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Review article

# Plant responses to cold: transcriptome analysis of wheat

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## Summary

Temperature and light are important environmental stimuli that have a profound influence on the growth and development of plants. Wheat varieties can be divided on the basis of whether they require an extended period of cold to flower (vernalization). Varieties that have a requirement for vernalization also tend to be winter hardy and are able to withstand quite extreme subzero temperatures. This capacity, however, is not constitutive and plants require a period of exposure to low, non-freezing temperatures to acquire freezing tolerance: this process is referred to as cold acclimation. Cold acclimation and the acquisition of freezing tolerance require the orchestration of many different, seemingly disparate physiological and biochemical changes. These changes are, at least in part, mediated through the differential expression of many genes. Some of these genes code for effector molecules that participate directly to alleviate stress. Others code for proteins involved in signal transduction or transcription factors that control the expression of further banks of genes. In this review, we provide an overview of some of the main features of cold acclimation with particular focus on transcriptome reprogramming. In doing so, we highlight some of the important differences between cold-hardy and cold-sensitive varieties. An understanding of these processes is of great potential importance because cold and freezing stress are major limiting factors for growing crop plants and periodically account for significant losses in plant productivity.

## Introduction

Temperature and light are important abiotic stimuli that provide plants with diurnal and seasonal cues which enable them to adapt to environmental change. The autumn—winter decline in temperature and light that occurs in temperate regions act as cues enabling plants to anticipate the change in season and consequently prepare for the arrival of freezing temperatures by inducing or enhancing cold stress tolerance mechanisms. Wheat and related temperate cereal species, which are able to grow under widely different climatic conditions (Dubcovsky and Dvorak, 2007), show broad genetic variability with respect to the capacity to withstand chilling and freezing conditions (Fowler and Gusta, 1979; Monroy *et al.*, 2007)—plant species, such as rice, maize and tomato, are damaged by chilling temperatures and have no capacity to withstand freezing. Among the temperate cereals (e.g., barley, rye and wheat), there are both winter-hardy and winter-sensitive varieties. Winter-hardy cereals are able to withstand quite extreme subzero temperatures, while tender varieties are unable to withstand such conditions. However, the capacity to withstand subzero temperatures is not constitutive and even hardy plants require a period of exposure to low, non-freezing temperatures to acquire freezing tolerance. This process is referred to as cold acclimation. Non-acclimated wheat of the cultivar Norstar, for example, is killed at freezing temperatures of about

–5 °C, while after cold acclimation plants of the same cultivar can survive temperatures as low as –20 °C (Jaglo et al., 2001).

Temperature also acts as a key stimulus controlling the timing of the developmental transition from vegetative to reproductive growth ensuring that plants flower when seasonal conditions are appropriate to do so. Vernalization is the process by which plants respond to an extended period (several weeks) of cold and become competent to flower—the commitment to flowering may require further environmental cues such as appropriate day length (Putterill *et al.*, 2004; Trevaskis *et al.*, 2007; Winfield *et al.*, 2009). Winter varieties are hardy and tend to have a requirement for vernalization, while spring varieties are tender and have no such requirement.

Although both cold acclimation and vernalization are responses to low temperature, the duration of cold exposure required to initiate these responses is quite distinct. A rapid induction of cold-protective proteins is essential for surviving the sometimes sudden declines in temperature that may occur as winter approaches, and only 1 or 2 days of low, non-freezing temperatures are usually sufficient to bring about cold acclimation (Sung and Amasino, 2005). This capacity is rapidly lost, however, on a return of warmer conditions. On the other hand, given that temperature often fluctuates in the autumn, it is vitally important that short-pronounced cold spells followed by a return of warmer temperatures are not mistaken for the end of winter. Thus, plants require an extended period of cold before they become fully vernalized and competent to flower. What is more, plants retain a 'memory' of this extended exposure to cold and remain committed to flowering as temperatures rise in the spring (Sung and Amasino, 2006).

Most temperate cereals, be they winter or spring varieties, exhibit some degree of chilling tolerance. There is some debate about whether this is a constitutive characteristic or whether it is in part, or completely, induced upon exposure to cold (Jan *et al.*, 2009). Cold acclimation and the acquisition of freezing tolerance, on the other hand, require the orchestration of many different, seemingly disparate physiological and biochemical changes (Steponkus, 1984; Thomashow, 1999; Ouellet *et al.*, 2001). These changes are, at least in part, mediated through the differential expression of many genes (Guy *et al.*, 1985; Thomashow, 1999; Monroy *et al.*, 2007; Kosova *et al.*, 2008; Kosmala *et al.*, 2009). These genes are thought to be induced either by cold *per se* or by the relative state of dehydration that is brought about by cold stress (Griffith and Yaish, 2004). Many of these cold-regulated genes have been identified by transcriptome analysis. In *Arabidopsis*, for example, several hundred transcripts have been reported to respond to cold (Chen *et al.*, 2002; Seki *et al.*, 2002; Provart *et al.*, 2003; Vogel *et al.*, 2005). Similarly, in the temperate grasses a large numbers of genes have been shown to be cold responsive [(Zhang *et al.*, 2009)—perennial ryegrass, (Svensson *et al.*, 2006)—barley]. In wheat, Monroy *et al.* (2007) identified over 450 genes that were regulated by cold. Although these genes have been identified on the basis of their response to a cold stimulus, in many cases their specific function has not been discovered and their role in cold acclimation, if any, remains unknown (Tsuda *et al.*, 2000). However, there are a good number of cold-regulated genes that have been assigned specific functions either as transcription factors that act up-stream in cold acclimation or as effector molecules that act to counter the potential damaging effects of cold stress.

In this article, we provide a general overview of the present understanding of cold acclimation and the acquisition of freezing tolerance. In addition to this, we provide information obtained from two separate microarray-based studies of wheat carried out in our laboratory. In these experiments, we explored the effect of low temperature on transcriptome reprogramming in three wheat cultivars: two winter varieties (Harnesk and Solstice) and a spring variety (Paragon). In one experiment, referred to hereafter as the 'cold-shock experiment', plants were rapidly transferred from 16 to 4 °C and held for 2 days—2 days of exposure was chosen because it has been reported that many COR genes accumulate maximally within this period (Ganeshan *et al.*, 2008). In a second experiment, designed to mimic a natural autumn to winter transition, plants were exposed to a gradual decline in temperature and light (quality and day length) over several weeks (hereafter, this is referred to as the 'cold acclimation experiment'). See Winfield *et al.* (2009) for details of experimental design and procedure.

We begin this review with a brief look at global changes in the transcriptome that occur when plants are exposed to cold and provide some general consideration on their potential significance. We then move on to consider in more detail various aspects of the cold acclimation process itself.

## Global changes in transcripts upon exposure to cold

It has long been known that many changes in gene expression occur when plants are exposed to cold stress

(Guy *et al.*, 1985; Thomashow, 1999). In microarray-based analysis of the *Arabidopsis* transcriptome, it has been estimated that between 4% [(Lee *et al.*, 2005)—exposure to cold of 24 h] and 20% [(Hannah *et al.*, 2005)—exposure to cold for up to 14 days] of the genome is cold regulated. In a microarray study of spring and winter wheat varieties, Monroy *et al.* (2007) reported there to be *c.* 8% of features that showed altered levels of expression in response to cold (>2-fold change). However, in this latter case, the features on the array were highly selected to represent regulatory genes and genes involved in signal transduction and so are not directly comparable with results using more general array platforms. It has also been shown that both up- and down-regulation of gene expression occur, but that, generally more genes are up-regulated than down-regulated. In *Arabidopsis*, Fowler and Thomashow (2002) reported that of 302 genes found to be cold responsive, 88 (27%) decreased in abundance.

In our study, using the Affymetrix GeneChip Wheat Array (62 000 features representing approximately 55 000 transcripts), the number of transcripts changing after a cold shock (2 days at 4 °C) was broadly similar for all three varieties: with 2.85% (Harnesk), 3.46% (Paragon) and 2.30% (Solstice) of the wheat genome as represented on the array showing a greater than twofold change (Figure 1a). Overall, this represents 3113 (up = 1711, down = 1402) features on the array that, for at least one of the cultivars, indicated a response to a cold shock. However, relatively few of these transcripts showed a common response profile in all three varieties (Figure 1b). One might tend to assume that the transcripts that did respond in a similar fashion in all three varieties (394 transcripts) are from genes involved in basal responses to cold; i.e., they may be the genes that determine basal responses to chilling. The responses that united the two winter varieties but distinguish them from Paragon (217 transcripts) would more likely be those that determine hardiness and the ability to tolerate freezing conditions; they might also be part of the armoury for providing better chilling tolerance.

Surprisingly, these simple assumptions are not completely borne out by a study of the two gene lists. Notably absent from the list uniting Harnesk and Solstice, the two winter varieties, were many of the antifreeze proteins (see later section) that obviously play a major role in freezing tolerance, while transcripts for ice recrystallization inhibitors were induced in all three cultivars when one might not have expected to see them in Paragon, the spring variety.

As highlighted in several recent articles (Fowler, 2008; Ganeshan *et al.*, 2008; Campoli *et al.*, 2009), a weakness of the majority of research to date is that it has been based on responses to rapid, dramatic changes in temperature that do not in any way represent conditions found in nature. In such studies, plants have been directly transferred from favourable conditions for active growth (*c.* 20 °C) and placed at low nonfreezing temperatures (usually 4 or 2 °C)—our 'shock' experiment was of this kind and permitted us to make comparisons with the results from other such studies. The changes observed under such conditions are unlikely to truly reflect those that occur when plants experience a gradual decline in light and temperature more typical of the change from autumn to winter. Gene lists of candidate cold-responsive

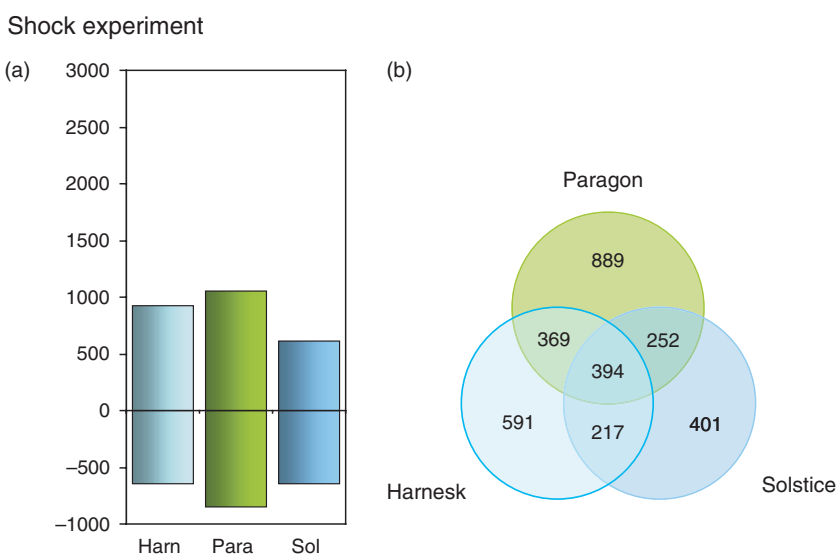


**Figure 1** (a) Statistically significant two-fold or greater changes ($P$ = 0.05) in transcript abundance in plants exposed to a 4 °C for 2 days. (b) Venn diagram showing the number of genes expressed in common between the three varieties after exposure to a 'cold shock'.

genes obtained from such cold-shock studies might also be misleading, therefore. For instance, in our shock experiment, we saw high levels of induction of some of the early light-inducible proteins (ELIPs) that might have been interpreted as a cold response given no other information. However, when plants were exposed to a slow decline in temperature and light, little or no response was seen from these genes (Figure 2). Experimental design, therefore, is fundamentally important in being able to identify candidate cold-responsive genes. A great deal of attention has been paid to the events occurring when plants are exposed to a rapid fall in temperature. Much less attention has been directed towards the elucidation of the molecular mechanisms underlying responses to gradual changes in ambient temperature that might be more representative of the conditions experienced during a typical autumn—winter progression.

A further criticism of many of the studies carried out to date is that analysis has been performed on a single tissue—usually leaf tissue. However, Ganeshan *et al.* (2008) clearly show that cold-responsive genes are differentially expressed between different tissues (crown and leaf) and point out that analysing only the changes that occur in a single tissue will provide an incomplete picture of the events taking place in cold-treated plants. What is more, in winter cereals, it has been shown that whole-plant survival is dependent on the survival of specific tissues within the crown (Tanino and Mckersie, 1985; Livingston *et al.*, 2006). The crown contains the meristematic regions from which all other tissues arise. The mature leaf tissue may well die back after suffering cold damage, but the immature, meristematic tissue of the crown must survive to re-establish growth when permissive conditions return. Our cold acclimation experiment was designed with both these criticisms in mind: temperature was gradually declined over several weeks, and leaf and crown tissue were assayed separately so that we could identify differential responses.

In our cold acclimation experiment, global changes in transcript abundance were markedly different between the two tissues, and between the spring and winter varieties (Figure 3a). That is, in comparisons of expression pattern between crown and leaf in any single variety, Harnesk and Solstice experienced many more changes in the leaves than in the crown. Paragon, the spring variety, showed the opposite relationship; that is, there were more changes in the crown than in the leaves. Comparing the expression patterns between the varieties, Paragon showed many more changes in crown tissue than the winter varieties and, conversely, showed many fewer changes in the leaf tissue. This last result is exactly opposite to what we saw in the cold-shock experiment. The cold-responsive genes in the two tissues were, in most cases, quite different (Figure 3b). Thus, the criticisms of experimental design put forward by Ganeshan *et al.* (2008) and Campoli *et al.* (2009) are supported by our results.

The differential response in crown tissue may probably be accounted for by the phase of growth in which the plants find themselves. Paragon, the spring wheat, possessed a highly expressed *VRN1* gene and was by definition committed to flowering: evidence for this is given in Winfield *et al.* (2009). Thus, the meristems in the crown

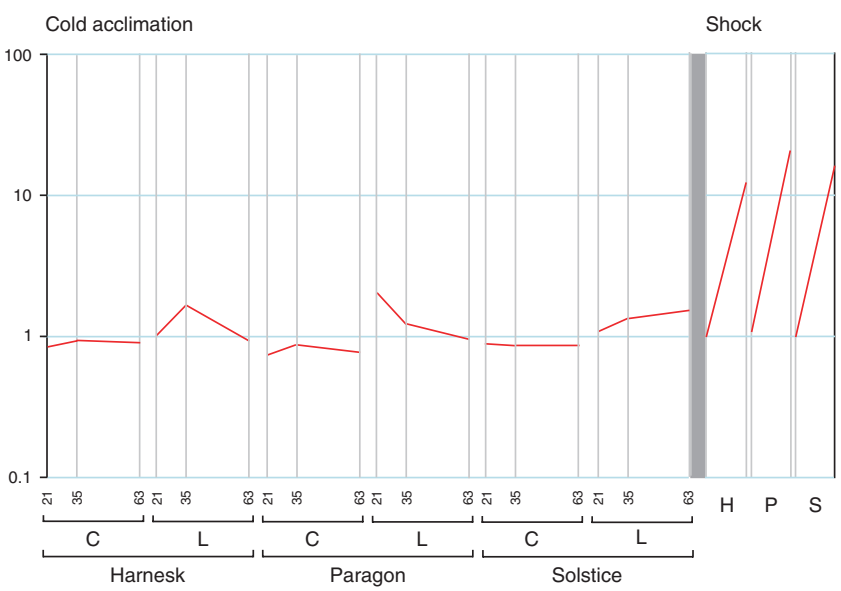


**Figure 2** An ELIP showing a distinct response to a 'cold shock', but no response to a slow decline in temperature. C, crown; L, leaf; H, Harnesk; P, Paragon; S, Solstice.

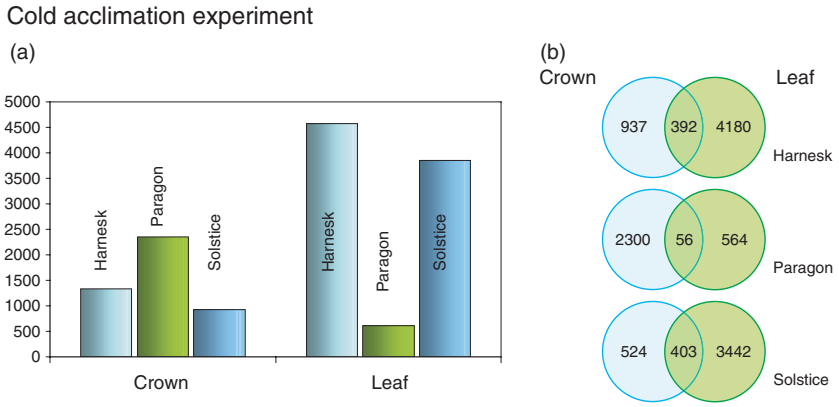


**Figure 3** (a) Statistically significant two-fold or greater changes ($P = 0.05$) in transcript abundance in plants exposed to a gradual decline in temperature, light intensity and day length; (b) Venn diagrams showing the number of statistically significant two-fold or greater changes ($P = 0.05$) in transcript abundance in plants exposed to a gradual decline temperature, light intensity and day length. The values refer to genes that changed in expression during the period 21–63 days (all comparisons were made: 21–35, 35–63 and 21–63 days).

of this variety were undergoing the many changes associated with the transition from vegetative to floral growth. The winter varieties, on the other hand, initially had very low levels of *VRN1* transcript and so would not have been committed to flowering. Although *VRN1* transcript accumulated across the course of the experiment, vernalization requirement would not have been satisfied until its end. Thus, the crown tissue of the winter varieties remained in the vegetative phase with fewer morphological and physiological changes taking place.

Interestingly, although the crown tissue is vital in terms of over-winter survival because it is the site of spring regrowth, in Harnesk and Solstice the majority of significant changes in transcript abundance occurred in the leaves (3a and b). This might indicate that effector molecules are produced in the leaves and then transported to the crown or that the protein products of only a relatively small number of genes are required to protect the tissues of the crown. However, given the much greater number of genes changing in the leaves of winter wheat varieties compared to Paragon, it would appear clear that their different capacity to cold acclimate and tolerate chilling and freezing temperatures is underpinned by the degree of transcriptome reprogramming that they are able to bring about as temperature drops.

The number of transcripts showing changes in abundance was much greater between the fifth and the 9th weeks than between the third and 5th weeks. This may simply be an artefact of the criterion used for selection of genes (i.e., they require to show a twofold change to be called) and although genes were induced early they had not accumulated above the threshold, or it may genuinely show that many genes were induced later in the time course. The latter interpretation seems the more likely because in the earlier part of the experiment, temperatures may not have fallen below the threshold required for induction of cold-responsive genes—by the 5th week average day/night temperature was 12 °C. That is, as temperatures gradually fall over an extended period, as might occur during autumn, plants respond by initiating a series of events that put in place those mechanisms required to protect them from potential damage. The temperature at which these events are initiated, the threshold temperature, is well above freezing and quite different between species and different cultivars of the same species (Fowler, 2008). For example, the cold-hardy rye cultivar Puma has a threshold temperature of 18 °C. Norstar, a winter wheat, has an inductive threshold of *c.* 15 °C and Manitou, a spring wheat, an inductive threshold of *c.* 8 °C (Fowler, 2008). This has the important corollary that hardy species/cultivars begin preparing for the stresses of winter earlier than tender species/cultivars. However, plants cannot fully acclimate until temperature drops well below the induction threshold, and the rate of acclimation is inversely proportional to temperature (Fowler, 2008; Ganeshan *et al.*, 2008, 2009). The capacity to acquire freezing tolerance is closely associated with a requirement for vernalization, and maximum freezing tolerance is attained when plants are fully vernalized.

After this general overview of the global changes in the transcriptome that occur as part of the process of cold acclimation and the acquisition of chilling and freezing tolerance, we will look at specific issues, such as signal perception and signal transduction, and will indicate where our studies give support or otherwise to the held conceptions. This is not always possible, of course, because not all are under transcriptional control. For instance, changes in the transcriptome occur in response to the cold stimulus, and therefore cannot be part of the initial perception itself. Thus, in the initial discussion of stimulus perception, little can be added from our studies. Figure 4 provides a

 

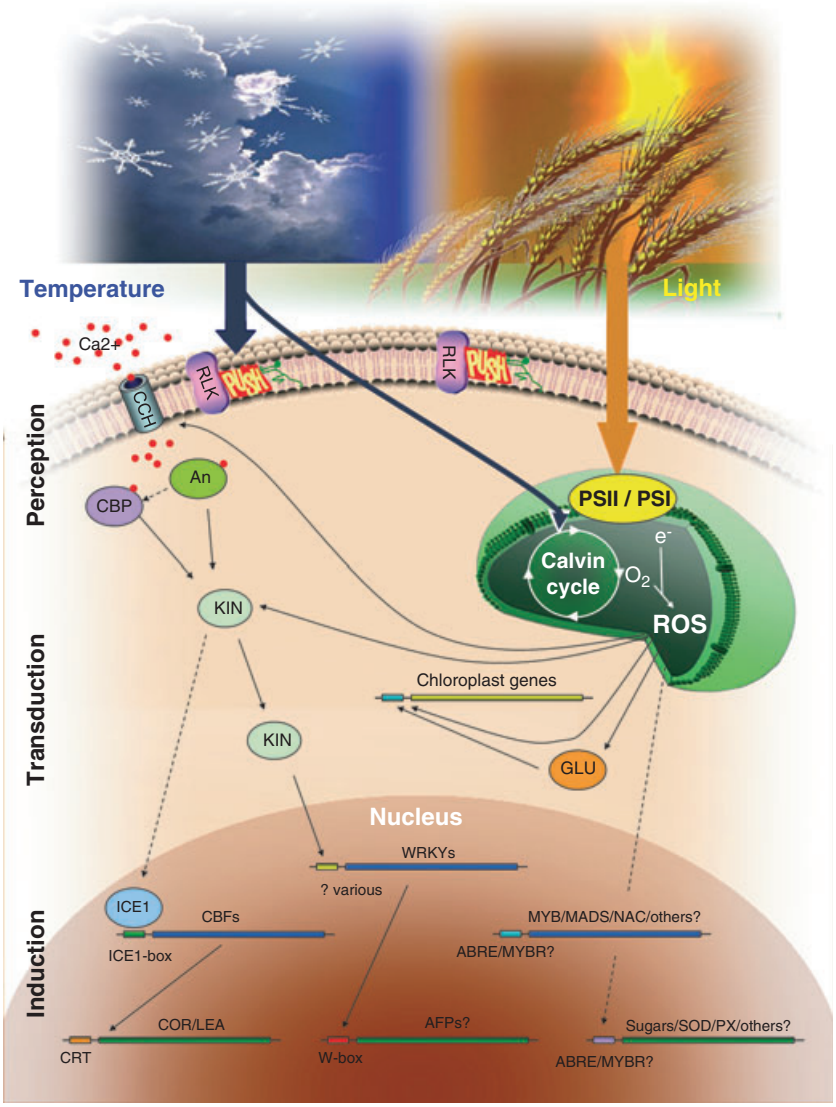


**Figure 4** Schematic representation of the cold response in plant cells. The coloured rods in the nucleus represent genes; those with the coding region coloured blue are transcription factors, those coloured green represent genes for effector molecules. Each gene is represented with only one *cis*-acting promoter region, but several may be present. Ice1 is a constitutive protein that is localized to the nucleus; post-translation mechanisms are involved in its activation (see Chinnusamy *et al.* (2007) for a review of this pathway). Membrane-bound kinases (RLK) might be involved in signal transduction as a result of the mechanical stress resulting from membrane rigidification. The annexins (An) and calcium-binding proteins (CBP) are activated by binding calcium (•). ABRE, ABA response element; CBP, calcium-binding proteins, CCH, calcium channel; GLU, glutathione; KIN, kinases and phosphatases; RLK, receptor-like kinase, ROS, reactive oxygen species.

schematic representation of the main points that are touched upon in this review.

## Perception of the cold stimulus

### *Membranes*

In the paradigm of information processing by cells, stimulus perception is followed by signal transduction and culminates in an appropriate physiological response. Although the temperature sensor(s) in plants has not as yet been identified unambiguously (Penfield, 2008), it has been hypothesized that temperature-mediated alterations in membrane fluidity/rigidity may be the primary sensing event in the perception of a temperature stimulus (Orvar *et al.*, 2000; Los and Murata, 2004); a decrease in temperature lowers membrane fluidity, whereas a temperature increase results in membranes becoming more fluid (Alonso *et al.*, 1997). Studies showing that it is possible to induce or retard the expression of cold-responsive genes through the application of pharmacological compounds that modify membrane fluidity support this hypothesis. Commonly, benzyl alcohol is used to increase membrane fluidity, while dimethyl sulphoxide (DMSO) is used to artificially rigidify membranes. For example, in Alfalfa (*Medicago sativa*) the low-temperature expression of *COLD-ACCLIMATION SPECIFIC 30* (*CAS30*) was blocked by benzyl alcohol and resulted in a reduction in freezing tolerance (Orvar *et al.*, 2000). Conversely, in *Brassica napus*, the addition of DMSO-induced *BN115*—an orthologue of COR15a in *Arabidopsis* (Jaglo *et al.*, 2001)—in the absence of cold treatment (Sangwan *et al.*, 2001). Vaultier *et al.* (2006) used fatty acid desaturase mutants to

 

provoke similar changes in membrane rigidity without the associated side effects of pharmacological compounds and produced similar results.

*Photosystems*

Plants can perceive variations in day length, light quality and light intensity. Intriguingly, light may also be of considerable importance in temperature perception because photosynthetic processes are usually the first to be influenced by changing temperatures (Ensminger *et al.*, 2006; Kocova *et al.*, 2009), and it has been shown that the ability of plants to develop frost resistance is associated with the presence of light and photosynthetic activity during cold acclimation (Svensson *et al.*, 2006).

Photosynthesis is highly sensitive to changes in environmental conditions because it needs to maintain a balance between the energy absorbed by photosystems I and II (PSI and PSII) and that consumed by the metabolic reactions of the plant (that is, there is a requirement to maintain homeostasis between energy source and sink). The primary reaction of photosynthesis carried out by PSI and PSII is to trap light energy and transform it into redox potential energy. Biochemical reactions convert this to stable reducing power in the form of NADPH. The photophysical and photochemical reactions of PSI and PSII are extremely rapid and independent of temperature. Biochemical reactions, on the other hand, are much slower and extremely temperature sensitive and are slowed as temperature decreases. This leads to uncoupling of the two systems, and electrons from PSI are transferred to oxygen thereby generating reactive oxygen species (ROS); e.g., superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical.

Reactive oxygen species are particularly interesting in that they may play two distinct roles in cold stress. They are thought to be one of the main causes of stress-induced damage to DNA, proteins and lipids, and chilling-resistant species and cultivars are thought to possess more efficient antioxidant systems than sensitive ones (Kocova *et al.*, 2009). A second aspect, however, is that they are thought to play a key role in mediating stress-related signal transduction events. Thus, the rate of light-induced ROS production might play a central role in cold stress perception (Suzuki and Mittler, 2006). Certainly, the ability of plants to develop cold resistance is associated with the presence of light and photosynthetic activity during the cold acclimation period (Gray *et al.*, 1997; Crosatti *et al.*, 1999; Wanner and Junttila, 1999; Svensson *et al.*, 2006; Franklin and Whitelam, 2007; Franklin, 2009), and it has been shown that cold treatment in the light results in the up-regulation of about twice the number of cold-responsive genes compared to the number induced under the same conditions in the dark (Soitamo *et al.*, 2008). In meadow fescue (*Festuca pratensis*), it has been reported that about 50% of the proteins that change in abundance during cold acclimation are involved in photosynthesis (Kosmala *et al.*, 2009).

Chloroplasts utilize light as a source of energy and react to variations in light intensity by adapting metabolism to the redox state of the electron transport chain (Pfannschmidt *et al.*, 1999, 2001). Increased PSII excitation pressure is one of the primary stimuli promoting expression of cold-regulated genes (Gray *et al.*, 1997; Ndong *et al.*, 2001). Consequently, exposure to cold in the absence of light reduces the induction of several cold-regulated genes (Crosatti *et al.*, 1999; Kobayashi *et al.*, 2004). Barley *albina* mutants that are unable to properly develop chloroplasts show under-expression of cold-responsive genes (Svensson *et al.*, 2006).

*DNA-nucleosome composition*

Very recently, a major step forward in our understanding of cold perception in *Arabidopsis* has been made. Kumar and Wigge (2010) have shown that a particular histone protein variant, H2A.Z, plays a key role in the perception of temperature and might be responsible for the coordinated expression of many temperature-sensitive genes. It is suggested that H2A.Z is present in chromatin immediately downstream of promoters of temperature-responsive genes and that this creates a physical barrier to RNA polymerase II and gene expression. As temperatures rise, H2A.Z is removed from these sites permitting expression of the genes involved. With regard to genes that are expressed at low temperature, it is proposed that H2A.Z occupancy may prevent the binding of repressors. Importantly, the authors give evidence for the involvement of H2A.Z in temperature-dependent regulation of gene expression in yeast, suggesting that this is an evolutionary conserved mechanism.

## Calcium as the secondary messenger

Whatever the actual mechanism of perception, one of the earliest consequences of detecting change in temperature is thought to be $Ca^{2+}$ influx into the cytosol (Chinnusamy *et al.*, 2006; Kaplan *et al.*, 2006a). This may be mediated through membrane rigidification–activated mechanosensitive $Ca^{2+}$ channels and/or induced through the presence of stress-induced reactive oxygen species. That $Ca^{2+}$ influx is

an important initial event in the monitoring of temperature change that has been shown through experiments in which the administration of calcium chelators and calcium channel blockers has been shown to prevent cold acclimation (Monroy *et al.*, 1997). The spatial and temporal patterns of $Ca^{2+}$ influx are although to be characteristic for particular stimuli and are referred to as $Ca^{2+}$ signatures (DeFalco *et al.*, 2010). The information contained in these characteristic $Ca^{2+}$ signatures is interpreted through an array of $Ca^{2+}$ -binding proteins (CBPs) that act as $Ca^{2+}$ sensors (Kaplan *et al.*, 2006a). The three main $Ca^{2+}$ sensors in plants are calmodulin (CaM) and calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs). These proteins, which contain a highly conserved EF-hand motif that binds $Ca^{2+}$, have been shown to participate in the orchestration of calcium-directed signal transduction networks (Yang *et al.*, 2004). As a consequence of binding $Ca^{2+}$, CBPs undergo a conformational change that enables them to interact with and regulate (activate or inactivate) target proteins (DeFalco *et al.*, 2010). In turn, these downstream effectors initiate a series of events that results in the large-scale reprogramming of gene expression that is seen in cold acclimation. It would be fascinating, therefore, if one could identify specific CBPs that are potentially involved in the initiation of specific cold-related gene cascades. Unfortunately, at the transcriptional level, we cannot draw any clear conclusions about any particular CBP. In our experiments, transcripts identified as CBPs behaved in a range of different ways, many of which were not indicative of them being involved in cold responses. However, given their importance in myriad signal sensing and transduction mechanisms, it is not surprising that we observed a range of different responses among the various CaM and CaM-binding proteins that were assayed on the array. Some, such as CaM4-1, showed up-regulation only in the leaf tissue of Paragon in the cold acclimation experiment and so are unlikely to play a role in cold acclimation. Several features on the array identified as putative calmodulins behaved similarly in all three varieties: that is, in the cold acclimation experiment they were up-regulated between week 3 and week 5 and then by week 9 had returned to their initial level: in the cold-shock experiment these transcripts also increased. Another putative calmodulin-binding protein was differentially up-regulated in the leaf tissue of the two winter varieties given a slow decline in temperature, but showed no response to a cold shock. One particularly interesting CLP showed a large decline in transcript abundance in all three varieties in the shock experiment (15- to 30-fold decline) and very little response under any other condition: a $\beta$-glucanse gene and *WINV2* (an invertase gene) were two of the very few genes that were coregulated with this.

## Cold-responsive genes

Cold usually precedes freezing in nature and induces many physiological and biochemical changes in the cells of freezing-tolerant plant species that enable them to survive unfavourable conditions. Low temperature affects water and nutrient uptake, membrane fluidity and protein and nucleic acid conformation, and dramatically influences cellular metabolism either directly through the reduction in the rate of biochemical reactions or indirectly through the large-scale reprogramming of gene expression. A large number of low temperature–induced genes have been identified and characterized in plants (Tsuda *et al.*, 2000; Zhang *et al.*, 2009) and are referred to as Late Embryogenesis–Abundant (LEA), Dehydrin (DHN), Responsive To Abscisic Acid (RAB), Low Temperature–Responsive (LT) and Cold-Responsive (COR) genes. As a majority of these genes belong to the Lea family that commonly encode highly hydrophilic proteins, they are usually referred to as *COR/LEA* genes or simply *COR* genes. A positive correlation exists between the level of *COR* gene expression and that of freezing tolerance (Grossi *et al.*, 1998; Baldi *et al.*, 1999; Ohno *et al.*, 2001; Vagujfalvi *et al.*, 2003). For example, the over-expression of the wheat COR/LEA protein WCS19 in *Arabidopsis* improves freezing tolerance, although only of cold-acclimated leaves (Dong *et al.*, 2002).

Among these gene products, many are structural proteins that are directly involved in protecting the plants from stress (e.g., protein chaperones, osmoprotectants, ice-binding proteins), while others are regulatory genes (e.g., transcription factors, protein kinases and enzymes involved in the synthesis of plant hormones) (Table 1). Individual transcription factors are thought to control many target genes through direct binding to *cis*-acting elements in the promoter regions of the target genes. The transcription factors and the genes controlled by them are collectively referred to as a 'regulon'. One of the most studied regulons involved in cold responses is the CBF regulon driven by CBF transcription factors.

## CBF/DRE regulon

A common feature of cold acclimation is the rapid induction of genes encoding CBF-like transcription activators

**Table 1** Categories of genes induced by cold stress

| Genes induced by cold stress | |
|---|---|
| Signal transduction* | Effectors† |
| Annexins | Antioxidants |
| Calcium-binding factors | Chaperones |
| Kinases | Enzymes of fatty acid metabolism |
| Phosphatases | Enzymes of osmolyte biosynthesis |
| Transcription factors | LEA proteins |
| | Lipid transfer proteins |
| | mRNA-binding proteins |
| | Osmotin |
| | Protease inhibitors |
| | Water channel proteins |

Some of these transcripts show rapid accumulation while others accumulate gradually; in addition, transcripts may be transient or may persistent.

*Some transcription factors have been over-expressed in transgenic plants and shown to generate stress-tolerant phenotypes (for examples, see Pellegrineschi *et al.* (2004) and Shen *et al.* (2003).

†Some effector molecules have been expressed in transgenic plants and shown to function in stress tolerance (for examples, see Ndong *et al.* (2002) and Houde *et al.* (2004).

(Jaglo *et al.*, 2001; Thomashow, 2001; Thomashow *et al.*, 2001). In *Arabidopsis*, these are named *CBF1*, *CBF2* and *CBF3* (rather confusingly, these are also referred to as dehydration-responsive elements and named *DREB1b*, *DREB1c* and *DREB1a*, respectively). A role for *CBF* genes in the enhancement of freezing tolerance has been established through over-expression experiments. Constitutive expression of the *CBF* genes in transgenic *Arabidopsis* plants results in the induction of *COR* gene expression and an increase in freezing tolerance without a low-temperature stimulus (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2000). Significantly, multiple biochemical changes that are associated with cold acclimation and thought to contribute to increased freezing tolerance, such as the accumulation of simple sugars and the amino acid proline, occur in non-acclimated transgenic *Arabidopsis* plants that constitutively express *CBF3* (Gilmour *et al.*, 2000). Thus, it has been proposed that the *CBF* genes act to integrate the activation of multiple components of the cold acclimation response (Gilmour *et al.*, 2000). This is referred to as the CBF regulon. The CBF regulon has been extensively studied in *Arabidopsis* (Nakashima and Yamaguchi-Shinozaki, 2006) and has been shown to be present in many species, both dicots and monocots (Dubouzet *et al.*, 2003; Takumi *et al.*, 2003; Kume *et al.*, 2005; Oh *et al.*, 2007). Even plant species that suffer damage at chilling temperatures and that are completely unable to tolerate freezing, such as tomato, maize and rice, also possess components of the CBF cold-response pathway (Jaglo *et al.*, 2001; Nakashima and Yamaguchi-Shinozaki, 2006). The CBF transcription factors, which are members of the larger AP2/EREBP family of DNA-binding proteins (Campoli *et al.*, 2009), recognize the cold- and dehydration-responsive DNA regulatory element designated the C-repeat/dehydration-responsive element (CRT/DRE). These elements, which have a conserved 5-bp core sequence of CCGAC, are present in the promoter regions of many cold- and dehydration-responsive genes. The *CBF* genes are induced within 15 min in plants being exposed to low non-freezing temperatures, and after about 2 h one begins to see the induction of cold-regulated genes that contain the CRT/DRE regulatory element (Gilmour *et al.*, 1998).

The *CBF* genes belong to a multigene family that has been divided into several groups. Plants belonging to the *Poaceae* (the grasses) contain *CBF*s that have been classified into ten groups, the members of which share a common phylogenetic origin and similar structural characteristics. Six of these groups (IIIc, IIId, IVa, IVb, IVc and IVd) are found only in the *Pooideae* (a subfamily of the grasses that contains the temperate cereals wheat, barley and rye). In wheat, there are up to 25 different *CBF* genes (Badawi *et al.*, 2007): a cluster of these genes is found on the long arm of homoeologous group 5 chromosomes. This corresponds with a major QTL for frost resistance, the *Fr2* locus. Expression studies reveal that five of the *Pooideae*-specific groups (CBFIIId, IVa, IVb, IVc and IVd) display higher constitutive and low temperature–inducible expression in winter cultivars compared to spring cultivars (Badawi *et al.*, 2007; Sutton *et al.*, 2009). The higher constitutive and inducible expression within these CBF groups may play a predominant role in the superior low-temperature tolerance capacity of winter cultivars and is possibly the basis of genetic variability in freezing tolerance within the *Pooideae* subfamily.

In our studies, we saw a range of responses from *CBF* genes—on the array, there were features representing 18 different *CBF*s. Five showed no response at all in either of the two experiments (*CBFII-5*, *CBFIIIc-D3*, *CBFIIIc-B10*, *CBFIIId-15* and *CBFIVd-D22*). The other thirteen *CBF* genes showed a response under one or both of the experimental conditions (Table 2). In the shock experiment, only *CBFI-Va-A2* and *CBFIVb-D20* were differentially expressed between spring and winter varieties; they accumulated in Harnesk and Solstice but not in Paragon. In the cold acclimation experiment, several transcripts responded

**Table 2** The response of *CBF* genes in plants exposed to a slow decline in temperature compared to those occurring when plants experience a cold shock

| Gene name | Accumulation pattern | |
|---|---|---|
| | Cold Acclimation expt. | Shock expt. |
| *CBFII-5* | X | X |
| *CBFIIIa-6* | ✔↓ H+S+P | X |
| *CBFIIIc-D3* | X | X |
| *CBFIIIc-B10* | X | X |
| *CBFIIId-12 (CBF12)* | ✔↑ H+S (c) | X |
| *CBFIIId-15* | X | X |
| *CBFIIId-A19* | ✔↓↑ H+S (c,l), P (c) | ✔↑ H+S+P |
| *CBFIVa-A2 (CBF2)* | ✔↓↑ H+S (c,l) | ✔↑ H+S |
| *CBFIVb-D20 (CBF1)* | ✔↑ H+S | ✔↑ H+S |
| *CBFIVb-21* | ✔↑ H+S (c,l) | X |
| *CBFIVc-14* | ✔↑ H+S (c,l), P (c) | ✔↑ H+S+P |
| *CBFIVc-B14* | ✔↑ H+S (c,l), P (c) | ✔↑ H+S+P |
| *CBFIVd-4* | ✔↓↑ H+S (c), ↓ H+S+P (l) | ✔↑ H+S+P |
| *CBFIVd-B9 (CBF9)* | ✔↑ P (l) | X |
| *CBFIVd-A22* | ✔↑ H+S (c) | X |
| *CBFIVd-B22* | ✔↓↑ H+S (c), ↓ H+S (l), ↑ P (c) | X |
| *CBFIVd-D22* | X | X |
| *CBF1** | ✔↑ P (l) | X |

A tick (✔) indicates that the transcript responded to the temperature stimulus, a cross (X) indicates that no response was observed. Arrows show whether the transcript was up- (↑) or down-regulated (↓). H, Harnesk; P, Paragon; S, Solstice, c, crown; l, leaves.*The *CBF1* transcript was recognized on the basis of its similarity to the barley (*Hordeum vulgare*) *HvCBF1* gene and so does not follow the nomenclature used for the other *CBF* genes.

differentially between the winter and spring cultivars (Table 2). The most dramatic differential response was for *CBFIIId-12*: in the crown tissue of the two winter wheat varieties transcript increased more than 10-fold, while in Paragon it showed no response. It showed no response in leaf tissue of any of the three varieties. Two transcripts, *CBFIVd-B9* and *CBF1* responded only in Paragon during the cold acclimation experiment; that is, in leaf they increased over the course of the experiment.

Surprisingly, there were no other transcripts with similar profiles (90% similarity) of accumulation to those of any of the CBF transcription factors. Therefore, there appears to be no direct correlation between accumulation of CBFs and the genes that they control. The CBFs for which no response was observed might either be involved in pathways unrelated to cold stress, or they may have accumulated in a rapid, transient fashion, or they may be controlled in a non-transcriptional fashion.

The considerable cross-talk that occurs between temperature-regulated and light-regulated pathways (Franklin, 2009) has been shown to occur in the expression of the CBF regulon. Some of the CBF transcription factors have been shown to be regulated in a light-dependent, diurnal fashion under growth at 20 °C (Badawi *et al.*, 2007). In addition, it has been reported that light quality signals (red/far red ratio), mediated through the phytochromes and cryptochromes, regulate the expression of the CBF regulon (Franklin and Whitelam, 2007).

In *Arabidopsis*, a major gene acting upstream and controlling the expression of the CBF regulon is *ICE1* (*INDUCER OF CBF EXPRESSION 1*). The product of this gene is a MYC-type basic helix–loop–helix transcription factor that binds MYC recognition sites (the ICE1-box) in the promoter of *CBF3* and induces its expression. The *ice1* mutant is defective in the cold induction of *CBF3*, is sensitive to chilling stress and completely unable to cold acclimate (Chinnusamy *et al.*, 2007). Conversely, the constitutive over-expression of *ICE1* in transgenic *Arabidopsis* enhanced the expression of *CBF2*, *CBF3* and *COR* genes during cold acclimation and increased freezing tolerance. *ICE1* is a constitutively expressed gene and post-translation modification of its protein product, which is localized to the nucleus, is required for *CBF* induction. A similar mechanism is probably present in other species, because over-expression of *ICE1* in transgenic rice improves cold tolerance (Xiang, 2003), and *ICE1*-like genes (*TaICE41* and *TaICE87*) that have been shown to bind the MYC elements in the promoters of certain *CBF* genes have been found in wheat (Badawi *et al.*, 2008). The over-expression of either *TaICE41* or *TaICE87* in transgenic *Arabidopsis* enhanced freezing tolerance, although only upon cold acclimation. The increased freezing tolerance in transgenic *Arabidopsis* was associated with a higher expression of the cold-responsive activators AtCBF2 and AtCBF3 and of several cold-regulated genes. Unfortunately, there is no probe set for *ICE*-like genes on the Affymetrix wheat array, so we were unable to monitor its abundance in our experiments. However, these are reported to be constitutively expressed (Badawi *et al.*, 2008), so we may not have observed cold-related change in their abundance.

Although the CBF regulon appears to be one of the main regulatory pathways involved in cold acclimation, and it is certainly the most studied, it is by no means the only one. In *Arabidopsis*, for example, only about 12% of all cold-induced genes are thought to be responsive to the CBF regulon (Chinnusamy *et al.*, 2007), while in wheat at least one-third of the genes induced by cold are not responsive to CBF regulation (Monroy *et al.*, 2007). Obviously, there must be additional regulatory mechanisms involving other transcription factors and their regulons (Fowler and Thomashow, 2002; Vergnolle *et al.*, 2005).

 

### WRKY transcription factors

WRKY transcription factors are members of a large gene family that includes 74 members in *Arabidopsis* and over 100 in rice (Berri *et al.*, 2009). They are found almost exclusively in plants, although they are also found in some green algae (Eulgem *et al.*, 2000). They are characterized by the presence of one or two highly conserved 60 amino acid WRKY domains which contain a zinc finger motif that provides DNA binding; on the basis of the number and nature of their zinc-finger motifs, the genes are assigned to three separate groups. The WRKY domain binds sequence specifically to the W Box DNA element (C/T)TGAC(C/T) of target genes, which are defined as elicitor-responsive elements. Several defence-related genes in plants have over-representation of W boxes in their promoters—WRKY genes themselves have W boxes in their promoters and may be self-regulated to some degree. WRKY transcription factors have been reported to be involved in various physiological programmes and, in addition, to respond to pathogen attack. However, more recently they have been shown to be involved in responses to abiotic stimuli (Mare *et al.*, 2004), and it has been reported that WRKY transcription factors may be involved in cold hardening in wheat (Talanova *et al.*, 2009).

We saw evidence for cold induction of some WRKY transcription factors in our study. In the cold acclimation experiment, sequences identified as *WRKY5* and *WRKY10* showed transcript accumulation in the leaf tissue of Harnesk (over 40-fold) and Solstice (c.20-fold), but no change in Paragon (Figure 5). Neither of these transcription factors was induced after 2 days of a cold shock. This is worth of note, because these transcription factors would not have been evidenced under the experimental conditions where a short cold shock was applied. This might explain why these transcription factors have been so little studied with respect to cold acclimation. Interestingly Talanova *et al.* (2009) identified a WRKY transcription factor that responded rapidly (within 15 min) and dramatically ('by a factor of several tens') upon plants being placed at 4 °C; thereafter, over a period of several days, the transcript returned to basal levels. Obviously, we would not have observed this change in our cold acclimation experiment. Thus, there may be several different WRKY transcription factors that control different sets of genes involved in response to cold.

In our studies, several genes with obvious roles as stress-related effector molecules were co-regulated with the WRKY transcription factors (Figure 5). Perhaps, the most significant of these co-regulated transcripts were some of the glucanases, chitinases and thaumatin-like proteins that have been shown to play a significant role as effectors in freezing tolerance (see later section and Figure 6). Additionally, the following transcripts were also up-regulated in a similar fashion to some of the WRKY transcription factors: a Mlo3-like protein (Mlo3 in barley is a transmembrane protein involved in defence against fungal attack), gibberellin pathway paralogues that might play a role in signal transduction, and an oxalate oxidase-like protein (a germin—see later section) that could play a role in scavenging of reactive oxygen species.

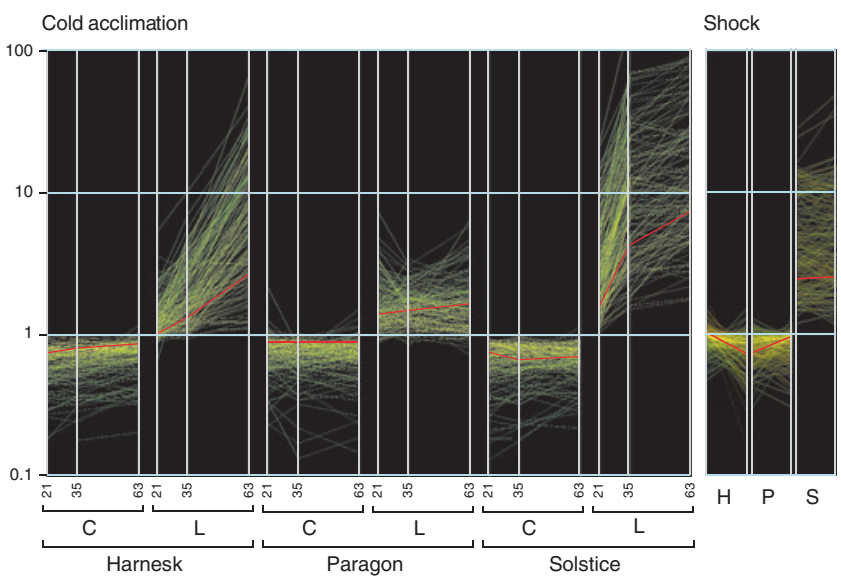


**Figure 5** Profile of accumulation for the transcription factor WRKY5 (red line) and the transcripts that were coregulated with it (>90% identity). C, crown; L, leaf; H, Harnesk; P, Paragon; S, Solstice.

 

### Effectors

Cold and freezing conditions give rise to several stresses in addition to their direct effect on biochemical reactions and the physical damage caused by ice formation. Thus, cold-induced effector molecules are quite varied in their respective functions (Table 1): osmoregulants—sugars, proline that may act to stabilize cell membranes (lipid metabolism, membrane proteins); chaperones that act to protect proteins from cold-induced structural change; inhibitors of ice formation; photosynthetic enzymes involved in establishing homoeostasis between photosystems I and II and the biochemical reactions of the Calvin cycle; enzymes involved in the up-regulation of respiration (Cook *et al.*, 2004); reactive oxygen species scavengers.

A major consequence of cold stress is dehydration and osmotic stress, and several of the *COR* genes are dehydrins. Dehydrins are a distinct biochemical group of LEA proteins (known as LEA D-11 or LEA II) characterized by the presence of a lysine-rich amino acid motif, the K-segment (Allagulova *et al.*, 2003; Kosova *et al.*, 2007). They are highly hydrophilic, soluble upon boiling and rich in glycine and polar amino acids. Their expression is induced by various environmental factors—heat, drought, salinity—that cause cellular dehydration (Kosova *et al.*, 2007). Extreme cold and frost can also lead to osmotic stress, and it has been shown that the induction and accumulation of dehydrins is an important part of the cold acclimation apparatus of winter cultivars of the cereals (Stupnikova *et al.*, 2002, 2004; Borovskii *et al.*, 2005). It is thought that they can act either as emulsifiers or chaperones in the cells, protecting proteins and membranes against unfavourable structural changes caused by dehydration. They have also been shown to bind to mitochondrial membranes in a seasonal-dependent manner: during the winter they accumulate, while during the spring they decline in abundance (Borovskii *et al.*, 2005). In our experiments, we saw very high induction (up to 40-fold increase) of some of the dehydrins. These increased in both tissues of all three varieties under both sets of experimental conditions, but in the cold acclimation experiment they accumulated less in the leaves of Paragon than in the leaves of the two winter varieties.

The well-characterized wheat cold-specific (*WCS*120) gene family belongs to the Cor/Lea superfamily (Fowler *et al.*, 2001). The WCS120 protein family members share homology with the Lea D11 dehydrins (Thomashow, 1999; Kosova *et al.*, 2007). As shown by biochemical, immunohistochemical, molecular and genetic analyses, this gene family is specific to the *Poaceae* (Sarhan *et al.*, 1997). They encode a group of highly abundant proteins ranging in molecular weight (MW) from 12 to 200 kDa; among these, the five major members, WCS200 (MW = 200 kDa), WCS180 (180 kDa), WCS66 (66 kDa), WCS120 (50 kDa) and WCS40 40 kDa), are inducible by cold treatment (Sarhan et al., 1997). Members of the WCS120 family of proteins are thought to play a significant role in frost tolerance because of their higher induction in winter-hardy compared to tender spring wheat plants (Vitamvas *et al.*, 2007; Vitamvas and Prasil, 2008). Indeed, because of their abundance it has been suggested that the WCS120 proteins could serve as molecular

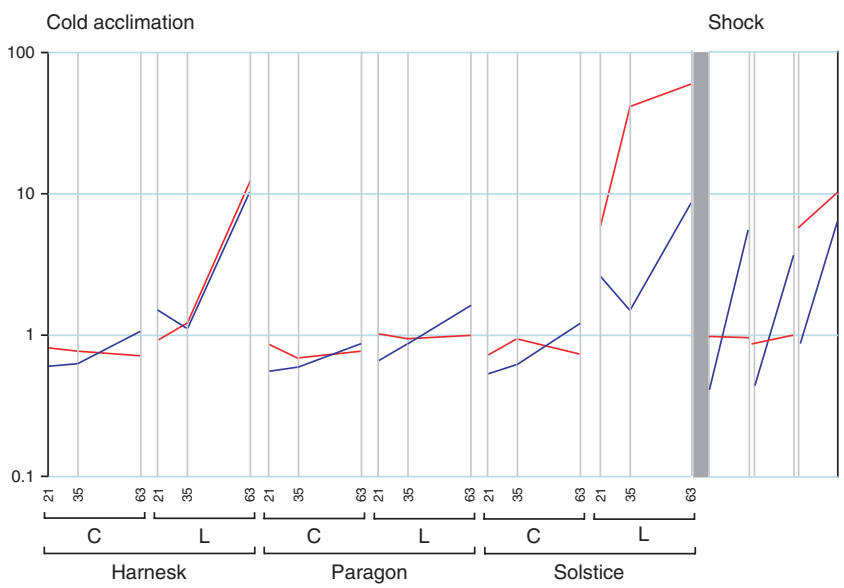


**Figure 6** Profile of transcript abundance for (a) glucanase Glb2b and (b) ice-recrystallization inhibitor 2. The two genes showed similar profiles of accumulation in the 'cold acclimation experiment' but only IRI responded to a cold shock.

 

markers for frost tolerance in the *gramineae* (Houde *et al.*, 1992). Unfortunately, of the various members of the *WCS120* family, only *WCS66* has a probe set on the Affymetrix Wheat Array GeneChip. The *WCS66* transcript accumulated in both the cold-shock and cold acclimation experiments, but accumulated to a greater degree in the leaves of winter wheat (12-fold and 5-fold in Harnesk and Solstice, respectively) than in the spring wheat (two-fold). In crown tissue, statistically significant differences in accumulation pattern were not observed.

A cereal-specific protein, Wheat Low Temperature–Responsive 10 (WLT10), that is induced by cold has been shown to differentiate hardy and tender wheat cultivars (Ohno *et al.*, 2001). A freezing-tolerant winter cultivar, M808, accumulated mRNA more rapidly and over a longer period than a tender spring variety (Chinese Spring). The increase in transcript abundance was temporary, but the peak occurred at the time when maximum freezing tolerance was attained (at 3 days under a cold-shock regime). Interestingly, the transcript was reported to accumulate to different levels under different light/dark regimes, once again indicating the importance of light in the perception of cold. In our cold acclimation study, *WLT10* transcripts accumulated principally in the leaf tissues with some evidence of slightly greater accumulation in the two winter varieties than in Paragon (13- to 15-fold increase in the winter varieties compared to a six-fold increase in Paragon). Induction occurred after the 5th week, there being a small decline in abundance prior to this. In the cold-shock experiment, there was a dramatic and similar increase in all three varieties.

*Oxygen free radicals*

Cold or chilling stresses have a dramatic effect on plant metabolism causing the disruption of cellular homeostasis and the uncoupling of major physiological processes leading to the accelerated formation of oxygen-based free radicals (Suzuki and Mittler, 2006). These radicals are toxic molecules capable of disrupting cell function, and they may even cause sufficient damage to result in cell death. Chloroplasts are highly sensitive to damage by the reactive oxygen species (ROS) that are generated by the reaction of chloroplastic $O_2$ and the electrons that escape from the photosynthetic electron transfer system (Foyer *et al.*, 1994). Cells possess antioxidants and antioxidative enzymes capable of interrupting cascades of uncontrolled oxidation in cellular organelles. Oxidative stress results from the imbalance between the formation of ROS and their neutralization by antioxidants. Various processes disrupt this balance by increasing the formation of free radicals in relation to the available antioxidants (Talukdar *et al.*, 2009). Under optimal conditions for growth, ROS are produced at a low level, but during stress their rate of production is greatly increased. The accumulation of enzymes and metabolites that cooperatively scavenge ROS is thus an important part of the cold acclimation process (Tao *et al.*, 1998). Antioxidants such as ascorbic acid and glutathione, and ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidise (APX), catalase (CAT), glutathione peroxidise (GPX) and peroxiredoxin (PrxR) are involved in stress-related removal of ROS. We observed changes in some of these genes. However, most showed no statistically significant change in abundance. Transcripts for various glutathione transferases and some peroxidases were interesting exceptions to this. Glutathione transferases, which are encoded by a large and diverse gene family in plants and perform a range of functions, may exhibit glutathione peroxide activity and may also play a role in stress-related signal transduction (Dixon *et al.*, 2002a,b). Interestingly, in the cereals GSTs are constitutively very highly expressed, representing up to 2% of all protein in the leaves (Dixon *et al.*, 2002b). This was clearly seen in our analysis, many of these genes having very high basal levels of expression in the leaves. They have also been reported to be transcriptionally controlled and to be induced by various abiotic stresses [see review by Dixon *et al.* (2002b)]. In our studies, some GSTs increased exclusively in the leaves of the two winter varieties, while others accumulated to a greater extent. Therefore, they may well be involved in the response to cold stress and be part of the mechanism to remove ROS.

Flavonoids are secondary metabolites derived from phenylalanine and acetate metabolism that perform a variety of essential functions in higher plants including playing an important role as antioxidants (Winkel-Shirley, 2002). Chalcone synthase and chalcone isomerase are key enzymes in flavonoid biosynthesis and in our experiments showed differential patterns of transcript accumulation between the winter and spring varieties. In the cold acclimation experiment, the transcript for naringenin-chalcone synthase exhibited a winter wheat–specific increase in leaf tissue (20-fold in Harnesk and only three-fold in Solstice, but this had a much higher initial basal level). A putative UDP-glucose: flavonoid 7-*O*-glycosyltransferase showed a similar profile of accumulation, while a transcript for a chalcone isomerase–like enzyme declined in the leaves of the winter varieties. These profiles might be indicative of the involvement of the flavonoid pathway in cold stress

 

responses. The transcript for a chalcone isomerase–like gene was constitutively much more highly expressed in the two winter varieties (c. 20-fold) than in Paragon; however, it showed down-regulation in all three cultivars in both experiments.

In red beet (*Beta vulgaris*), it has been found that a 5-*O*-glucosyltransferase (GT), an enzyme involved in the synthesis of the pigment betacyanin, is induced by wounding, bacterial infiltration and as a consequence of oxidative stress (Sepulveda-Jimenez *et al.*, 2005). They concluded that ROS act as a signal to induce *BvGT* expression, necessary for betanin synthesis and that betacyanins act as ROS scavengers. We observed differential expression of betanidin-5-*O*-glucosyltransferase between the spring and winter varieties with marked accumulation (up to 20-fold) in the leaf tissue of the latter only. A particularly interesting set of genes appeared to be co-regulated with this (Pearson correlation of at least 95%): there were several glucanases, which are thought to act as antifreeze proteins (see next section), a number of transcripts for pathogen-related proteins and genes for other enzymes involved in pigment biosynthesis that might themselves play a role in ROS-scavenging or ROS-induced signal transduction.

*Antifreeze proteins*

Once cold-acclimated, cold-hardy cultivars of wheat are able to tolerate temperatures as low as −25 °C (Yoshida *et al.*, 1997), while some of the forage grasses are able to withstand temperatures as low as −30 °C (Moriyama *et al.*, 1995). Under freezing conditions, cell membranes are thought to be the main sites of injury (Thomashow, 1999; Uemura *et al.*, 2006). Freezing tolerance, therefore, is closely related to the mechanisms by which plant cells avoid injury to the cellular membranes (Uemura *et al.*, 2006; Yamazaki *et al.*, 2009). A major part of this depends on the capacity to withstand extracellular ice formation and the ability to prevent its formation within the cell. Extracellular freezing results in freeze-dehydration because of the movement of water from the cytoplasm to the growing ice crystals and freeze-induced dehydration is thought to be the major factor causing injury to the plasma membrane (Yamazaki *et al.*, 2009). Ice formation also produces mechanical stress with deformation and apposition of cellular membranes that can lead to cell rupture and loss of semi-permeability. A key pre-emptive function of cold acclimation, therefore, is to put in place mechanisms to stabilize membranes against potential freezing injury (Uemura *et al.*, 2006; Yamazaki *et al.*, 2009). This includes the production of antifreeze proteins that either retard ice formation or limit its growth, osmoprotectants that protect membranes and proteins from the effects of dehydration and the modification of cell membrane composition. The best studied of these mechanisms is that related to the inhibition of ice formation and growth through the production of a range of antifreeze proteins (AFPs).

*Chitinases, glucanases, thaumatin-like proteins*

As temperatures drop below freezing, ice formation initiates in the extracellular spaces and xylem vessels because the extracellular fluid generally has a lower solute concentration and consequently a higher freezing point than the intracellular fluid (Pearce, 1986; Pearce and Ashworth, 1992). During cold acclimation, freezing-tolerant plants accumulate antifreeze proteins in anticipation of the arrival of freezing conditions. These proteins, which principally accumulate in the apoplast (xylem-lumena, cell wall and intercellular spaces), include a diverse range of proteins which have the common characteristic of being highly similar to pathogen-related (PR) proteins (Griffith and Yaish, 2004). These are the chitinases, glucanases and thaumatin-like proteins (Antikainen *et al.*, 1996; Pihakaski-Maunsbach *et al.*, 1996; Bishop *et al.*, 2000; Stahl and Bishop, 2000; Griffith and Yaish, 2004). All three of these protein groups belong to large gene families, the members of which have undergone extensive evolutionary change and functional diversification (Bishop *et al.*, 2000; Stahl and Bishop, 2000). Thus, they have evolved to perform many biological roles including responses to abiotic and biotic stress (Karlsson and Stenlid, 2008).

Pathogen-related proteins are released into the apoplast in response to infection and act together to enzymically degrade fungal cell walls and to inhibit the action of fungal enzymes. Similarly, antifreeze proteins are also targeted to the apoplast (Griffith and Yaish, 2004) and form complexes of various composition (Yaish *et al.*, 2006). However, rather than interfering with the growth of pathogens, they have the capacity to bind to ice crystals and inhibit their growth (Moffatt *et al.*, 2006). They also inhibit ice recrystallization, a process that occurs when temperatures fluctuate about that of freezing resulting in the migration of water molecules from small ice crystals to larger ones (Knight *et al.*, 1984). Although it does not appear that chitinase- and glucanase AFPs contain a particular ice-binding domain, the characteristic that distinguishes them from pathogen-related proteins is their capacity to assume a three-dimensional structure that presents an ice-binding surface (IBS) (Yeh *et al.*, 2000; Yaish

*et al.*, 2006). These bind ice crystals through hydrogen bonds and van der Waals forces, and in doing so inhibit their and growth and recrystallization (Yeh *et al.*, 2000; Griffith and Yaish, 2004). Interestingly, some of these AFPs have also retained their capacity to interact with pathogens and are thought to provide a pre-emptive defence against cold loving (psychrophilic) fungi, such as the snow moulds, that can be a serious problem in the cultivation of forage and cereal crops (Hoshino *et al.*, 2009).

In our study, the transcripts of several glucanases, chitinases and thaumatin-like proteins showed a winter wheat–specific increase in abundance totally consistent with a role as AFPs (Figure 6). That is, they showed a marked increase in abundance in the leaf tissue of the winter varieties, but no response in Paragon. In the leaf tissue of the two winter varieties, transcript abundance for *TaGLB2b* and *TaGLB2b* increased 160- and 57-fold, respectively: there was no response to a short cold shock.

*Ice recrystallization inhibition proteins (IRI)*

The two ice recrystallization inhibitor proteins, TaIRI-1 and TAIRI-2, belong to a class of ice-binding proteins thought to be specific to the grass subfamily, *Pooideae*, which includes wheat, barley and rye (Tremblay *et al.*, 2005). These bipartite proteins contain a short N-terminal leucine-rich repeat (LRR) domain which shows homology to that of receptor kinases and a C-terminal repeat domain that shows homology to the ice-binding domains of other anti-freeze proteins and that has been reported to exhibit strong ice recrystallization inhibitory properties (Sandve *et al.*, 2008). In our experiments, transcripts for the two ice recrystallization inhibition proteins, TaIRI-1 and TaIRI-2, greatly increased in abundance as a consequence of both a gradual decline in temperature and a cold shock (Figure 6). This latter detail distinguishes them from the chitinase, glucanase and thaumatin-like AFPs that showed no statistically significant changes on exposure to a cold shock (Figure 6). Additionally, their induction came later than that of the other AFPs, occurring between the fifth and the 9th weeks, rather than showing initial induction between the third and 5th weeks. The most marked response was in the leaves, particularly in the case of the *TaIRI-2* transcript, and there was much greater accumulation in the winter varieties than in Paragon. Interestingly, there were very few other transcripts that behaved in a similar fashion and so could be thought to be co-regulated. Among this group of genes, however, were *WLT10*, *BLT14-1* and *BLT14-2* proteins (which themselves are closely related to *WLT10*), a dehydrin, a xyloglucan endo-transglycosylase and an undefined plasma membrane protein (Unigene code Ta.4222). Finally, we observed a winter wheat–specific response in leaf tissue for the transcript for a putative polygalacturonase inhibitor. A protein of this type has been reported to act as an ice recrystallization inhibitor (Worrall *et al.*, 1998).

*Simple sugars*

It has long been considered that the accumulation of compatible solutes (organic osmoprotectants) in the cytoplasm contributes to freezing survival by reducing the rate and extent of cellular dehydration, by sequestering toxic ions, and/or by protecting macromolecules against dehydration-induced denaturation (Steponkus, 1984). Carbohydrates, in particular, are recognized as playing an important role in freezing tolerance (Livingston *et al.*, 2006), and the accumulation of simple sugars such as trehalose, raffinose and sucrose has been shown to be correlated with enhanced freezing tolerance (Wanner and Junttila, 1999; Pennycooke *et al.*, 2003; Kaplan *et al.*, 2006b). There have been several studies on the membrane stabilizing effect of various sugars suggesting a relationship between carbohydrate accumulation and freezing tolerance: trehalose (Crowe, 2002), raffinose (Pennycooke *et al.*, 2003), sucrose (Hincha and Hagemann, 2004) fructans (Livingston *et al.*, 2009). However, changes in sucrose levels have been shown to occur very rapidly—within 1 h at 4 °C—and this response did not appear to be driven by transcript abundance (Kaplan *et al.*, 2007).

In *Arabidopsis* and *Petunia*, raffinose is reported to show cold-related accumulation (Wanner and Junttila, 1999; Pennycooke *et al.*, 2003). This trisaccharide accumulates as a result of down-regulation of α-galactosidase, the enzyme responsible for its breakdown. Over the time course of our experiment, we observed a significant increase in galactinol synthase (GolS), the first enzyme in the pathway that leads to the synthesis of raffinose (Taji *et al.*, 2002). GolS is involved in carbon partitioning between sucrose and raffinose, a process that might be important in producing simple sugars as osmoprotectants. It has also been reported that cold-stimulated synthesis of GolS is under the control of the key cold- and dehydration-responsive transcription factor, DREB1a (CBF3) (Taji *et al.*, 2002; Maruyama *et al.*, 2009), and that galactinol and raffinose scavenge hydroxyl radicals as part of their function to protect plants from the potential oxidative damage that may results from chilling (Nishizawa *et al.*, 2008). There also appears to be a tendency of the two

winter varieties to increase transcript levels for sucrose phosphate synthase, an enzyme that shunts carbohydrate away from starch synthesis and into sucrose accumulation. It is important to note that both GolS and SPS are members of small gene families, the members of which respond differently to any particular stimulus (Taji *et al.*, 2002; Castleden *et al.*, 2004). In our cold acclimation experiments, transcripts for SPS1, 2 and 5 did not show any change. Transcripts for SPS 7 and 9 increased in abundance as temperature dropped, and there was differential expression between the winter and spring varieties. Similarly, the transcript for sucrose synthase 1 and 2 accumulated in both experiments and in all three varieties used in the study. In the cold acclimation experiment, they accumulated to a greater degree in Harnesk and Solstice than in Paragon.

*Annexins*

Annexins belong to a multi-gene family of multi-functional membrane- and $Ca^{2+}$-binding proteins. All annexins are soluble proteins that contain a highly conserved calcium-binding domain and a variable N-terminal region. The characteristic feature of these proteins is that they can bind membrane phospholipids in a reversible, $Ca^{2+}$-dependent manner. Their special feature is that they can behave as either cytosolic, peripheral or integral membrane proteins (Talukdar et al., 2009). They are principally cytosolic but, depending on local conditions of cytosolic free calcium, pH and membrane voltage, either attach to or insert into either plasma- or endomembranes (see reviews by Laohavisit and Davies (2009) and Talukdar et al. (2009)). They are thought to be involved in a diverse range of cellular functions. They may act as plant ion transporters that could account for channel activities in plasma membranes (Mortimer *et al.*, 2008; Laohavisit and Davies, 2009). They may also operate in signalling pathways involving cytosolic free calcium and reactive oxygen species (Mortimer *et al.*, 2008; Laohavisit and Davies, 2009; Talukdar *et al.*, 2009). Some of these properties have been reported for animal annexins (Gerke and Moss, 2002), but have not been experimentally demonstrated in plants (Talukdar et al., 2009). There is the interesting possibility that they could act in ROS detoxification during oxidative stress and may also be involved in ROS-mediated cell signalling. Annexins have been shown to have a role in the generation and propagation of calcium signals in nodule formation in Medicago truncatula (Talukdar et al., 2009). Breton et al. (2000) identified four cold-induced annexins in wheat and showed that they are intrinsic membrane proteins: their association with the membrane was shown to be calcium independent. In general, a rise in cytosolic $Ca^{2+}$ promotes relocation of annexins to membranes and as a consequence, they have been implicated in $Ca^{2+}$-driven signal transduction. In our study, an annexin, highly similar to annexin p33 of *Zea mays*, accumulated preferentially in the leaves of the two winter cultivars.

*Germins and germin-like proteins (GLPs)*

The germins and GLPs belong to the cupin superfamily based on the possession of a highly conserved β-barrel motif involved in metal binding (Zimmermann *et al.*, 2006; Davidson *et al.*, 2009). They are thought to play roles in calcium regulation, oxalate metabolism and responses to pathogenesis. True germins show oxalate oxidase activity and are found only in cereals. GLPs, on the other hand, are a much more diverse group of proteins that are encoded by a heterogeneous group of genes present in many land plants including monocots, dicots, gymnosperms and mosses. GLP is a term referring either to all germin motif-containing proteins with unknown enzyme activity or to those that do not possess oxalate oxidase activity. Interestingly, about two-thirds of the germins and GLPs analysed by Davidson *et al.* (2009) showed responses to various biotic and abiotic stresses. *They are all glycoproteins associated with the extracellular matrix and may either (i) have enzymic activity (oxalate oxidase or superoxide dismutase), (ii) be structural proteins or (iii) act as receptors.* For a recent review, see Davidson *et al.* (2009).

Germins and GLPs in our study showed a range of responses that differentiated the two tissues and the spring and winter varieties. An oxalate oxidase precursor accumulated preferentially in the leaves of the winter varieties, while a second accumulated preferentially in the leaf tissue of Paragon. Others accumulated in the leaves of all three varieties, but to a lesser extent in Paragon than in either Harnesk or Solstice. These GLPs might therefore be involved in basal responses to cold temperature, with the greater accumulation in winter varieties determining, in part, their enhanced capacity to tolerate extreme temperatures.

## Conclusion

### What determines the difference between winter and spring wheat?

Both spring and winter varieties of wheat, like most temperate plants, show some degree of chilling tolerance.

 

The initial responses that they show to cold, therefore, may be quite similar. In many articles, however, this point is somewhat over-looked; cold acclimation is presented as a process exclusively involved in the acquisition of freezing tolerance and authors often fail to consider the requirement of the plants to combat the stresses related to chilling temperatures. All falls in temperature below an optimum for biological processes will constitute stress, and plants need to mount a response to the stresses resulting from these chilling temperatures. A broad spectrum of responses is likely to be initiated as soon as plants experience suboptimal conditions, and these responses will augment as temperature becomes more extreme. Certainly, given a gradual decline in temperature, many of the early cold-induced changes in transcript abundance are unlikely to be part of the mechanism to protect plants from subzero temperatures and the growth of ice. More probably, they are responses to the general stresses (probably mainly oxidative stresses) of growth under suboptimal conditions and initially, these stresses might be as much to do with light as with the low temperature itself. Chilling tolerance, then, is a response to present stress resulting from both cold and light. The acquisition of freezing tolerance, on the other hand, is the putting in place of mechanisms for future temperature extremes. The cold-induced changes that unite spring and winter varieties are likely to be involved in maintaining cellular homeostasis. Cold responses either unique to or enhanced in hardy varieties will be those that anticipate the particular physical stresses experienced at zero and subzero temperatures such as extreme, life-threatening changes in membrane properties or damage as a result of ice growth and the extremes of osmotic stress associated with this. Thus, these are much more temperature-based phenomena.

The ability to withstand cold is an extremely variable character. There are chilling-sensitive and chilling-hardy plants, and there are also frost-tolerant and frost-intolerant plants. These categories should not be confused with the groupings 'winter' and 'spring' that indicate whether a plant requires to be vernalized to flower. However, it is frequently observed that winter varieties of wheat (i.e., those that require vernalization to flower) are hardier than spring types (Limin and Fowler, 2006). Because of this, it has been hypothesized that cold acclimation and the acquisition of freezing tolerance are closely associated with a requirement for vernalization (Prasil *et al.*, 2005). Winter varieties of wheat have a requirement for an extended cold period (several weeks) to become competent to flower. Spring varieties, on the other hand, do not have such a requirement. Vernalization requirement is controlled by the *Vrn1 loci* on the long arm of group 5 chromosomes: *Vrn-A1*, *Vrn-B1* and *Vrn-D1* on chromosome 5A, 5B and 5D, respectively. Spring varieties possess at least one dominant *Vrn1* allele, and this is constitutively expressed as was reported by us in a previous publication (Winfield *et al.*, 2009). Winter varieties have recessive alleles at all three *loci*. Molecular genetic studies have shown very tight genetic linkage between the *Vrn1 loci* and a major QTL, the frost tolerance 1 (*Fr1*) locus, associated with resistance to cold stress (Galiba *et al.*, 1997; Cattivelli *et al.*, 2002; Toth *et al.*, 2003). Indeed, the linkage of the two *loci* is so tight that the question has been raised of whether they are, in fact, the same *locus*. That is, is the marked correlation between winter habit and frost tolerance due either to pleiotropic effects of *VRN1* or because of the tight linkage between the *Vrn1* and *Fr1 loci*? However, most recent results indicate that they are two distinct, but closely linked *loci* (Kobayashi *et al.*, 2005).

Whatever the mechanism of control and the relationship between the *Fr1* and *Vrn1 loci*, it appears that some element of the vernalization pathway limits the expression of cold-responsive genes in spring varieties (Galiba *et al.*, 2009). Vernalization saturation is thought to act as a negative regulator of the cold acclimation genes (Gulick *et al.*, 2005; Monroy *et al.*, 2007). The expression of cold-responsive genes is initially the same in both spring and winter varieties (Monroy *et al.*, 2007), but spring varieties are unable to sustain the expression of these genes. It is suggested that this might be related to the developmental control of phase transition from vegetative to reproductive growth, plants competent to flower being unable to cold regulate. The ability of winter wheat varieties to induce and maintain the expression of frost tolerance genes dramatically decreases after the vegetative to reproductive transition, so that during the spring these plants deacclimate (Prasil *et al.*, 2005). In spring varieties, which as a result of possessing a dominant *VRN1* allele are already competent to flower, genes involved in cold acclimation may be induced, but are quickly repressed. Winter wheat varieties, on the other hand, require cold treatment to induce *VRN1*, and the cold-responsive genes are able to be expressed until vernalization saturation in achieved. Thus, they may be expressed over several weeks. In addition, winter cultivars may have a higher induction temperature for cold activated genes and so initiate their expression earlier than spring varieties.

NB a major differential characteristic of vernalization and acquisition of freezing tolerance is that plants retain a 'memory' of the former—plants do not de-vernalize once returned to warm conditions (this is necessary, or they might lose the capacity to flower as spring approaches)—but not of the latter—once the cold stimulus is removed, cold tolerance returns to its basal levels. Results from our analysis are consistent with this view. Under a growth regime in which there was a gradual decline in temperature over several weeks, but not as the result of a cold shock, there were many more cold-regulated genes in the winter varieties than in Paragon (Figure 3) and in many cases, the degree of transcript accumulation was also greater in the winter varieties (e.g., IRI genes; Figure 6) as reported previously by (Takumi *et al.*, 2003).

It seems evident that the different capacity of winter and spring varieties to cold acclimate and tolerate chilling and freezing conditions is underpinned, at least in part, by their differential ability to accumulate cold-responsive proteins. In some cases, this is absolute, with transcripts for certain genes accumulating only in winter varieties (for example, certain WRKY transcription factors and the anti-freeze proteins), while in other cases accumulation takes place in both winter and spring varieties, but to a lesser extent in the latter (e.g., several of the *COR* genes, dehydrins, and the ice-recrystallization proteins).

Agricultural production in many areas of the world is limited by cold and freezing temperatures. Determining what accounts for the differences in chilling and freezing tolerance between plant species and the molecular basis of cold acclimation is of basic scientific interest and has the potential to provide new approaches to improve the chilling and freezing tolerance of plants, both important agronomic traits. Higher yield could be achieved either by improving the freezing tolerance of over-wintering crops or by increasing the survival of freezing-sensitive crop plants following light frosts. Since 1929, little or no improvement in cold tolerance in cultivated varieties has been achieved (Fowler and Thomashow, 2002). Determining what accounts for the differences in chilling and freezing tolerance between different cultivars would provide the basis for approaches to improve these characteristics and so extend the range of varieties that might have many agronomic benefits, but which respond poorly in cold conditions (see intro. to Jaglo *et al.*, 2001). Given that certain transcription factors appear to have a major effect on the expression of suites of cold-responsive genes, many of which appear to be present in sensitive varieties and even species (Jaglo *et al.*, 2001), this might necessitate the adoption of 'regulon biotechnology' (Nakashima and Yamaguchi-Shinozaki, 2006).

A better understanding of the process of cold acclimation/acquisition of freezing tolerance and its relationship to vernalization of wheat at the transcriptome level and the identification of further components in the pathway should provide new opportunities for genetic improvement and management of this important temporal crop species.

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