	Low temperature induced protein	1.27	11.75 *	19.92 *	1.69	2.15 *
J882	TC63551	Inorganic pyrophosphatase	0.68	1.57 *	2.63 *	1.68 *	1.15
C290	TC92066	Ice recrystallisation inhibition protein	0.93	1.50 *	2.42 *	1.61 *	1.51
J680	TC79239	Ice recrystallisation inhibition protein	1.33	5.19 *	8.17 *	1.57 *	2.10 *
C108	TC93229	Unknown Protein F15K9.6	1.16	2.12 *	3.29 *	1.55 *	1.81 *
C205	TC64307	Ubiquitin	0.74	1.09	1.67 *	1.52 *	1.12
C28	TC92066	Ice recrystallisation inhibition protein	1.74	21.93 *	30.70 *	1.40 *	2.43 *
C196	TC67656	Remorin 2	1.13	1.18	1.62 *	1.38	1.56 *
J885	TC92901	Cold acclimation protein	1.93	13.91 *	18.70 *	1.34 *	2.59 *
C29	AL821437	Cold acclimation protein	1.72	3.17 *	3.60 *	1.14	1.95 *
J458	TC65326	Rubisco small subunit	0.60	0.54 *	0.60 *	1.10	0.66 *
C465	TC66723	Unknown protein	0.52	0.77	0.73 *	0.95	0.49 *
JB080	TC85188	Lipid transfer protein	0.63	0.73 *	0.63 *	0.85	0.54 *
C653	TC63643	Carbonic anhydrase	0.75	0.37 *	0.32 *	0.85	0.64 *
J517	TC91471	Blt101.2./ESI3 Lt, salt-stress induced protein	2.43	4.81 *	3.87 *	0.81 *	1.96 *
J275	TC87867	Blt101/ESI3 LT, salt-stress induced protein .	2.33	4.99 *	4.02 *	0.80 *	1.87 *
JB018	TC65343	Ribulose-1,5-bisphosphate carboxylase	0.77	0.44 *	0.33 *	0.76	0.59 *
J302	TC87869	Blt101/ESI3 LT, salt-stress induced protein	2.84	5.87 *	4.42 *	0.75 *	2.14 *
C302	CA630452	Hypothetical protein	2.13	5.87 *	4.42 *	0.75	1.60 *
J883	TC64211	Carbonic anhydrase	0.96	0.58 *	0.39 *	0.68	0.66 *

E32_F	TC65820	Nonspecific lipid-transfer protein	1.14	3.33 *	2.21 *	0.66 *	0.76
C86	TC84168	2-oxoglutarate/malate translocator	1.92	12.79 *	8.27 *	0.65 *	1.24
C170	TC63101	Hypothetical protein	1.40	1.57 *	0.81 *	0.52 *	0.72
F29_R	TC93925	LRK10-like receptor kinase	1.28	2.17 *	1.12	0.52 *	0.66
S15_F	CA698312	Putative aspartate--tRNA ligase	ND	1.26	0.65 *	0.51 *	ND
J893	CA621935	Alpha-soluble NSF attachment protein	ND	1.28	0.63 *	0.50 *	ND
J851	TC64697	C13 endopeptidase NP1 precursor	ND	1.30	0.63 *	0.49 *	ND
C75	BQ170273	Peptidase-like protein	1.40	2.03 *	0.98	0.48 *	0.68
J279	TC72165	Unknown Triticae protein	1.51	1.98 *	0.90	0.46 *	0.69 *
J666	TC69676	WCOR14a, cold-responsive protein	3.67	18.85 *	8.41 *	0.45 *	1.64 *
A02_F	TC70414	Cold regulated protein	2.28	1.95 *	0.75 *	0.39 *	0.88
J442	TC102138	E2 Ubiquitin ligase	2.37	1.73 *	0.62 *	0.36 *	0.85
J342	TC68241	Hypothetical protein	2.35	1.66 *	0.43 *	0.26 *	0.61
J626	TC63138	Cell wall-associated hydrolase	3.10	1.73 *	0.41 *	0.24 *	0.73
J854	TC87536	LEA/RAB-related COR	7.20	12.55 *	2.64 *	0.21 *	1.51 *

Table 4. Genes with Different Induction, Repression or Expression in Winter and Spring Wheat at 6 days of Cold Acclimation. TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6.0. Control W/S was calculated as the ratio of (Expression W/S) / (Induction W/S) for plants cold treated for six days. Spring and Winter Induction were measured directly as a ratio of signal from cold treated plants to control plants. Induction W/S was calculated as the ratio of Induction in winter and spring wheat. Expression W/S was measured directly as the ratio of signal from the two genotypes after six days of cold treatment. Statistical significance (*) criteria is P-value <= 0.05.

Clone	TIGR Accession	Annotation	Control W/S	Spring Induction	Winter Induction	Induction W/S	Expression W/S
J339	TC93659	WCS120 protein	1.78	2.15 *	11.63 *	5.41 *	9.62 *
C29	AL821437	Cold acclimation protein	0.98	5.24 *	20.29 *	3.87 *	3.80
J900	BQ161796	Calcium binding EF-hand protein	0.47	2.64	8.82	3.34 *	1.56
C161	TC96342	LT induced protein	1.70	14.80 *	47.56 *	3.21 *	5.46
J619	TC87319	Actin depolymerization factor	ND	1.32	3.96	3.01 *	ND
J374	TC87737	Dehydrin WZY1-1	3.42	2.41 *	7.20 *	2.98 *	10.20 *
J459	TC85842	Probable acclimation protein	2.10	11.93 *	34.35 *	2.88 *	6.06
E31_F	TC92493	COR39 cold-regulated gene	ND	2.43	6.84 *	2.82 *	ND
F29_R	TC93925	LRK10 receptor-like kinase	0.34	1.23	3.23 *	2.63 *	0.89
J536	TC84127	Rubisco large subunit	ND	0.77	1.67	2.18 *	ND
C274	TC93229	Expressed protein	ND	1.20	2.43	2.02 *	ND
J680	TC79239	Ice recrystalization inhibitor	1.23	4.62	8.85	1.91 *	2.36
J275	TC87867	ESI3/Blt 101 LT, NaCl induced	1.65	2.14 *	3.58 *	1.67 *	2.76
S06_F	TC91686	Lipoxygenase	0.45	0.93	0.96	1.03	0.47 *
C09	BU979312	Unknown protein,	0.65	0.87	0.87	1.00	0.64 *
J625	BJ276977	Expressed protein	0.55	0.81	0.77	0.96	0.53 *
J377	TC69422	Plastid ribosomal protein CL9	0.62	0.77	0.50 *	0.65	0.40 *
J330	TC87536	LEA/RAB- protein	3.40	5.82 *	3.50 *	0.60 *	2.04
J597	TC65328	Rubisco small subunit	0.48	0.43 *	0.23 *	0.52 *	0.25 *
J324	TC64862	Rubisco small subunit	0.81	0.99	0.50 *	0.51 *	0.41 *
J773	TC65728	HMG1/2-like protein	1.42	1.22	0.62	0.51 *	0.72
S09_F	TC82882	LHCI-680 PS I antenna protein	1.00	1.31 *	0.62 *	0.47 *	0.47 *
J883	TC64211	Carbonic anhydrase	0.49	0.94	0.42 *	0.45 *	0.22 *
JB036	TC64840	Rubisco small subunit	1.10	1.05	0.47 *	0.45 *	0.49 *
C301	TC68028	PE-PGRS homolog MAG24-1	0.93	0.72	0.32 *	0.44	0.41 *
J382	TC87552	RRM-containing protein	ND	ND	0.70 *	ND	0.42 *
J884	TC70334	Peroxidase	1.10	2.04	0.85	0.42 *	0.46
J649	TC90819	Hypothetical protein	0.94	0.85	0.35	0.41 *	0.39 *
J458	TC65326	Rubisco small subunit	0.71	0.54 *	0.21 *	0.40 *	0.28 *
J393	TC64827	Hypothetical protein	1.23	0.99	0.36 *	0.36 *	0.44 *
JB025	TC84155	Probable heat shock protein	1.33	1.08	0.37 *	0.34 *	0.46 *
C26	TC83130	LHCI-680 PS I antenna protein	1.59	1.17	0.38 *	0.33 *	0.52 *
JB018	TC65343	Rubisco small subunit	0.84	0.68	0.21 *	0.31 *	0.26 *
C77	TC63643	Carbonic anhydrase	0.83	1.00	0.31 *	0.31 *	0.25 *
S41_F	TC86043	Chlorophyll a/b-binding protein	1.57	4.16 *	1.09	0.26 *	0.41 *
B34_F	TC83306	Chlorophyll a/b-binding protein	ND	2.26 *	0.57 *	0.25 *	ND
F33_F	TC86043	Chlorophyll a/b-binding protein	ND	2.93 *	0.71 *	0.24 *	ND
C177	TC63105	Chlorophyll a/b-binding protein	1.97	2.08 *	0.46 *	0.22 *	0.43 *
J444	TC63552	Chlorophyll a/b-binding protein	1.81	4.64 *	0.97	0.21 *	0.38 *
J676	TC83195	Latex-abundant protein putative	2.06	3.85	0.78	0.20 *	0.42
B24_F	TC86051	Chlorophyll a/b-binding protein	ND	5.75 *	0.77	0.13 *	ND

Table 5. Genes with Different Induction, Repression or Expression in Winter and Spring Wheat at 36 days of Cold Acclimation. TIGR accessions are the most similar sequence in the TIGR Wheat Gene Index, V6.0. Control W/S was calculated as the ratio of (Expression W/S) / (Induction W/S) for plants cold treated for 36 days. Spring and Winter Induction were measured directly as a ratio of signal from cold treated plants to control plants. Induction W/S was calculated as the ratio of Induction in winter and spring wheat. Expression W/S was measured directly as the ratio of signal from the two genotypes after 36 days of cold treatment. Statistical significance (*) criteria is P-value <= 0.05.

genes that are induced, repressed and relatively unchanged relative to a 1.5-fold threshold.

Most genes that were differentially induced or repressed between the cultivars were different at only one time point. Only 15 genes had different expression in the two cultivars at more than two time points and one gene (J275, a LT10/ESI3 gene) was differentially expressed at all three time points, these genes had general patterns of expression that were parallel in that they were induced or repressed in both cultivars but more strongly induced or repressed in one cultivar than the other. However one gene, F29_R, an LRK-10 like receptor kinase, had different patterns of expression in winter and spring wheat rather than simply a different degree of induction or repression. The F29_R gene is repressed in both spring and winter wheat after one day of cold acclimation (treatment/control ratio of 0.63 and 0.48, respectively) but after six days of acclimation it is induced two-fold in the spring cultivar but not induced in the winter cultivar. At 36 days of cold acclimation the gene is three-fold induced in winter wheat but is expressed at control levels in spring wheat.

In addition to the comparison of gene induction and repression, we measured the steady state level of mRNAs in winter and spring wheat by direct comparison of target cDNA from the two cultivars after six and 36 days of cold acclimation. This comparison detected differences in expression that are not detected by comparison of gene induction and repression in the two cultivars. This may be the case if the levels of expression are not the same in control plants for the two cultivars and because of reduced experimental variance since the comparison is based on the comparison of two sample sets rather than four sets that are required to compare gene induction between the two cultivars. Though many of the same genes were detected by the two methods of comparison, the direct

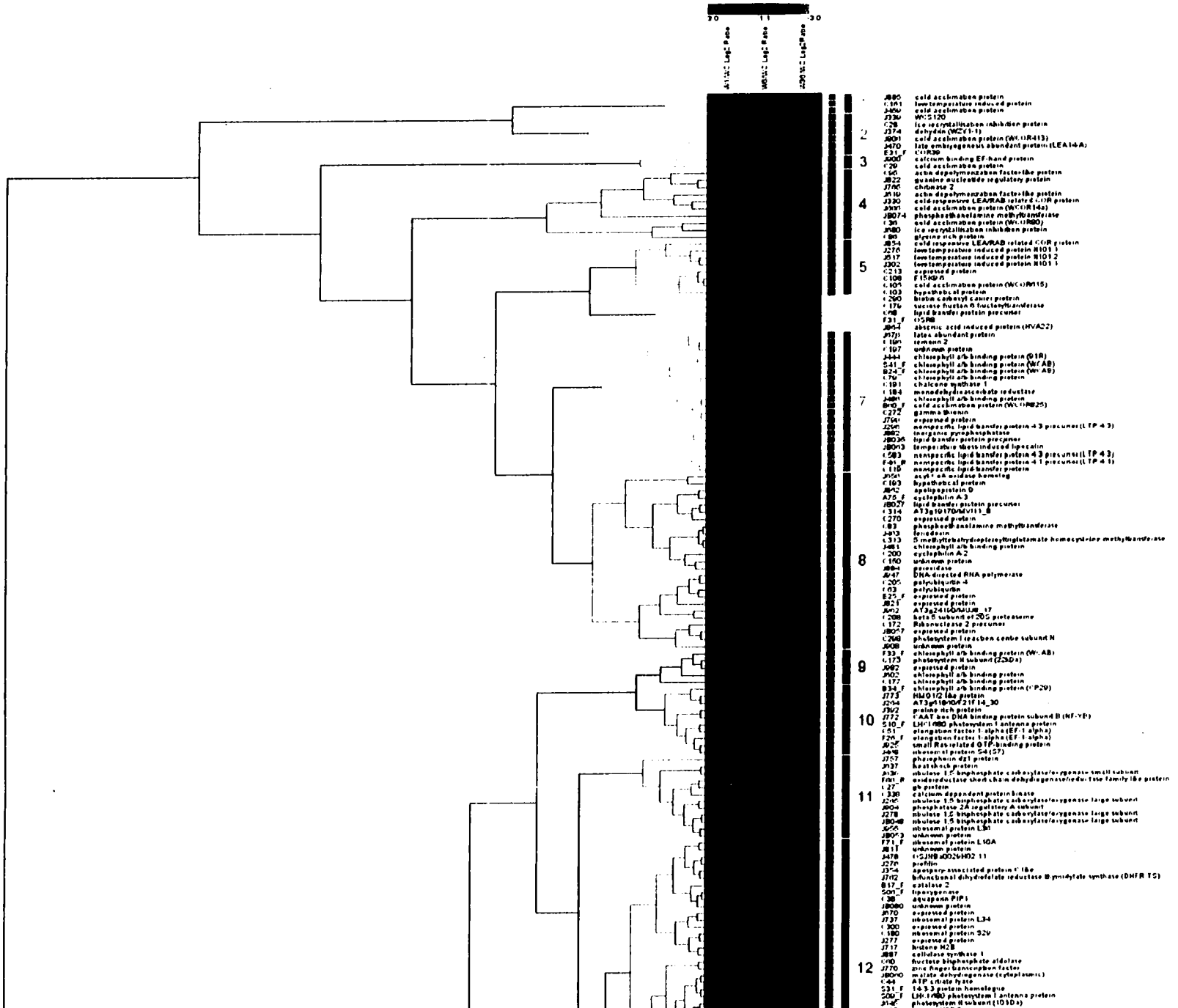
comparison of the two cultivars allowed the detection of an additional 14 differentially expressed genes in the two cultivars at six days, eight of these were more strongly expressed in winter than in spring (Tables 4 and 5, column Expression W/S). Four genes were differentially expressed in the two cultivars at 36 days of cold treatment, all of which were more strongly expressed in Glenlea than in Norstar.

Though most genes that were seen to be more strongly induced in one cultivar were also had higher levels of steady state mRNA levels in that cultivar, this was not universally the case. This indicates that the relative level of expression in non-acclimated controls in the two cultivars is also different. The relative level of gene expression of the control plants was calculated from other direct hybridization measurements (Tables 4 and 4). These data demonstrate a more complex comparison of genes in the two cultivars. For example, several genes including J302, J517, J275, C86, J666 and J854, have higher levels of induction in spring wheat than in winter wheat measured between one day control and six days of acclimation but have higher expression in winter than in spring wheat, presumably because they had higher basal levels of expression in the non-acclimated controls on day one. In most cases, the induction ratio and the expression ratios for genes are not equal, indicting that the level of expression in the control plants are not equal in the two cultivars. These analyses indicate that differential expression in control plants between cultivars may indicate a genetic predisposition to a different degree of FT. This approach to gene discovery compliments that of comparison of gene induction rates described above. In most cases direct comparison of mRNA levels was in agreement with the comparison of gene induction but it also detected additional genes whose induction rates were too small to be detected or were somewhat masked by apparently elevated expression levels in the control plants of one of the cultivars. It also

demonstrates the utility of multiple comparisons in detecting key indicators of genotypes with superior cold tolerance.

5.4 Cluster Analysis

Cluster analysis was done to characterize changes in gene expression over the time course of the experiment and to further characterize differences in gene expression between the freezing tolerant winter wheat and the less freezing tolerant spring wheat. Genes with detectable expression for all three time points in winter wheat and which had an expression ratio significantly different than one ($P < 0.05$) for at least one time point (compared to non-cold acclimated control plants) were used for cluster analysis. There was no threshold imposed for the expression ratio. Genes with the same functional annotation that were not members of the same contig were kept as independent sets of values. Average-linkage hierarchical clustering analysis of the three-point cold-acclimation time course for was done for 215 genes that were characterized in winter wheat and 136 genes in spring wheat and results are in Figures 2 and 3, respectively. To facilitate the detection of differences and similarities of gene expression in the two cultivars, the combined data sets for the two cultivars which included 125 common genes were clustered together and the results are in Figure 4. Genes in the combined data set from winter and spring wheat grouped into 23 clusters with similar patterns of expression. The majority of clustered gene expression patterns in Norstar and Glenlea are similar as might be expected based on analysis of gene expression at individual time points discussed above. Clusters 1 to 11, 14 and 15 show patterns of relatively strong gene induction during cold acclimation. The sub patterns of each cluster highlights subtle differences in gene expression. Clusters 12, 13, 16, 22, and 23 are comprised of genes



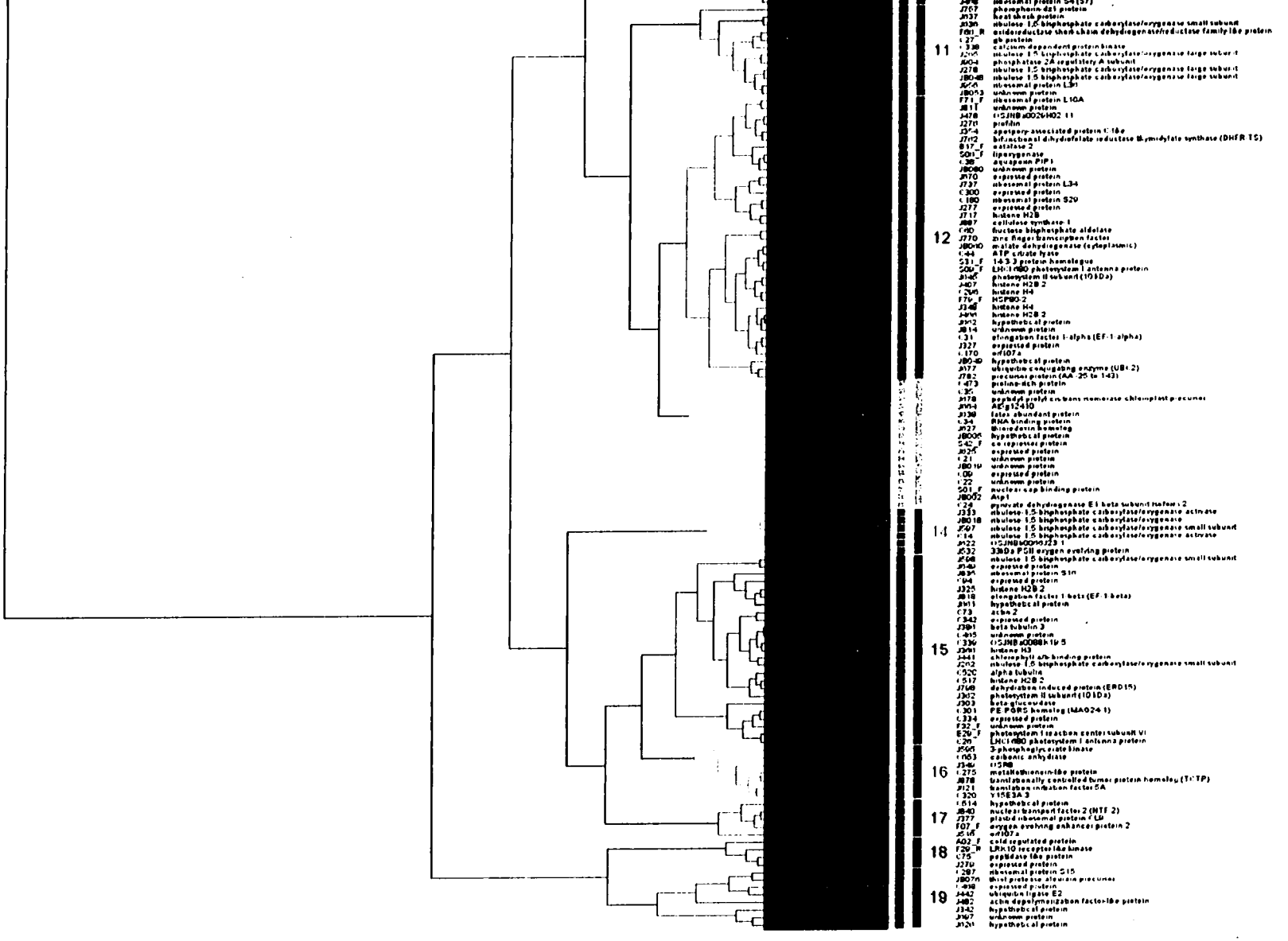
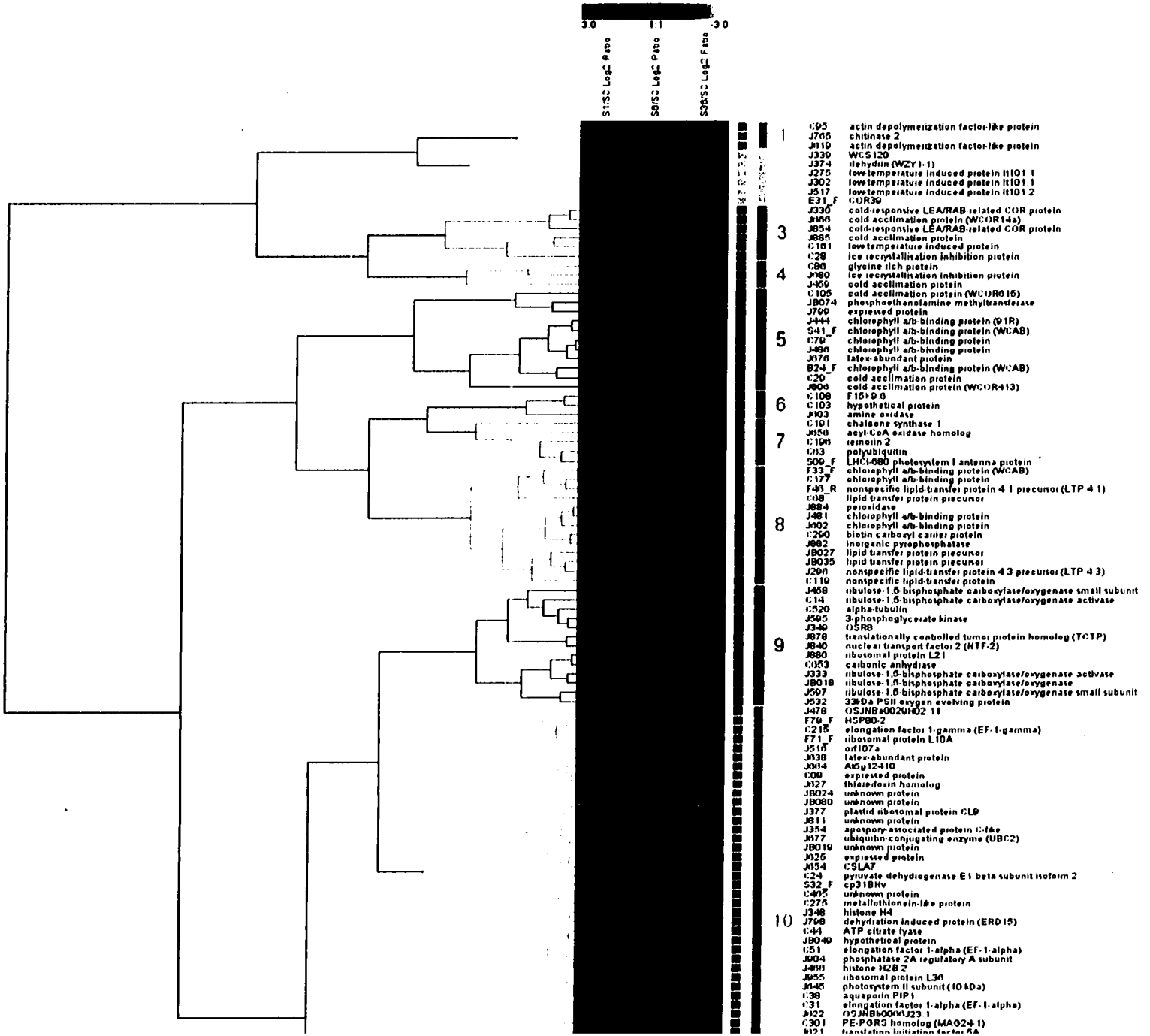


Figure 2. Average linkage clustering analysis dendogram of winter wheat (cv Norstar) genes for which all three time points are present (W1/WC, W6/WC, W36/WC), and for which at least one of these time points is statistically significative ($P < 0.05$). No fold-induction/repression threshold was set. Cluster assignment was done subjectively by looking at the expression profiles of the genes from each assigned cluster. All ratio values are given in Log_2 format. The ratio cutoff was chosen to give a maximum of information for the majority of the genes. Ratios are Log_2 transformed. Some genes with very high induction/repression (>3.0-fold) show as saturated.


3.0
1.1
-3.0
S1/S0: Log2 Ratio
S2/S0: Log2 Ratio
S3/S0: Log2 Ratio
1
3
4
5
6
7
8
9
10
C95 actin depolymerization factor-like protein
J765 chitinase 2
J419 actin depolymerization factor-like protein
J339 WCS120
J374 dehydrin (WZY1-1)
J275 low-temperature induced protein lt101.1
J302 low-temperature induced protein lt101.1
J517 low-temperature induced protein lt101.2
E31_F COR39
J330 cold-responsive LEA/RAB-related COR protein
J466 cold acclimation protein (WCOR14a)
J854 cold-responsive LEA/RAB-related COR protein
J885 cold acclimation protein
C101 low-temperature induced protein
C28 ice recrystallisation inhibition protein
C86 glycine rich protein
J480 ice recrystallisation inhibition protein
J469 cold acclimation protein
C105 cold acclimation protein (WCOR615)
JB074 phosphoethanolamine methyltransferase
J799 expressed protein
J444 chlorophyll a/b-binding protein (91R)
S41_F chlorophyll a/b-binding protein (WCAB)
C79 chlorophyll a/b-binding protein
J486 chlorophyll a/b-binding protein
J870 late-abundant protein
B24_F chlorophyll a/b-binding protein (WCAB)
C20 cold acclimation protein
J866 cold acclimation protein (WCOR413)
C108 F15I9.6
C103 hypothetical protein
J403 amine oxidase
C101 chalcone synthase 1
J850 acyl-CoA oxidase homolog
C101 remorin 2
C83 polyubiquitin
S09_F LHCI-680 photosystem I antenna protein
F33_F chlorophyll a/b-binding protein (WCAB)
C177 chlorophyll a/b-binding protein
F46_R nonspecific lipid-transfer protein 4.1 precursor (LTP 4.1)
C08 lipid transfer protein precursor
J884 peroxidase
J481 chlorophyll a/b-binding protein
J402 chlorophyll a/b-binding protein
C260 biotin carboxyl carrier protein
J882 inorganic pyrophosphatase
JB027 lipid transfer protein precursor
JB035 lipid transfer protein precursor
J290 nonspecific lipid-transfer protein 4.3 precursor (LTP 4.3)
C119 nonspecific lipid-transfer protein
J458 ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
C14 ribulose-1,5-bisphosphate carboxylase/oxygenase activase
C520 alpha-tubulin
J565 3-phosphoglycerate kinase
J340 OSR8
J878 translationally controlled tumor protein homolog (TCTP)
J840 nuclear transport factor 2 (NTF-2)
J880 ribosomal protein L21
C053 carbonic anhydrase
J333 ribulose-1,5-bisphosphate carboxylase/oxygenase activase
JB018 ribulose-1,5-bisphosphate carboxylase/oxygenase
J597 ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
J532 33kDa PSII oxygen evolving protein
J478 OSJNBa0029H02.11
F79_F H5P80-2
C215 elongation factor 1-gamma (EF-1-gamma)
F71_F ribosomal protein L10A
J515 orf107a
J438 late-abundant protein
J004 At5g12410
C09 expressed protein
J427 thioredoxin homolog
JB024 unknown protein
JB080 unknown protein
J377 plastid ribosomal protein CL9
J811 unknown protein
J354 apospory-associated protein C-like
J577 ubiquitin-conjugating enzyme (UBC2)
JB019 unknown protein
J426 expressed protein
J454 OSLA7
C24 pyruvate dehydrogenase E1 beta subunit isoform 2
S32_F cp31BHv
C405 unknown protein
C275 metallothionein-like protein
J348 histone H4
J798 dehydration induced protein (ERD15)
C44 ATP citrate lyase
JB049 hypothetical protein
C51 elongation factor 1-alpha (EF-1-alpha)
J804 phosphatase 2A regulatory A subunit
J488 histone H2B.2
J855 ribosomal protein L30
J445 photosystem II subunit (10 kDa)
C38 aquaporin PIP1
C31 elongation factor 1-alpha (EF-1-alpha)
J422 OSJNBb0009J23.1
C301 PE-PORS homolog (MAG24.1)
J421 translation initiation factor 5A

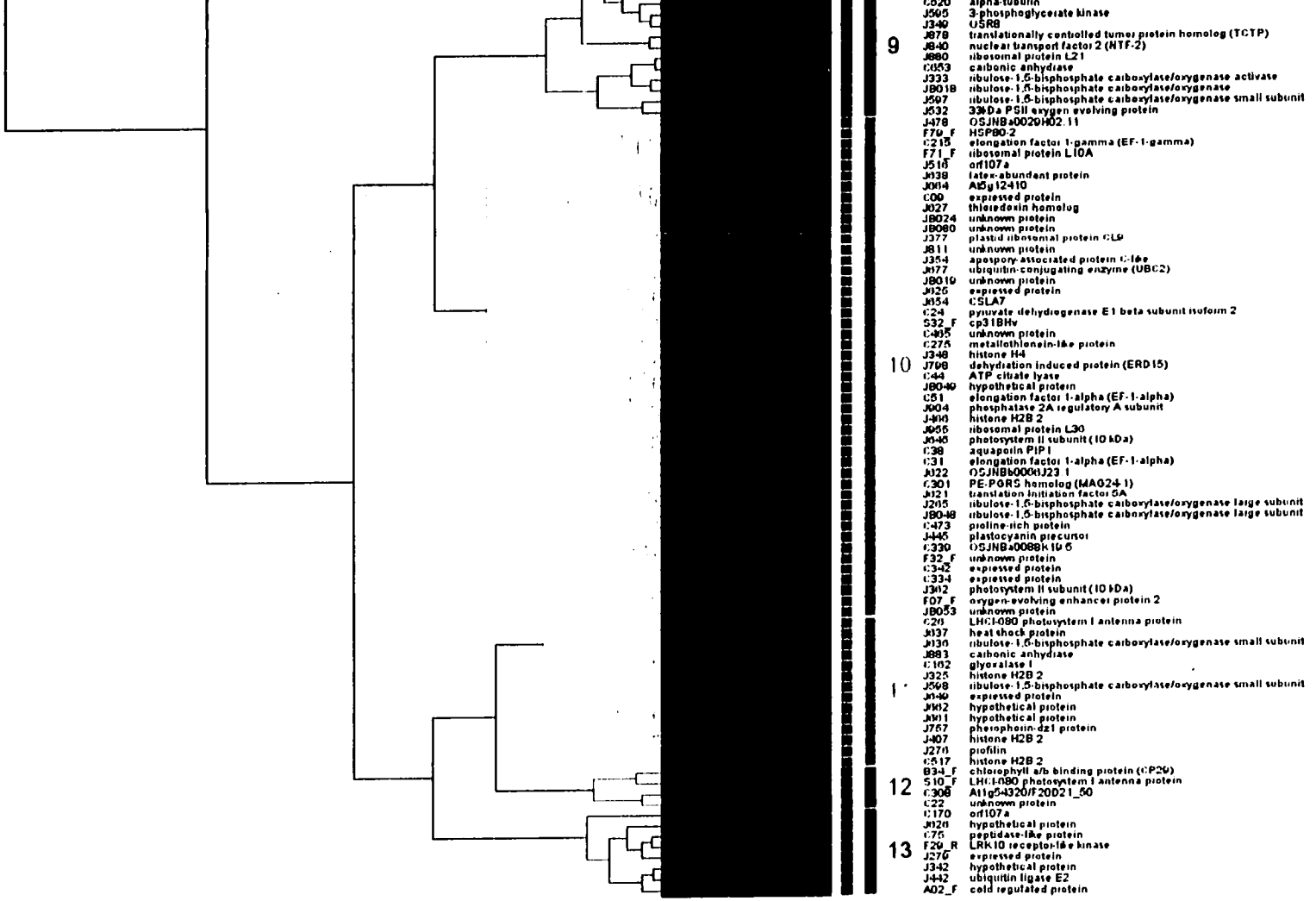

9
10
1
12
13
C620 Alpha-tubulin
J505 3-phosphoglycerate kinase
J340 U5R8
J878 translationally controlled tumor protein homolog (TCTP)
J840 nuclear transport factor 2 (NTF-2)
J880 ribosomal protein L21
C053 carbonic anhydrase
J333 ribulose-1,5-bisphosphate carboxylase/oxygenase activase
JB018 ribulose-1,5-bisphosphate carboxylase/oxygenase
J597 ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
J532 33kDa PSII oxygen evolving protein
J478 OSJNBa0029H02.11
F79_F H5P90.2
C215 elongation factor 1-gamma (EF-1-gamma)
F71_F ribosomal protein L10A
J516 orf107a
J038 latex-abundant protein
J004 At5g12410
C09 expressed protein
J027 thioredoxin homolog
JB024 unknown protein
JB080 unknown protein
J377 plastid ribosomal protein CL9
J811 unknown protein
J354 apospory-associated protein C-like
J077 ubiquitin-conjugating enzyme (UBC2)
JB019 unknown protein
J026 expressed protein
J154 CSLA7
C24 pyruvate dehydrogenase E1 beta subunit isoform 2
S32_F cp31BHv
C485 unknown protein
C275 metallothionein-like protein
J348 histone H4
J708 dehydration induced protein (ERD15)
C44 ATP citrate lyase
JB049 hypothetical protein
C51 elongation factor 1-alpha (EF-1-alpha)
J004 phosphatase 2A regulatory A subunit
J-806 histone H2B 2
J955 ribosomal protein L30
J045 photosystem II subunit (10 kDa)
C38 aquaporin PIP1
C31 elongation factor 1-alpha (EF-1-alpha)
J022 OSJNBb0006J23.1
C301 PE-PGRS homolog (MAG24-1)
J021 translation initiation factor 5A
J205 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
JB048 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
C473 proline-rich protein
J445 plastocyanin precursor
C330 OSJNBa0088K19.6
F32_F unknown protein
C342 expressed protein
C334 expressed protein
J012 photosystem II subunit (10 kDa)
F07_F oxygen-evolving enhancer protein 2
JB053 unknown protein
C20 LHCII-080 photosystem I antenna protein
J037 heat shock protein
J036 ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
J883 carbonic anhydrase
C162 glyoxalase I
J325 histone H2B 2
J598 ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
J049 expressed protein
J062 hypothetical protein
J001 hypothetical protein
J767 pterophorin-dz1 protein
J-807 histone H2B 2
J270 profilin
C517 histone H2B 2
B34_F chlorophyll a/b binding protein (CP29)
S10_F LHCII-080 photosystem I antenna protein
C306 At1g54320/F20D21_50
C22 unknown protein
C170 orf107a
J026 hypothetical protein
C75 peptidase-like protein
F29_R LRK10 receptor-like kinase
J276 expressed protein
J342 hypothetical protein
J-442 ubiquitin ligase E2
A02_F cold regulated protein

Figure 3. Average linkage clustering analysis dendogram of spring wheat (cv Glenlea) genes for which all three time points are present (S1/SC, S6/SC, S36/SC), and for which at least one of these time points is statistically significative ($P < 0.05$). No fold-induction/repression threshold was set. Cluster assignment was done subjectively by looking at the expression profiles of the genes from each assigned cluster. The ratio cutoff was chosen to give a maximum of information for the majority of the genes. Ratios are Log_2 transformed. Some genes with very high induction/repression (>3.0-fold) show as saturated.

4
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23

Figure 4. Average linkage clustering analysis dendogram of the combined winter wheat (cv Norstar) and spring wheat (cv Glenlea) genes for which all three time points are present in at least one cultivar (W1/WC, W6/WC, W36/WC, and/or S1/SC, S6/SC, S36/SC), and for which at least one of these time points is statistically significative ($P < 0.05$). No fold-induction/repression threshold was set. Cluster assignment was done subjectively by looking at the expression profiles of the genes from each assigned cluster. The ratio cutoff was chosen to give a maximum of information for the majority of the genes. Ratios are Log_2 transformed. Some genes with very high induction/repression (>3.0-fold) show as saturated.

with combination of induction and repression at different time points. Clusters 17 to 21 include genes that were predominantly repressed during cold acclimation. The high degree of similarity in the expression patterns of the genes within a cluster suggests that they may be regulated by common signaling pathways.

Those genes whose expression patterns differ in Norstar and Glenlea are readily observed in that they do not cluster together in Figure 4. The analysis of co-clustering of the 128 genes for which complete data exists for the spring cultivar spring and winter cultivar revealed that 48 genes show highly similar patterns of expression in the two cultivars in that they grouped into the same cluster, 53 genes showed similar patterns of expression but had some quantitative differences at one or more time point as seen by clustering into different but similar clusters. Different patterns of expression were observed for 21 genes in the two cultivars, as observed by the grouping of those genes into distinct clusters with clearly different patterns of expression (Figure 5). The observed qualitative or quantitative changes in expression patterns strongly suggest that important differences exist in cold signaling pathways between cultivars. Therefore, these genes are candidates for the elucidation of these pathways, as they may encode for products that function in FT. Below is a brief discussion of genes with important differences in expression in the two cultivars and their possible role in the cold acclimation process.

5.5 Previously known cold regulated genes

Microarray analysis identified a large number of genes with relatively strong induction during cold acclimation, in some cases genes showed greater than 20-fold

3.0
W1/WC Log2 Ratio
W6/WC Log2 Ratio
W36/WC Log2 Ratio
1:1
S1/SC Log2 Ratio
S6/SC Log2 Ratio
S36/SC Log2 Ratio
-3.0
C79
J444
J070
C190
F33_F
J48_T
J002
J510
J507
A02_F
C75
F29_R
J275
J342
J520
J442
C170
S09_F
J878
C22
C09
chlorophyll a/b-binding protein
chlorophyll a/b-binding protein (PIR)
latex-abundant protein
remorin 2
chlorophyll a/b-binding protein (WCAB)
chlorophyll a/b-binding protein
chlorophyll a/b-binding protein
orf107a
ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
cold regulated protein
peptidase-like protein
LRK10 receptor-like kinase
expressed protein
hypothetical protein
hypothetical protein
ubiquitin ligase E2
orf107a
LHCI-680 photosystem I antenna protein
translationally controlled tumor protein homolog (TCTP)
unknown protein
expressed protein

Figure 5. Selected genes that showed distinct clustering patterns in samples from winter and spring wheat. Columns one to three show treatment/control ratios of the winter wheat Norstar, cold treated at 4°C for one, six, and 36 days. Columns five to seven show the induction ratio of the spring wheat Glenlea, cold treated at 4°C for one, six, and 36 days, respectively. Ratios are Log_2 transformed.

induction. Many of these genes are in clusters one to six for winter wheat in Figure 2, and include a large number of the previously described genes or homologues of previously described cold acclimation-induced genes in plants such as WCS120 (Houde et al. 1992), actin depolymerizing factor-like (Danyluk et al. 1996), WCOR615, WCOR80, WCOR825 (Danyluk 1997), J330 cold-responsive LEA/RAB (Tsuda et al. 2000), phosphoethanolamine methyltransferase (Frenette, Charron et al. 2002), WCOR14a (N'Dong et al. 2002), WCOR413 (Breton et al 2003), ice recrystallization inhibition proteins (Worrall et al. 1998). The microarray expression data for these genes is similar to what was previously obtained by Northern blot analysis when identical time points where assayed and thus confirms the accuracy of the array analysis. Other strongly induced genes in these clusters were identified in other species, but have not been previously reported to be cold-regulated in wheat.

5.6 Photosynthesis and carbon fixation

A large number of genes encoding proteins involved in photosynthesis (PS) and carbon fixation had altered expression in response to cold treatment and a number of these were differentially expressed in the winter and spring cultivars. Many of these genes are members of gene families that showed similar patterns of expression. The degree of induction caused by LT treatment varies from gene to gene, but there is a general tendency for PS related genes to be repressed. The most accentuated differences between spring and winter wheat occur after 36 days of growth at low temperature when PS related genes tend to be down regulated in winter wheat relative to spring wheat. Genes, encoding the rubisco small subunit, carbonic anhydrase, and PSI antenna proteins are repressed in winter wheat after 36 days of cold treatment whereas they are either

induced or expressed at control levels in spring wheat (Tables 1 and 4). Chlorophyll a/b-binding proteins, in contrast to other genes encoding proteins involved in photosynthesis, were up regulated after one and six days of cold treatment in both cultivars. They remained significantly induced in the spring cultivar at 36 days, whereas in the winter cultivar these genes returned to control levels after 36 days of cold acclimation.

A shift of warm-grown plants to cold temperatures can result in an imbalance between the light energy absorbed through photochemistry versus the energy utilized through metabolism (Huner et al. 1998). This imbalance is the result of the light harvesting and O_2 generation reactions that are temperature independent, while LT conditions slow the kinetics of the carbon fixation pathway. The excess light excitation can lead to oxidative damage due to higher level of unutilized electrons. Excess electrons can damage photosystem subunits, particularly the PSII D1 reaction center subunit, leading to an increased production of reactive oxygen species (ROS).

Transcripts for light harvesting complex chlorophyll binding proteins of the outer antenna of PSII and the inner antenna of PSI have been shown to be regulated in *Arabidopsis* by cold treatment; however their pattern of regulation were quite different from those observed in our experiments (Strand et al, 1997). Though the experimental conditions and the days of sampling in the *Arabidopsis* work were not identical to the experiments reported here, the comparison of patterns of expression indicate a different response to cold in these two species and between the two wheat cultivars. In *Arabidopsis*, these genes show a rapid decline in transcript levels at one day and a gradual and partial recovery over 21 days (Strand et al, 1997). In wheat, transcripts show little change after one and six days of acclimation but at 36 days the spring wheat shows a small increase in transcript levels and the winter wheat has a reduction of transcripts for

these genes. Though *Arabidopsis* acclimates to cold, its level of tolerance is similar to cold acclimated spring wheat i.e. -4 to -8°C, thus the difference in expression for genes involved in photosynthesis may be significant indicators for patterns associated with higher FT. The biochemical significance of the decreased gene expression for photosynthesis related genes in winter wheat warrants further investigation. It may reflect a decrease in the light harvesting apparatus which would reduce the imbalance between light harvesting and dark reactions, it may also reflect reduced damage and consequently reduced recycling of proteins of the photosynthetic apparatus of winter wheat compared to spring wheat.

5.7 Oxidative Stress

In our study, peroxidase (J884) is 1.8-fold and 2.4-fold more strongly induced in spring wheat than in winter wheat at one day and at 36 days respectively; in winter wheat the gene is slightly induced at six days but is not induced at one or 36 days. Peroxidase plays a role in detoxification of peroxide, an active oxygen species that is enhanced by cold stress, and changes in peroxidase expression in cold stressed plants have been previously described in several species (*e.g.* Anderson et al 1995). The disruption of photosynthesis by cold increases the reduction state of components of the photosynthetic apparatus that contributes to increased reactive oxygen species. In cold-treated *Arabidopsis* a peroxidase was shown to be transiently induced early and was again up-regulated at seven days of cold acclimation (Fowler et al 2002). Although longer term treatments were not included in the study, the *Arabidopsis* pattern is more similar to that observed in spring wheat than winter wheat. Similarly, catalase is slightly repressed in winter wheat at six days, though it is not statistically different from spring wheat. These

patterns show a similarity between spring wheat and Arabidopsis and suggest that winter wheat has additional responses to avoid oxidative stress. The decreased turnover of components of the photosynthetic apparatus may also reflect this adaptation. The data presented here are only suggestive since peroxidases exist as a gene family of at least nine members in Arabidopsis (Ostergaard et al 1998) and the gene expression pattern differs among gene family members (Fowler et al. 2002). Further studies are necessary to describe fully the response of peroxidase genes during cold acclimation in wheat.

5.8 PIP Aquaporins

PIP aquaporins are plasma membrane intrinsic proteins that facilitate the passive transport of water across plasma membranes, and have also been shown to function in the diffusion of carbon dioxide into plant cells (Terashima I. and Ono K, 2002). The PIP1 gene family has five members in Arabidopsis and may represent more than 1% of the total plasma membrane protein content. In our experiments, a PIP1 gene (C38) showed sustained decrease in expression in spring wheat to about 65% of control level during cold treatment up to 36 days, whereas winter wheat showed a similar initial decrease in expression but at 36 days expression had returned to control levels (Table 1). Some PIP1 family members have been shown to be induced in salt stressed barley plants, whereas others were repressed (Katsuhara M., et al 2002). The over expression of a PIP1 transgene in *Nicotiana tabacum* improved plant growth under normal growth conditions but was deleterious for drought stressed plants (Aharon., et al., 2003). The decreased expression of PIP1 in cold treated plants suggests that the diffusion of water in the plant during cold acclimation is reduced and that winter wheat recovers from cold treatment induced changes in the aquaporin gene C38 more rapidly than spring wheat. PIPs are

abundant proteins in plant membranes and the TIGR assembly of wheat ESTs indicates that there are at least 20 gene family members. This gene family may provide insight to the plants' response to cold and serve as a useful marker for cold tolerance.

5.9 Protein degradation

Several genes involved with protein degradation including peptidases, polyubiquitin, a component of the proteosome and an E2 ubiquitin ligase (ESTs C63, C73, C75, C205, C208, C325, J442, J660, J677, J851, JB076) have modest changes in expression, with increases or decreases to between 1.5- to 2.7-fold levels of control plants during the cold acclimation. There are subtle differences in expression between spring and winter wheat that may reflect differential regulation of the protein degradation pathways during cold adaptation (Table 1).

5.10 Signal transduction and novel genes

Many genes encoding regulatory type proteins (J822, J713, J900, S31_F, J832, J770, C176, C338, S42_F, J904, C34, C319, J878, J840, J621,C288, C652, F29_R, J772, J925, J293) had altered expression in cold treated plants, some of which had significantly different expression in the two cultivars. Most of these genes had less than two-fold changes in expression, though some genes in this class had high levels of induction. J822 which encodes a putative guanidine binging regulatory protein was induced 9.7-fold and 11.8-fold induction in spring and winter wheat, respectively at one day of cold acclimation. J900 which encodes an EF-hand calcium binding protein was induced 2.9- and nine-fold at 36 days of LT treatment in spring and winter wheat respectively. F29_R which encodes a LRK10-like protein kinase was induced 3.2-fold in winter wheat at 36

days of LT treatment and C288 which encodes a protein kinase was induced 2.8-fold in winter wheat at six days of LT treatment. A number of other genes in this class including protein kinases, protein phosphatases, transcription factors, RNA binding protein, a 14-3-3 homolog and a Ras related GTP binding protein had altered expression in at least one cultivar one at least one time point (Table 1), though significant differences were not detected between the two cultivars. In many cases the transcripts were not detected at all time points in both cultivars for these genes, so comparative data is incomplete. Regulatory genes tend to have low levels of expression and may often have mRNAs that are below the level of detection of our experiments. Altered expression includes both gene induction and repression and interpretation of such changes in expression require further characterization of candidate genes. Those genes with differential expression between the two cultivars are high priority candidates for further characterization.

6.0 CONCLUSIONS

Microarray analysis led to the discovery of 43 novel genes which were differentially regulated between spring and winter cultivars that had not previously been reported to be LT-regulated in wheat. In addition to genes described above, they include an inorganic pyrophosphatase, 2-oxoglutarate/malate translocator, NADH dehydrogenase, a putative aspartate-tRNA ligase, c13 endopeptidase, a cell wall-associated hydrolase, a ribosomal protein, a golgi-localized protein, a NSF attachment protein, a latex-abundant protein and many genes encoding proteins of unknown function (Table 1). These genes and their products are good candidates for function analysis using bioinformatics, biochemical and genetics approaches. In addition, a large number of new cold-regulated genes were detected that were not necessarily differentially regulated between the two cultivars. The identification of this large number of novel genes using the transcription profiling demonstrates that microarray analysis for genotype comparisons is an extremely useful approach to gene discovery. The use of larger microarrays will provide more information on gene interactions during cold acclimation and will play a critical role in elucidating the different low temperature signaling pathways that lead to increased FT in wheat.

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