

Transcriptional responses of winter barley to cold indicate nucleosome remodelling as a specific feature of crown tissues

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Received: 25 October 2010 / Revised: 31 January 2011 / Accepted: 2 February 2011 / Published online: 1 March 2011
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Abstract We report a series of microarray-based comparisons of gene expression in the leaf and crown of the winter barley cultivar Luxor, following the exposure of young plants to various periods of low (above and below zero) temperatures. A transcriptomic analysis identified genes which were either expressed in both the leaf and crown, or specifically in one or the other. Among the former were genes responsible for calcium and abscisic acid signalling, polyamine synthesis, late embryogenesis abundant proteins and dehydrins. In the crown, the key organ for cereal overwintering, cold treatment induced transient changes in the transcription of nucleosome assembly genes, and especially *H2A* and *HTA11*, which have been implicated

in cold sensing in *Arabidopsis thaliana*. In the leaf, various heat-shock proteins were induced. Differences in expression pattern between the crown and leaf were frequent for genes involved in certain pathways responsible for osmolyte production (sucrose and starch, raffinose, γ -aminobutyric acid metabolism), sugar signalling (trehalose metabolism) and secondary metabolism (lignin synthesis). The action of proteins with antifreeze activity, which were markedly induced during hardening, was demonstrated by a depression in the ice nucleation temperature.

Keywords Barley · Differentially expressed genes · Cold acclimation · Crown · Leaf · Metabolic pathways

Electronic supplementary material The online version of this article (doi:10.1007/s10142-011-0213-8) contains supplementary material, which is available to authorized users.

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Introduction

Both the survival and productivity of crop plants are heavily dependent on the environmental conditions in which they are grown. Low temperature is one of the most influential factors limiting where temperate plants can be cultivated. To survive periods of cold, plants have developed two distinct strategies—avoidance and acclimation. The former describes a capacity to prevent the freezing of sensitive tissues by, for example, preventing the formation of ice nucleators (Wisniewski and Fuller 1999). Acclimation relates to the process of acquiring certain non-heritable, but beneficial modifications in structure and function as a response to external stress (Kacperska 1999). The first stage of cold acclimation (CA), sometimes also referred to as the first phase of hardening (1PH), begins at low temperatures above 0°C, while the second stage (second phase of hardening (2PH)) requires lower, but still non-lethal temperatures.

As exposure to sub-zero temperatures represents such an important environmental stress in temperate cropping areas,

considerable efforts have been devoted to identifying the genes and their products responsible for tolerance active during both the first (e.g. Campoli et al. 2009; Ganeshan et al. 2008; Kocsy et al. 2010; Monroy et al. 2007) and the second (Herman et al. 2006; Livingston and Henson 1998) phases of CA. Many of the cold-induced changes are triggered by stress signals generated by a small number of regulatory mechanisms (reviewed by Yamaguchi-Shinozaki and Shinozaki 2006). Although the identity of the temperature sensors is not yet known (Penfield 2008), potential targets for its perception include changes in membrane fluidity (Sangwan et al. 2002; Uemura et al. 2006; Vaultier et al. 2006; Wang et al. 2006), photosystem II excitation pressure (Gray et al. 1997; Ndong et al. 2001) or specific histone variants (Kumar and Wigge 2010). Secondary messengers, such as Ca^{2+} , Ca^{2+} -dependent protein kinases, mitogen-activated protein kinases and reactive oxygen species can induce various transcription factors (TFs). This induction is known to drive the expression of a large number of “cold-regulated” (*COR*) genes, the products of which are associated with frost tolerance (Century et al. 2008). The C-repeat binding factor (CBF) transduction pathway is the most well defined in *Arabidopsis thaliana* (e.g. Gilmour et al. 2004; Novillo et al. 2004; Van Buskirk and Thomashow 2006) as well as in wheat and barley (e.g. Campoli et al. 2009; Cattivelli et al. 2002; Skinner et al. 2005; Sutton et al. 2009), although other CA regulatory mechanisms have been identified such as WRKY TFs in wheat (Skinner 2009; Talanova et al. 2009; Winfield et al. 2010) or certain chloroplast factors in barley (Svensson et al. 2006).

Cold-induced alterations in plant metabolism reflect the increases in amount and activity of enzymes involved in the production of osmolytes, in detoxification cascades and in secondary metabolism (Fowler and Thomashow 2002; Renaut et al. 2006). Since sugar accumulation is generally insufficient to provide full osmotic adjustment, it has been suggested that they may also act as signalling molecules (Iordachescu and Imai 2008). The small nitrogenous molecules proline (Hare et al. 1998) and γ -aminobutyric acid (GABA) are both involved in the CA response (Guy et al. 2008; Mazzucotelli et al. 2006). Heat-shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins represent a major class of cryo-protective molecules (Kovacs et al. 2008; Timperio et al. 2008; Tommasini et al. 2008). Other proteins strongly induced by low temperature stress include antifreeze and ice recrystallisation inhibition proteins, since these all limit the extent of ice formation in the apoplastic space (Griffith et al. 1992; Griffith and Yaish 2004; Winfield et al. 2010; Wisniewski et al. 1999).

A prominent morphological feature of winter-sown cereal plants is the meristematic structure lying at the

junction of the root and the stem, called the crown. This structure is responsible for the re-establishment of growth when permissive conditions return in the spring (Winfield et al. 2010) and thus makes a significant contribution to winter survival (Olien 1967). Survival depends on the viability of specific tissues within the crown (Livingston et al. 2006). Despite this knowledge, the majority of CA gene expression studies to date have ignored this important organ. However, the crown’s physiological, transcriptomic, proteomic and metabolomic responses to cold stress have begun to be explored (Ganeshan et al. 2008; Livingston et al. 2006; Pearce et al. 1998; Sutton et al. 2009; Winfield et al. 2010), with some attention being paid to both 2PH (Herman et al. 2006; Livingston and Henson 1998; Zámečník et al. 1994) and the adjustment of the post-acclimation transcriptome (Skinner 2009).

Here, we tested the hypothesis if specific molecular mechanism(s) characterise the cold response of the crown, the key organ for winter survival. A time course experiment covering 1PH and 2PH was carried out to compare leaf and crown transcriptomes of winter barley cv. Luxor. As well as identifying what genes were cold-regulated in both organs, sequences (and their corresponding metabolic processes) have been revealed which were specifically activated in the crown or the leaf as a response to cold stress.

Materials and methods

Stress treatment

Pre-germinated winter barley cv. Luxor seedlings were raised in semi-sterile conditions under a 12 h photoperiod (irradiation intensity $\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and a day/night temperature of 18/13°C. When the second leaf was fully expanded, the seedlings were exposed for up to 3 weeks at +3/2°C (day/night temperature)—the first phase of hardening (1PH), at an irradiation intensity of $\sim 120 \mu\text{mol m}^{-2} \text{s}^{-1}$. A set of seedlings were also exposed after the 21 day acclimation period to -3°C for 1 day (the second phase of hardening, 2PH). The plants (the second leaf and crown separately) were sampled in the middle of the lit period after 0, 1, 3, 7 and 21 days at +3/2°C and after the day at -3°C . The plant tissue was snap-frozen in liquid nitrogen and stored at -80°C , before being used for RNA extraction based on the TRIZOL reagent (Invitrogen, CA, USA). The RNA was purified by passing through an RNeasy column in the presence of DNase (Qiagen, Hilden, Germany). RNA quality was assessed by both agarose gel electrophoresis and analysis in an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Each biological sample was represented by three independent replicates, each of which consisted of a bulk of four seedlings.

Expression profiling

Each RNA sample was hybridised to the Affymetrix 22 K Barley1 GeneChip Genome Array (Close et al. 2004). GeneChip® hybridization quality was ensured by using standard controls supplied by Affymetrix, and B2 oligonucleotides were added to each hybridization cocktail. PolyA controls (*lys*, *phe*, *thr*, *dap*) and hybridization controls (*BioB*, *BioC*, *BioD* and *Cre*) were used to monitor labelling and hybridization. Raw intensity values were normalised by applying the Robust Multi-array Average method (Irizarry et al. 2003), using the R package Affymetrix library (Irizarry et al. 2006). R^2 correlation coefficients were calculated among the three biological replicates. The R package Affymetrix library was also used to apply the MAS 5.0 algorithm to the raw data to generate a detection call (“present”, “marginal” or “absent”) for each probe set, and this output was used as an initial filter, so that only those calls for which all three replicates were recorded as “present” in at least one sample were retained. The filtered data were analysed using Genespring GX 7.3 (Agilent Technologies, Santa Clara CA) software. Differentially expressed genes (DEGs) were identified by means of a Hochberg false discovery rate corrected Welch *t* test (Benjamini and Hochberg 1995). Expression differences were considered significant when their *p* value was <0.05, and their induction (or repression) ratio was ≥ 2 .

Clusters of genes sharing similar expression patterns were identified using a K-means cluster analysis, based on Spearman correlations. Over-represented functional gene categories within a given cluster were determined by a search of FunCat (Ruepp et al. 2004; <http://mips.gsf.de/projects/funcat>). Blast searches were performed within www.harvest.ucr.edu, and an E-value cut-off of e^{-10} was chosen. Expression fold changes (FC) in leaf and crown were calculated and analysed using MapMan software (<http://mapman.gabipd.org>; Thimm et al. 2004; Usadel et al. 2005).

Freezing test, dry weight, ice nucleation and osmotic potential

Freezing damage was assessed by measuring electrolyte leakage, using a modified version of the protocol developed by Prášil and Zámečník (1998). The plant material (two replicates, each consisting of 16 leaf discs or four crowns) was cooled to -30°C at 9°C h^{-1} in a controlled alcohol bath. The plant material of each replicate was placed inside a thin glass tube, which was then seeded with ice crystals at $-1.5 \pm 0.2^{\circ}\text{C}$ (plants sampled at -3°C were seeded at $-3 \pm 0.2^{\circ}\text{C}$) and held for 30 min at $-1.5 \pm 0.2^{\circ}\text{C}$ (plants sampled at -3°C were held at $-3 \pm 0.2^{\circ}\text{C}$). The frozen samples were thawed slowly in ice trash and then

immersed for 2 h in distilled water at 25°C . Samples were removed from the freezing bath after reaching the 12 target temperatures (temperature sensors were placed inside the tubes). The extent of electrolyte leakage was calculated from the ratio between the electrical conductivity of the solution measured before and after boiling the same sample. Lethal temperature (LT_{50}) was defined as the temperature responsible for 50% electrolyte leakage. For the evaluation of crown regrowth, the same protocol was used, but here, intact plants were cooled, then replanted and cut above the crown, and their survival rate calculated during the next week. Here, LT_{50} was defined as the temperature at which 50% of the plants died. LT_{50} s for both electrolyte leakage and crown regrowth were estimated from the inflection point of the sigmoidal relationship between electrolyte leakage/plant survival and freezing temperatures. The resulting data were statistically evaluated following Janáček and Prášil (1991). Dry weights (DWs) were calculated from the mean of 20 crowns or detached leaves at each sampling point once their fresh weight had reached constant weight at 85°C . Leaf areas obtained from scans of individual detached leaves were divided by the leaf’s DW to obtain a specific leaf area. Osmotic potential was estimated by means of the dew-point method, using a Wescor C-52 chamber connected to a HR-33T psychrometer–hygrometer (Wescor, Logan, UT, USA). The plant material was hermetically sealed within a plastic syringe, and its osmotic potential measured from the sap released after the loss of turgor pressure following a freeze/thaw cycle. The osmotic potential and DW data were evaluated with STATISTICA v6.1 (StatSoft, USA) software. Ice nucleation activity and melting temperature were assessed using a differential scanning calorimeter (TA 2920, TA Instruments, Inc., New Castle, DE, USA). The measurements were taken from hermetically sealed 5-mm diameter leaf discs and crown pieces, following a cooling/warming cycle of $10^{\circ}\text{C min}^{-1}$. The samples were freshly harvested, but their surface was free of free moisture. During controlled warming ($10^{\circ}\text{C min}^{-1}$), the extrapolated onset temperature of endothermic heat flow peak was defined as the melting temperature. The resulting data were evaluated using Universal Analysis 2000 software (TA Instruments-Waters LLC, New Castle, DE, USA) and STATISTICA v6.1 (StatSoft, USA) software.

Results

Monitoring cold acclimation

A clear increase in freezing tolerance was observed during the 21 days of CA. The LT_{50} s of both the leaf and crown declined rapidly over the first 3 days, then more slowly up to day 21. CA was further enhanced when the 21-day

acclimated plants were exposed for a further day at -3°C (Fig. 1a). Although the crown regrowth method showed a similar trend in LT_{50} up to the day 21 of 1PH and was followed by a slight decrease during 2PH (Fig. 1b), the LT_{50} s were lower, indicating improved survival. Thus, plasma membrane injury appears to be a good although imperfect indicator of plant survival at low temperatures. The dry weight of the crown increased after 1 week of hardening and continued up to day 21 (Fig. 2), and a similar, but not statistically significant trend was apparent for leaf DW (expressed as specific leaf area; Fig. 2). An important component of the cold acclimation process is the accumulation of osmotically active sugars, sugar alcohols and amino acids (Rapacz et al. 2000). The osmotic potential of the leaf increased from -1.15 to -0.7 MPa over the first 3 days of acclimation and then slowly declined over the remainder of the acclimation period to -1.02 MPa; exposure to -3°C produced a further decline to -1.23 MPa

(Fig. 3). In contrast, in the crown, osmotic potential declined gradually from -0.95 to -1.44 MPa over the whole cold acclimation period, while exposure to -3°C increased it slightly to -1.40 MPa. The most rapid change in osmotic potential occurred between days 3 and 7 of acclimation. The ice nucleation temperature of the leaf was lower than that of the crown throughout the cold acclimation period. Thus, for the leaf, this varied from -10.4 ± 0.2 to $-13.9\pm 1.1^{\circ}\text{C}$ during the measurement period, while in the crown, the equivalent temperatures were $-9.6\pm 0.2^{\circ}\text{C}$ and $-13.2\pm 2.3^{\circ}\text{C}$. The linear regression between the ice nucleation temperature and the length of acclimation time was highly significant ($p\leq 0.05$) for both the leaf and the crown (Fig. 4). The melting temperature of the crown decreased significantly between days 3 and 7 of 1PH and decreased slightly more during the transition to 2PH (Fig. S1). Overall, the response of crown to low temperature seemed to be more significant than the equivalent response of the leaf.

Fig. 1 **a** The degree of freezing tolerance of the leaf and crown of winter barley cv. Luxor as determined by an electrolyte leakage method. Plants were exposed to 12 target temperatures. Points shown represent the mean ($n=2$) freezing tolerance (LT_{50} value) in each of the six treatments (C: no cold exposure; 1, 3, 7 and 21 days exposure to $+3^{\circ}\text{C}$; 21 days exposure to $+3^{\circ}\text{C}$, followed by 1 day at -3°C). Means assigned a different letter differ significantly from one another at $p\leq 0.05$. **b** The low temperature survival of the crown during the first phase of hardening (1PH) and the second phase of hardening (2PH). Plants were exposed to 12 target temperatures. After thawing, they were cut above the crown and left to recover for 1 week. LT_{50} was defined as the temperature at which 50% of the plants died. Points shown represent the mean ($n=4$) LT_{50} in each of the following six treatments: C no cold exposure; 1, 3, 7 and 21 days exposure to $+3^{\circ}\text{C}$; and 21 days exposure to $+3^{\circ}\text{C}$, followed by 1 day at -3°C . Standard deviations are only shown by bars when their size exceeded that of the circlet. Means assigned a different letter differ significantly from one another at $p\leq 0.05$

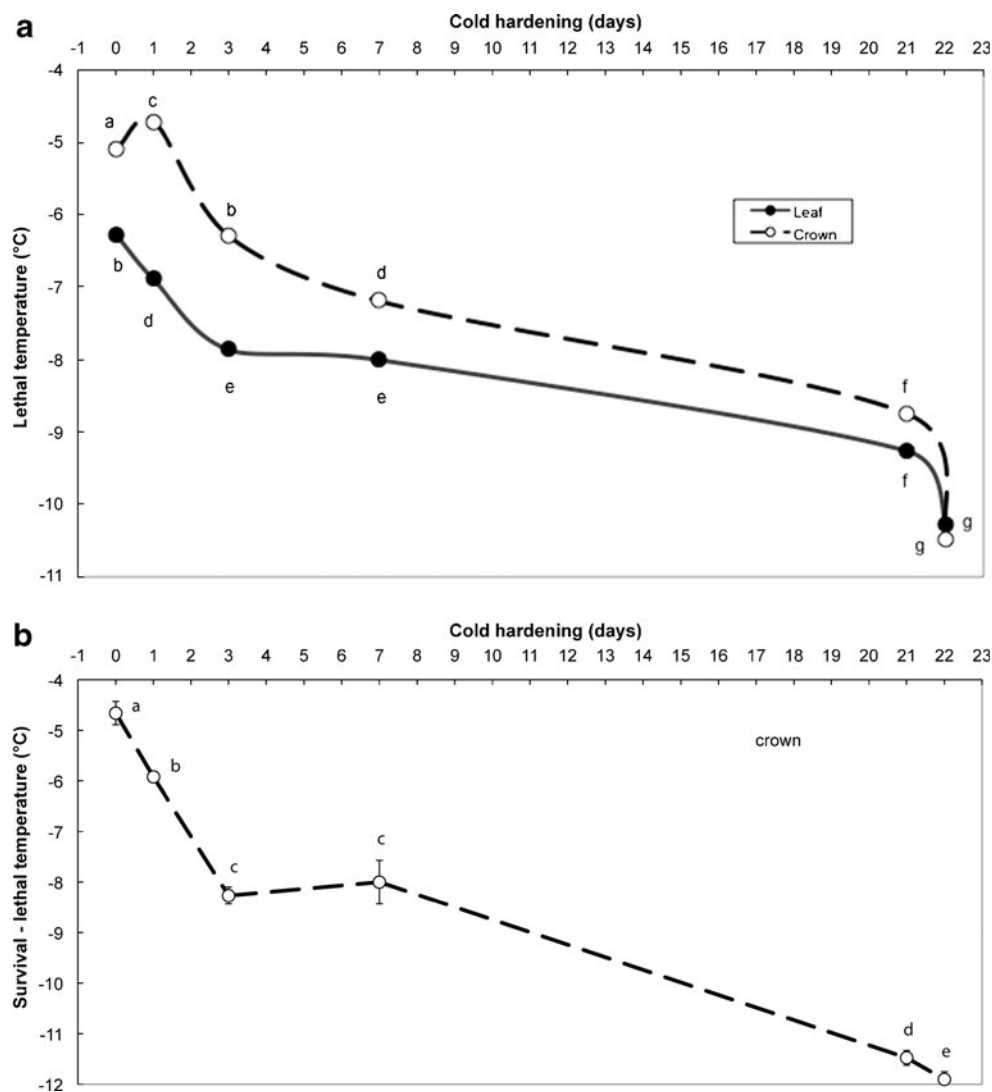
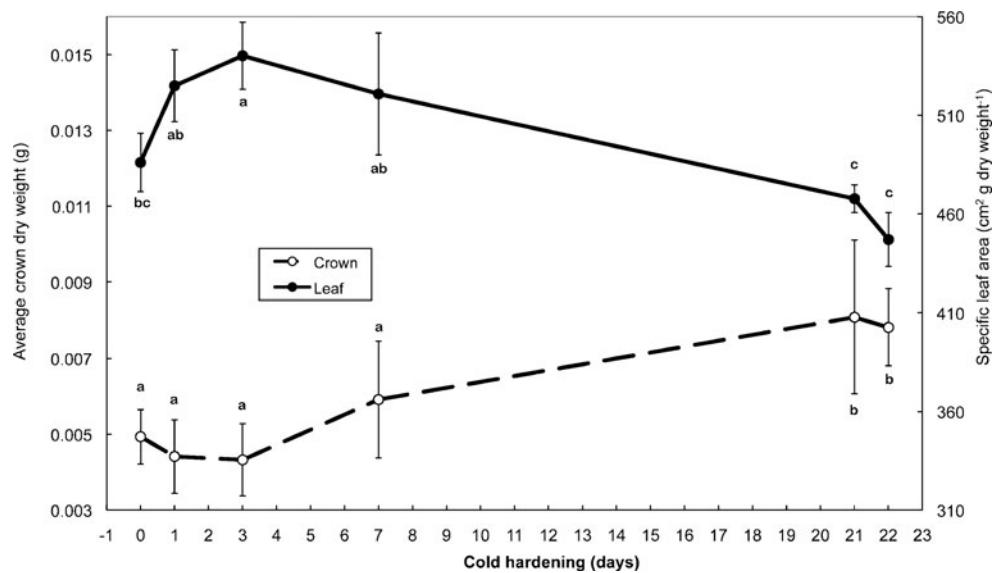


Fig. 2 Accumulation of dry matter in the crown and leaf of non-acclimated and acclimated (21 days at +3°C, followed by 1 day at -3°C) winter barley cv. Luxor. The mean dry weight (DW) of 20 crowns was expressed in g per crown. Specific leaf area ($n=20$) was calculated from leaf area/DW. The contrasting trends shown by the two curves can be interpreted as meaning that in both organs, dry matter accumulated during hardening. Means assigned a *different letter* (separately for the leaf and crown) differ significantly from one another at $p \leq 0.05$. Bars represent standard deviation



Differentially expressed genes (DEGs)

R^2 coefficients between the three replicates of each sample ranged from 0.97 to 0.99. The proportion of “present” calls was 64.0% in the leaf and 68.9% in the crown samples. Most of the crown DEGs were detectable following 3 days of hardening (1,456), but as for the DEGs identified following 1 day of hardening, more than 50% of them were down-regulated (52% after 1 day of hardening, 55% after 3 days). However, upon transition to 2PH, up to 82% of the crown DEGs were up-regulated, including various transcription factors responsible for production of cold protective compounds. About one half of all the leaf DEGs were up-regulated during 1PH and nearly three quarters during 2PH.

The three separate cluster analyses carried out were based on DEGs common to both the leaf and crown (1,371 genes, nine clusters, Fig. S2), those which were leaf-specific (4,827 genes, nine clusters, Fig. S3) and those which were crown-specific (999 genes, nine clusters, Fig. S4).

DEGs common to both the leaf and crown Clusters 1, 4 and 7 (Fig. 5) contain genes which were up-regulated following a 1 day exposure to +3°C, then further up-regulated by the transfer to -3°C. FunCat analysis identified five over-represented categories, namely 01. metabolism, 20. cellular transport, 32. cell rescue, defense and virulence, 34. interaction with the environment and 36. systemic interaction with the environment.

Fig. 3 Time course of osmotic potential in the leaf and crown of non-acclimated and acclimated (21 days at +3°C, followed by 1 day at -3°C) winter barley cv. Luxor, as measured by the dew-point method. Means ($n=3$) assigned a *different letter* (separately for the leaf and crown) differ significantly from one another (p value ≤ 0.05)

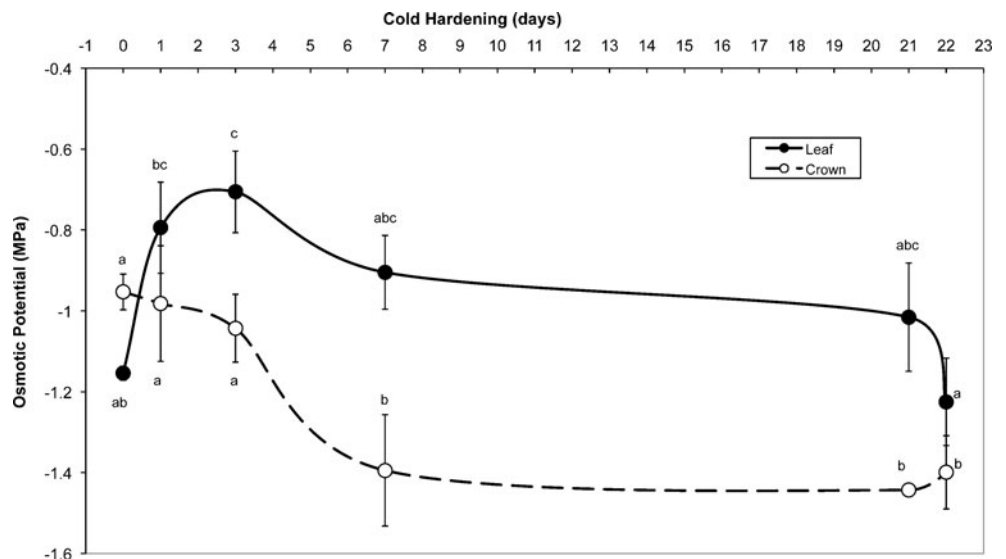
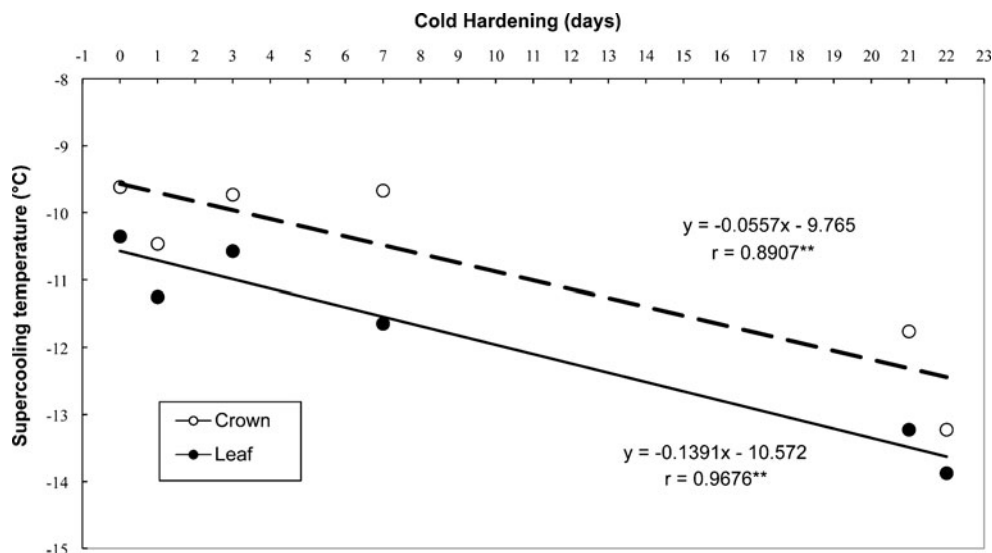


Fig. 4 The ice nucleation temperature of the leaf and crown of non-acclimated and acclimated (21 days at +3°C, followed by 1 day at -3°C) winter barley cv. Luxor. The ice nucleation temperature was assessed by differential scanning calorimetry. Each point represents the mean of three independent measurements



Downstream protein components are believed to translate the information encoded by transient changes in calcium concentrations into stress specific cellular adaptation responses (D’Angelo et al. 2006). Two candidate protein families in this transduction process have been identified recently, specifically the calcineurin B-like calcium sensors (CBLs) and the CBL-interacting serine-threonine protein kinases (CIPK, D’Angelo et al. 2006). Barley homologues of CIPK1 (*At3g17510*), CIPK12 (*At4g18700*), CIPK16 (*At2g25090*) and CBL1 (*At4g17615*) were present in these clusters. In addition, the calcium-transporting plasma-type ATPase 8 (ACA8, *At5g57110*) was co-regulated with CIPK and CBL. Clusters 1, 4 and 7 also contain a number of genes (LEAs, dehydrins and drought-related transcription factors) known to be

responsive to changes in either water status or ambient temperature, as well to treatment with exogenous abscisic acid (ABA).

Clusters 2 and 9 (Fig. 5) show a similar expression trend as clusters 1, 4 and 7, with the exception that they were not further responsive at sub-zero temperature. Some of the sequences in clusters 2 and 9 share homology with genes already assigned a role in the cold acclimation process. Among these was *SEX1* (Starch Excess 1; *At1g10760*), a gene which, when mutated in *A. thaliana*, induces cold sensitivity resulting from a failure to break down starch (Yano et al. 2005). Also represented are the homologues of *COR413-PM* (*At2g15970*), *COR314-TM2* (*At1g29390*) and the ABA-responsive genes *ATHVA22E* (*At5g50720*) and *RAB18* (*At5g66400*). Homologues of the drought

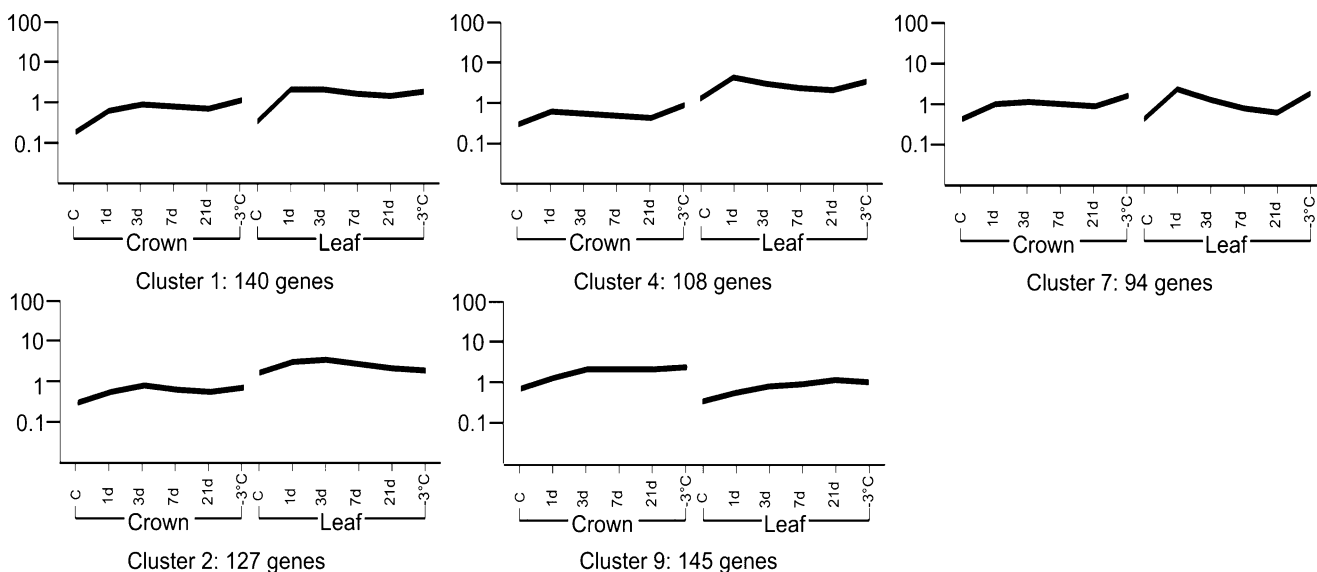


Fig. 5 Clusters 1, 2, 4, 7 and 9 of genes regulated in both the leaf and crown in response to cold acclimation. X axis: the number of days of exposure to low temperature and organ type; Y axis: gene expression

level. The curve represents the mean expression level of all the genes in the respective cluster

stress response genes *STO* (*At1g06040*), *ATOSM34* (*At4g11650*) and *OST1* (*At4g33950*) were also represented, along with those of three genes involved in proline metabolism, namely *P5CS* (δ 1 pyrroline-5-carboxylate synthase; *At2g39800*) a gene encoding a key proline synthesis enzyme, proline oxidase (*At5g38710*) and Δ -OAT (ornithine δ -aminotransferase; *At5g46180*) and the gene involved in ABA metabolism (zeaxanthin epoxidase, *At5g67030*). Cuevas et al. (2008) have noted that the accumulation of putrescine is essential for cold acclimation in *A. thaliana*, since mutants defective for its synthesis show low tolerance to freezing. The clusters also contained an *ADC2* homologue (arginine decarboxylase; *At4g34710*), a gene responsible for a key enzyme in the putrescine pathway.

Leaf-specific DEGs A demonstration that the leaf and crown are morphologically and physiologically distinct from one another was that nearly 5,000 DEGs were leaf-specific. These clustered into nine clades. Members of clusters 2 and 9 (839 genes) were all up-regulated after 1 day exposure to +3°C (Fig. 6). FunCat analysis identified five over-represented categories, namely 01. metabolism, 14. protein fate, 20. cellular transport, 32. cell rescue, defense and virulence and 34. interaction with the environment. Genes encoding chloroplast-localised proteins were also over-represented, supporting the notion that plastid-localised proteins are important for cold acclimation (Svensson et al. 2006). Several HSP homologues, in particular HSP17.4 (*At1g54050*), HSP70 (*At3g12580*) and HSP101 (*At1g74310*), as well as transcription factors

responsive to salinity stress (*STO*, *At1g06040*) and heat stress (*HSF3*, *At5g16820*) also belonged to these two clusters, as did a homologue of the dehydrin encoding gene *DHN* (*At1g54410*) and the papain family protease encoding gene *RD21* (*At1g47128*).

The expression of the 292 genes in cluster 3 increased in the leaf both when the temperature was reduced to +3°C, and then again to -3°C (Fig. 6). Four functional categories were over-represented, namely 14. protein fate, 32. cell rescue, defense and virulence, 34. interaction with the environment and 40. cell fate. The cluster also contained genes encoding calmodulin (*CAM1*, *At5g37780*), calmodulin-dependent kinases (*CPK7*, *At5g12480* and *CPK1*, *At3g17510*) and a calmodulin-binding heat-shock protein (lipase3, *At5g37710*), along with the transcription factor *SLT1* (*At2g37570*) and various cold-regulated genes (*ERD4*, *At1g30360*; *ERD7*, *At2g17840*; *LEA*, *At2g46140*; *RCI2A*, *At3g05880*; *HSP 17.6II*, *At5g12020*).

The 781 genes within clusters 5 and 7 were transiently down-regulated immediately following the first day of exposure to +3°C. Thereafter, their expression increased gradually over the rest of the acclimation period, to reach a level higher than that of the same genes in the non-cold treated samples. Their subsequent transfer to -3°C triggered at most a slight reduction in expression (Fig. 6). As for the genes present in clusters 2 and 9, there was an over-representation of genes belonging to 01. metabolism, 14. protein fate, 20. cellular transport, 34. interaction with the environment, as well as of several calcium-dependent protein kinases, calmodulins and HSPs. Homologues were recognised of the osmotic regulator *ATOSM34* (*At4g11650*),

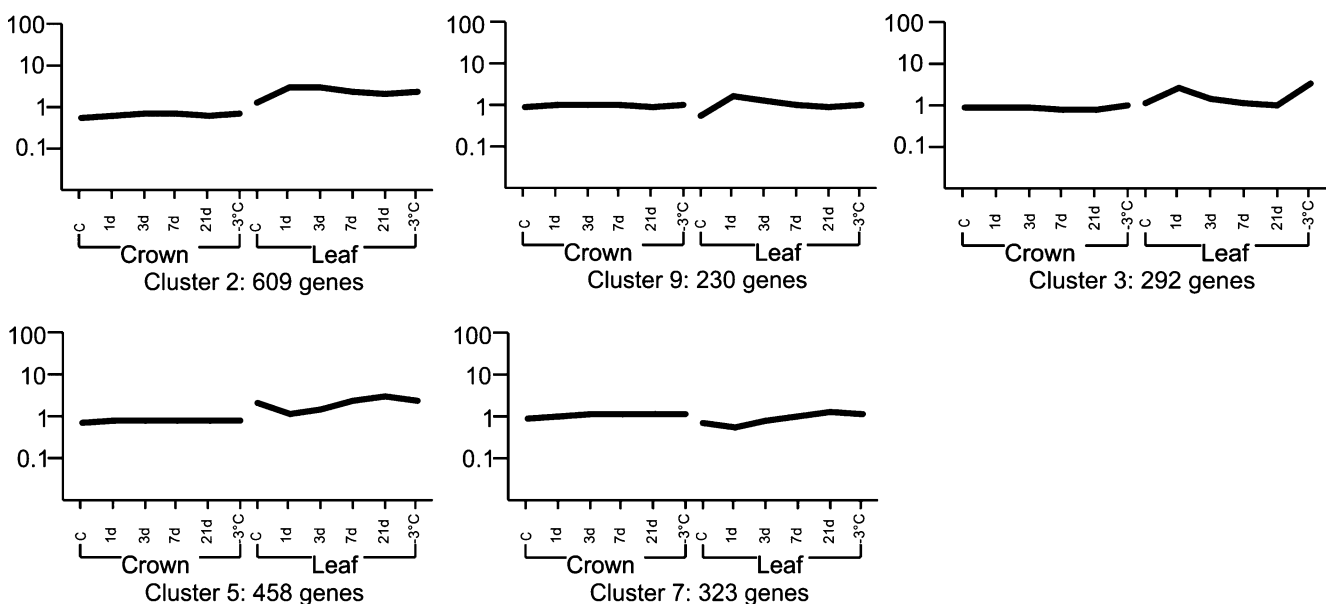


Fig. 6 Clusters 2, 3, 5, 7 and 9 of genes regulated specifically in the leaf in response to cold acclimation. *X axis*: the number of days of exposure to low temperature and organ type; *Y axis*: gene expression

level. The curve represents the mean expression level of all the genes in the respective cluster

P5CS which encodes a key enzyme in the proline synthesis pathway, three genes involved in glycine betaine synthesis (ALDH10A9, *At3g48170*; ALDH7B4, *At1g54100*; ALDH2C4, *At3g24503*), the two osmotically responsive genes LOS1 and LOS2 (*At1g56070*, *At2g36530*) and AREB3 (*At3g56850*), which encodes an ABA-responsive DNA binding protein.

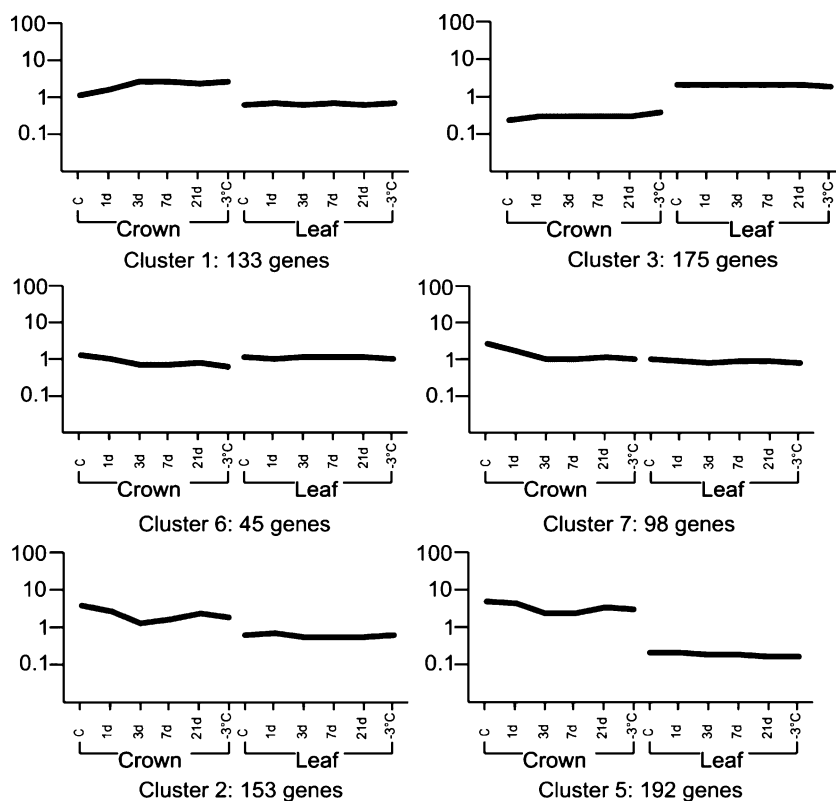
Crown-specific DEGs The expression of most of the 999 crown-specific DEGs was characterised by a slower response, with 3 days of cold acclimation being required to reach a peak (or trough) of expression. Only the genes in cluster 3 showed any increase in expression after 1 day at +3°C (Fig. 7). Cluster 1 captured a number of homologues of genes involved in the response to external stimuli, including an ABA-responsive DNA binding factor (ABF3/DPBF5, *At4g34000*) and an auxin response factor (ARF16, *At4g30080*). The presence of the latter suggested a role for auxin in cold stress sensing, as also noted in *A. thaliana* (Hannah et al. 2005). There is also some evidence for an interaction between ABA and auxin during cold acclimation (Brady et al. 2003; Suzuki et al. 2001).

The expression level of the 345 genes in clusters 2 and 5 fell gradually at the beginning of the cold acclimation period, but, from the third day onwards, it rose again; upon exposure to -3°C, their level of expression was marginally

reduced (Fig. 7). The over-represented functional categories included 01. metabolism, 10. cell cycle and DNA processing, 16. protein with binding function or cofactor requirement and 42. biogenesis of cellular components. Many of the genes in these clusters are involved in nucleosome assembly—for instance, those encoding histones H1.2 (*At2g30620*), H2A (*At4g27230*), H2B (*At5g22880*, *At2g37470*), H3 (*At5g10400*, *At5g10390*), H3.2 (*At1g5600*, *At1g13370*), H4 (*At5g59970*), HTB4 (*At5g59910*), HTB11 (*At3g46030*), HTA11 (*At3g54560*) and HTA12 (*At5g02560*). Of these, H2A and HTA11 have been shown to be directly involved in the perception of temperature during nucleosome remodelling (Kumar and Wigge 2010).

Many of the genes grouped into cluster 3 are involved with photosynthesis and so were, as expected, expressed at a high level in the leaf. Some were marginally up-regulated in the crown after a short exposure to +3°C (Fig. 7). Those in clusters 6 and 7 were gradually down-regulated over the course of the first 3 days of cold acclimation, after which their expression level remained relatively constant (Fig. 7). As in other clusters, the over-represented functional categories in these two clusters were 01. metabolism, 02. energy and 32. cell rescue, defense and virulence, which is rather typical of the general plant response to environmental stress.

Fig. 7 Clusters 1, 2, 3, 5, 6, and 7 of genes regulated specifically in the crown in response to cold acclimation. *X axis*: the number of days of exposure to low temperature and organ type; *Y axis*: gene expression level. The curve represents the mean expression level of all the genes in the respective cluster



Metabolic pathways potentially activated during cold acclimation

MapMan software (<http://mapman.gabipd.org>; Thimm et al. 2004; Usadel et al. 2005), which was developed as a means of translating transcriptomic into metabolic data, was employed to identify the potential metabolic consequences of cold-induced gene expression changes. We considered three key contrasts, namely untreated vs. 1 day at +3°C, untreated vs. 3 days at +3°C, and 21 days at +3°C vs. 21 days at +3°C followed by 1 day at −3°C. Mean FC values of the genes involved in the metabolic pathways described below are given in Tables 1 (leaf) and 2 (crown). The gene chip is designed to include multiple probe sets for each target gene, but, in some cases, these detected distinct expression profiles, suggesting the presence of gene isoforms. Thus, the probe sets which generated a uniform expression profile were considered as fully redundant, whereas those which generated distinct profiles were taken to have detected distinct isoforms. Because reference to MapMan enabled gene function in barley to be predicted, the spectrum of isoforms present in barley may be different to what has been described in *A. thaliana* (see Tables 1 and 2).

Sucrose and starch metabolism The expression of a number of genes involved in sucrose/starch metabolism responded to the cold acclimation treatment. The largest changes in expression in the leaf occurred after 3 days of acclimation, while the further temperature reduction to −3°C had very little effect. Most of these genes were up-, rather than down-regulated by the cold treatment. Of the four vacuolar invertases which responded within 3 days of acclimation, three were up-, and the fourth was down-regulated. The expression of cell wall invertases (two isoforms present) was greatly induced by the cold treatment, while that of the two isoforms of leaf neutral invertase was repressed. Lowering the temperature to below freezing led to further rises in the expression level of the vacuole and cell wall invertases. The up-regulation of the two sucrose synthase (SUS) isoforms after 3 days of CA suggested an enhanced level of sucrose cleavage activity. The increase in sucrose phosphate synthase and starch synthase transcript abundance during cold acclimation suggested that sucrose-6-phosphate and amylose may be accumulated early during the cold stress period. Starch is cleaved *in planta* by a combination of alpha- and beta-amylases and phosphorylase. Although at least three alpha- and beta-amylase genes were among the DEGs, this was not the case for phosphorylase, whose expression declined after 3 days of cold treatment. After 1 day, the expression of hexose transporter, and after 3 days that of a triose phosphate translocator was observed. The transcriptomic data, as a body, suggest that both the cold and the freezing treatments

produced an accumulation of maltose and glucose in the leaves.

In the crown, the expression of genes involved in sucrose/starch metabolism was only marginally affected by low temperature. Among the few genes significantly up- or down-regulated was a sucrose transporter (up-regulated), while a number of genes encoding enzymes involved in sucrose degradation (one isoform of cell wall invertase; vacuolar invertase; neutral invertase; fructokinase) were all down-regulated; this suggests that sucrose was exported rather than cleaved into fructose and glucose. Only at the beginning of the cold acclimation process was one isoform of vacuolar invertase up-regulated. Some starch degradation enzymes were down-regulated (alpha-glucosidase), but others were up-regulated (alpha-amylase; beta-amylase), which would be consistent with the accumulation of maltose—believed to be a cryoprotectant as well as a precursor for soluble sugar metabolism (Kaplan et al. 2006)—at the beginning of the stress period. Both maltose and glucose were likely accumulated after 3 days of cold acclimation. The exposure to sub-zero temperature resulted in the further up-regulation of some of the genes involved in sucrose degradation (cell wall invertase) and starch degradation (beta-amylase; alpha-amylase), suggesting a further accumulation of glucose and maltose as the response to freezing.

Trehalose metabolism The expression of starch degradation genes agrees well with that of the two trehalose metabolism genes *TPS* (trehalose-6-phosphate synthase) and *TPP* (trehalose-6-phosphate phosphatase), which reacted differentially to cold acclimation. Here, *TPS* was down-regulated by low temperature in both the crown and the leaf. Additionally, three further putative *TPS* isoforms were also down-regulated in the leaf at the beginning of the acclimation period. In contrast, one of the two *TPP* isoforms was down-regulated in both crown and leaf in response to low temperature exposure, while the other was up-regulated at the beginning of the acclimation period in the leaf and by the sub-zero temperature in both the leaf and the crown. These observations are consistent with the accumulation of trehalose, the absence of trehalose-6-phosphate and the breakdown of starch.

Raffinose metabolism The expression of a number of genes encoding enzymes involved in raffinose metabolism—such as galactinol synthase and raffinose synthase—was affected by the acclimation treatment. In the leaf, the transcript abundance of one isoform of galactinol synthase was reduced by the treatment, while that of two other isoforms was increased. The expression level of none of the isoforms was further modified by exposure to −3°C. Similarly, one raffinose synthase gene was up-regulated, while that of a putative isoform was down-regulated after 3 days of

Table 1 Fold change (FC) values of differentially expressed genes encoding key leaf enzymes

Metabolism, leaves	Annotation	Probe set ID	<i>Arabidopsis</i> Accn	FC: C vs 1 day (3°C)	FC: C vs 3 days (3°C)	FC: 21 days (3°C) vs 1 day (-3°C)
<i>sucrose and starch</i>	Vacuolar invertase	Contig4521_s_at;	AT1G12240.1;	30.91	12.86	NDEG
		HVSMEn0014H06r2_s_at	AT1G62660.1			
		Contig13653_at;	AT1G62660.1;	35.49	16.55	NDEG
		Contig2492_s_at;	AT1G62660.1;			
		Contig2499_s_at;	AT1G62660.1;			
		Contig3392_at;	AT1G62660.1;			
	Cell wall invertase	HA11P12u_s_at	AT1G62660.1			
		rbah48h06_s_at	AT1G62660.1	100.19	13.42	NDEG
		Contig2495_s_at	AT1G62660.1	NDEG	0.37	3.57
	Neutral invertase	Contig6787_at	AT3G13790.1	2.56	2.72	NDEG
		Contig11241_at	AT3G52600.2	175.58	44.15	9.45
	Sucrose synthase	Contig7027_at;	AT1G56560.1;	0.38	0.29	NDEG
		S0000700018E12F1_s_at	AT1G22650.1			
		Contig11836_at	AT1G22650.1	0.36	0.27	NDEG
	Sucrose phosphate synthase	Contig361_s_at;	AT3G43190.1;	NDEG	2.96	NDEG
		Contig689_s_at	AT3G43190.1			
	Starch synthase	Contig460_s_at	AT3G43190.1	NDEG	2.01	NDEG
		Contig16475_at	AT4G10120.2	4.81	3.89	NDEG
	Alpha-amylase	Contig6496_at;	AT5G11110.1;	2.53	2.64	NDEG
		HVSMec0008G05r2_s_at	AT5G20280.1			
Beta-amylase	Contig1808_at	AT5G24300.1	2.32	NDEG	NDEG	
	Contig14542_at	AT1G69830.1	2.03	NDEG	NDEG	
Phosphorylase	Contig14990_at	AT4G09020.1	5.17	2.54	NDEG	
	Contig7088_at	AT4G25000.1	2.36	3.24	NDEG	
Hexose transporter	Contig11522_at	AT4G17090.1	16.16	5.52	7.01	
	Contig6517_at	AT3G46970.1	NDEG	0.39	NDEG	
Triose phosphate translocator	Contig6135_at;	AT5G16150.3;	0.31	NDEG	NDEG	
	Contig6135_s_at	AT5G16150.3				
SUSIBA2	Contig3114_at	AT5G46110.1	NDEG	0.48	NDEG	
<i>raffinose</i>	Galactinol synthase	Contig7243_at;	AT4G26640.2;	0.40	NDEG	NDEG
		rbaal15j13_s_at	AT4G26640.2			
		Contig3812_at	AT1G56600.1	0.39	0.36	NDEG
	Raffinose synthase	Contig3810_at	AT1G09350.1	4.54	2.72	NDEG
		Contig3811_at;	AT2G47180.1;	3.60	3.55	NDEG
	Raffinose synthase, putative	Contig3810_s_at	AT1G09350.1			
		Contig26196_at	AT5G40390.1	8.61	5.52	2.35
	Alpha-galactosidase	Contig23948_at;	AT5G20250.3;	NDEG	0.35	NDEG
		Contig4459_at;	AT3G57520.1;			
	UGE	HV11P22r_s_at	AT3G57520.1			
Contig4187_at		AT5G08370.2	0.15	0.29	NDEG	
Contig10284_at		–	0.47	NDEG	NDEG	
Contig10066_at		AT4G10960.1	4.07	2.61	NDEG	
<i>trehalose</i>	TPS	Contig9721_at	AT1G12780.1	NDEG	0.15	NDEG
		Contig12019_at	AT1G78580.1	NDEG	0.38	NDEG
	TPS, putative	Contig11529_at;	AT1G68020.2;	0.36	NDEG	NDEG
		Contig3375_at	AT1G68020.2			
	TPP	Contig3373_s_at;	AT1G70290.1;	0.48	NDEG	NDEG
	HVSMek0022L04r2_s_at	AT1G68020.2				
	Contig4657_at	AT1G06410.1	0.38	NDEG	NDEG	
	Contig24583_at	AT5G51460.1	0.24	NDEG	NDEG	

Table 1 (continued)

Metabolism, leaves	Annotation	Probe set ID	<i>Arabidopsis</i> Accn	FC: C vs 1day (3°C)	FC: C vs 3days (3°C)	FC: 21days (3°C) vs 1day (-3°C)
		Contig14870_at; Contig2825_at; HS16E24u_s_at	AT4G12430.1; AT1G35910.1; AT1G35910.1	3.71	NDEG	6.75
<i>proline</i>	P5CS	Contig3814_at	AT2G39800.1	26.53	78.98	NDEG
<i>GABA</i>	GAD	Contig1380_s_at; Contig1388_at	AT2G02000.1; AT2G02010.1	9.65	10.07	NDEG
		Contig1385_at	AT5G17330.1	NDEG	0.29	NDEG
		Contig1373_at	AT5G17330.1	NDEG	NDEG	3.60
<i>ABA</i>	9-cis-epoxycarotenoid dioxygenase	Contig4988_at	AT4G19170.1	0.19	NDEG	NDEG
		HT11N18r_s_at	–	NDEG	NDEG	3.95
	Zeaxanthin epoxidase	Contig3181_at	AT5G67030.1	NDEG	2.31	NDEG
<i>lignin</i>	PAL1	Contig1795_at; Contig1800_at; Contig1800_s_at; Contig1800_x_at	AT2G37040.1; AT2G37040.1; AT2G37040.1; AT2G37040.1	2.12	NDEG	8.02
		Contig1799_s_at	AT2G37040.1	5.70	5.55	2.81
		Contig1803_at; HVSME0015M15r2_s_at	AT2G37040.1; AT2G37040.1	NDEG	0.41	NDEG
		Contig1805_s_at	AT2G37040.1	NDEG	NDEG	3.62
	4CL	Contig4674_at; Contig4677_at	AT1G51680.1; AT1G65060.1	3.32	3.88	NDEG
		Contig4676_at	AT1G51680.1	2.16	NDEG	2.03
	HCT	Contig6770_at; Contig6770_s_at	AT5G48930.1; AT5G48930.1	5.02	3.34	3.60
		Contig7815_s_at; HVSME0012H17r2_at	AT5G48930.1; AT5G48930.1	NDEG	2.77	NDEG
	CCR1	Contig8527_at	AT1G15950.1	2.19	NDEG	NDEG
		Contig14426_at	AT1G15950.1	NDEG	NDEG	2.27
	CAD	Contig19854_at; Contig19854_s_at; HM05N11r_at	AT4G37980.1; AT4G37980.1; AT4G37990.1	6.27	5.31	NDEG
		HVSME0081I20r2_s_at; Contig20411_at	AT4G37980.1; AT4G39330.1	2.12	NDEG	5.10
		rbags23e03_at	AT4G39330.1	4.74	4.09	NDEG
		Contig13997_at	AT4G37980.1	0.41	0.29	NDEG
		Contig4260_at	AT4G37980.1	NDEG	NDEG	5.00
	COMT	Contig2947_at	AT5G54160.1	0.32	0.19	NDEG
	CCoAOMT	Contig24054_at; Contig406_s_at	AT3G62000.1; AT4G34050.1	0.38	0.48	NDEG
	Laccase	Contig10632_at; Contig10632_s_at	AT3G09220.1; AT3G09220.1	6.85	3.87	2.13

Each FC value refers to the mean expression fold change of one isoform

ABA abscisic acid, *CAD* cinnamyl alcohol dehydrogenase, *CCoAOMT* caffeoyl-CoA 3-O-methyltransferase, *CCR1* cinnamoyl-CoA reductase 1, *COMT* caffeic acid *o*-methyltransferase, *GABA* gamma-aminobutyric acid, *GAD* glutamate decarboxylase, *HCT* hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase, *NDEG* not differentially expressed gene, *PAL1* phenylalanine ammonia-lyase 1, *P5CS* delta 1 pyrroline-5-carboxylate synthase, *SUSIBA2* sugar signalling in barley 2, *TPP* trehalose-6-phosphate phosphatase, *TPS* trehalose-6-phosphate synthase, *4CL* 4-coumarate:CoA ligase, *UGE* UDP-glucose 4-epimerase

Table 2 Fold change (FC) values of differentially expressed genes encoding key crown enzymes

Metabolism, crowns	Annotation	Probe set ID	<i>Arabidopsis</i> Accn	FC: C vs 1 day (3°C)	FC: C vs 3 days (3°C)	FC: 21 days (3°C) vs 1 day (-3°C)	
<i>Sucrose and starch</i>	Sucrose transporter	Contig4612_at	AT1G09960.1	NDEG	2.60	NDEG	
	Vacuolar invertase	Contig2495_s_at	AT1G62660.1	NDEG	0.41	NDEG	
		Contig4521_s_at; HVSMEn0014H06r2_s_at	AT1G12240.1; AT1G62660.1	2.12	NDEG	NDEG	
	Cell wall invertase	Contig7811_s_at; Contig6539_s_at	AT3G13790.1; AT3G52600.2	NDEG	0.43	NDEG	
		Contig11241_at	AT3G52600.2	NDEG	NDEG	3.43	
	Neutral invertase	Contig6108_at	AT1G22650.1	NDEG	0.44	NDEG	
	Fructokinase	Contig101_at	AT2G31390.1	NDEG	0.40	NDEG	
	Alpha-glucosidase	Contig7937_s_at	AT5G11720.1	NDEG	0.40	NDEG	
	Alpha-amylase	Contig7088_at	AT4G25000.1	NDEG	2.08	2.15	
	Beta-amylase	Contig11522_at	AT4G17090.1	2.41	NDEG	6.00	
<i>Raffinose</i>	Galactinol synthase	Contig3810_at; Contig3811_at	AT1G09350.1; AT2G47180.1;	12.18	15.44	2.89	
		Contig3810_s_at	AT1G09350.1	11.83	12.18	2.24	
	Raffinose synthase	Contig26196_at	AT5G40390.1	3.15	2.35	3.65	
<i>Trehalose</i>	TPS	Contig12019_at	AT1G78580.1	0.44	0.27	NDEG	
	TPP	Contig24583_at	AT5G51460.1	0.31	0.29	NDEG	
		Contig14870_at; Contig2825_at; HS16E24u_s_at	AT4G12430.1; AT1G35910.1; AT1G35910.1	NDEG	NDEG	2.63	
<i>Proline</i>	P5CS	Contig3814_at	AT2G39800.1	5.62	6.21	NDEG	
<i>GABA</i>	GAD	Contig1380_s_at; Contig1388_at	AT2G02000.1; AT2G02010.1	NDEG	2.43	NDEG	
		Contig1373_at	AT5G17330.1	0.28	0.10	NDEG	
<i>ABA</i>	9-cis-epoxycarotenoid dioxygenase	HT11N18r_s_at	–	NDEG	NDEG	2.99	
	ABA 8-hydroxylase	Contig11708_at	AT1G48400.1	3.39	2.60	2.18	
<i>Lignin</i>	PAL1	Contig1795_at; Contig1800_at; Contig1800_s_at; Contig1800_x_at	AT2G37040.1; AT2G37040.1; AT2G37040.1; AT2G37040.1	NDEG	0.39	NDEG	
		Contig1805_s_at	AT2G37040.1	0.38	0.23	NDEG	
		4CL	Contig4676_at	AT1G51680.1	NDEG	NDEG	2.11
		CCR1	Contig6799_at	AT1G15950.1	NDEG	0.35	NDEG
		CAD	HVSMEn0081120r2_s_at; Contig20411_at	AT4G37980.1; AT4G39330.1	NDEG	0.45	NDEG
	COMT	Contig2532_at; Contig5311_at; Contig5313_s_at; Contig6251_at	AT5G54160.1; AT5G54160.1; AT5G54160.1; AT5G54160.1	2.02	NDEG	NDEG	
		Contig2947_at	AT5G54160.1	NDEG	0.37	NDEG	
		Laccase	Contig10632_at; Contig10632_s_at	AT3G09220.1; AT3G09220.1	2.10	2.59	3.04

Each FC value refers to the mean expression fold change of one isoform

ABA abscisic acid, *CAD* cinnamyl alcohol dehydrogenase, *CCoAOMT* caffeoyl-CoA 3-O-methyltransferase, *CCR1* cinnamoyl-CoA reductase 1, *COMT* caffeic acid *o*-methyltransferase, *GABA* gamma-aminobutyric acid, *GAD* glutamate decarboxylase, *HCT* hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase, *NDEG* not differentially expressed gene, *PAL1* phenylalanine ammonia-lyase 1, *P5CS* delta 1 pyrroline-5-carboxylate synthase, *TPP* trehalose-6-phosphate phosphatase, *TPS* trehalose-6-phosphate synthase, *4CL* 4-coumarate:CoA ligase

acclimation. These observations are consistent with the accumulation of galactinol in the leaf as a response to low temperature, and with the accumulation of raffinose both at the beginning of the +3°C and the -3°C treatments. The early down-regulation of an alpha-galactosidase gene is suggestive of the accumulation of melibiose in the leaf. In the crown, the two galactinol synthase isoforms were both up-regulated by the cold stress, as was the raffinose synthase gene. These data suggest that the crown is able to accumulate galactinol and raffinose in response to cold acclimation.

Proline and GABA metabolism Together with the possible accumulation of soluble sugars, that of both proline and GABA probably also contributed to the decrease in osmotic potential which occurred during acclimation (Fig. 3). The expression of *P5CS* increased rapidly during acclimation in both the leaf and the crown, suggesting the steady accumulation of proline in both organs. However, proline synthesis probably ceases at sub-zero temperatures. The gene encoding glutamate decarboxylase (*GAD*) was down-regulated in the crown at +3°C, while two isoforms were up-regulated after 3 days of acclimation. In contrast, in the leaf, *GAD* was up-regulated, with one of its isoforms down-regulated after 3 days of acclimation. One of the *GAD* isoforms was induced in the leaf at -3°C. The expression data suggest that GABA was accumulated at the beginning of 1PH and 2PH in the leaf but not in the crown.

ABA metabolism *NCED*, the gene encoding 9-*cis*-epoxycarotenoid dioxygenase was down-regulated in the leaf (but not in the crown) after just 1 day of acclimation, but was induced at -3°C in both the leaf and the crown. This behaviour suggests that ABA was accumulated as a response to the dehydration associated with exposure to sub-zero temperatures. A detailed study of *NCED* expression during periods of water stress has demonstrated a tight correlation between transcript abundance, protein level and ABA content in dehydrated leaves and roots of bean, indicating a regulatory role for *NCED* in ABA biosynthesis (Qin and Zeevaert 1999).

Lignin metabolism Little is known of the role (if any) of lignin metabolism during cold acclimation, but the expression profiles of many genes involved in lignin synthesis were changed markedly by this treatment in barley. Particularly notable were some of the genes involved in secondary metabolism, especially those responsible for the enzymes participating in lignin synthesis. The transcriptomic data indicated a global up-regulation of lignin synthesis in the leaf but its down-regulation (or absence of effect) in the crown. In the leaf, the accumulation of cinnamic acid was suggested by the

up-regulation during acclimation of two *PAL1* (phenylalanine ammonia-lyase 1) isoforms (the third being down-regulated), while all three isoforms were induced by exposure to -3°C; at the same time there was little or no change in the abundance of *C4H* (cinnamate 4-hydroxylase) transcript. The genes encoding key enzymes for the synthesis of lignin precursors, namely *PAL*, *HCT* (hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase), *CCR1* (cinnamoyl-CoA reductase 1) and *CAD* (cinnamyl alcohol dehydrogenase) were all up-regulated after 1 day of cold acclimation. After the third day of acclimation, all but *CCR1* remained up-regulated, while upon the plants' removal to -3°C, the expression of all five genes was boosted. At +3°C, both *COMT* (caffeic acid *o*-methyltransferase) and *CCoAOMT* (caffeoyl-CoA 3-*O*-methyltransferase) were down-regulated in the leaf. In the crown, genes involved in the synthesis of lignin precursors were mostly down-regulated following exposure to +3°C (*PAL1* after 1 day and 3 days, *CCR1*, *CAD* and *COMT* after 3 days). Of these, only *COMT* was up-regulated at the beginning of acclimation (C vs 1d). *4CL* (4-coumarate:CoA ligase) was up-regulated following exposure to -3°C. The cold-induced up-regulation of the gene encoding laccase occurred in both the leaf and crown (Tables 1 and 2). On the other hand, the peroxidase genes presumably involved in lignin polymerization were down-regulated.

Discussion

The application of microarray technology has generated a large volume of gene expression data associated with CA in barley and other cereals. While much benefit can be gained from the wealth of information available in relation to gene identity and pathways in *A. thaliana*, some caution needs to be exercised in extrapolating this to barley. To date, much of the focus of cereal transcriptome analysis has been at the level of the whole plant (e.g. Campoli et al. 2009; Kocsy et al. 2010; Monroy et al. 2007) or in the leaf (e.g. Atienza et al. 2004; Badawi et al. 2007), although it is clear from physiological studies that in the temperate cereals, the critical organ for winter survival is the crown (summarised by Livingston et al. 2005). The modulation of the crown transcriptome in response to low temperature has received some attention (Ganeshan et al. 2008; Herman et al. 2006; Pearce et al. 1998; Skinner 2009; Sutton et al. 2009; Winfield et al. 2010), but there has been little, if any, focus on any specific mechanism(s) which differentiate the responses to cold of the crown from the leaf. The present study represents an attempt to contrast gene expression in the winter barley leaf with that in the crown over the course

of CA and subsequent sub-zero temperature hardening. We explored the notion that the crown possesses specific cold responsive features, which can explain its key role in winter survival.

The development of freezing tolerance during the course of 1PH has been documented previously (Fowler 2008; Gusta et al. 2001), and that freezing tolerance can be further improved by exposure to non-lethal sub-zero temperature has been known for many years (Trunova 1965). The 2PH is associated with extensive physiological, morphological and molecular changes, particularly because the very low temperatures are accompanied by a redistribution of water to the extracellular space (Herman et al. 2006). The cell dehydration associated with exposure to sub-zero temperatures was signalled in the present experiments by the induction during 2PH, in both the leaf and the crown, of *NCED*, which encodes a key enzyme of ABA biosynthesis, as well as by the expression of certain dehydration responsive genes. Crown tissue DW continued to increase from the end of the first week of 1PH until day 21 and was accompanied by a fall in osmotic potential; this behaviour is suggestive of the accumulation of cryo-protective compounds in this key organ for the purpose of winter survival.

The nucleation temperature of intrinsic ice nuclei decreased by approximately 3.6°C in both leaf and crown during the acclimation process, consistent with the behaviour of oilseed rape (Gusta et al. 2004). The theoretical ice nucleation temperature for low molecular weights calculated according to Franks (1985) varied from -7.4 to -10.9°C in the crown, and from -8.5 to -11.8°C in the leaf. During the first 7 days of acclimation, the measured ice nucleation temperature values (Fig. 4) lay within the theoretical range. However, by 21 days of acclimation, as well as for the measurement taken after the further 1 day at -3°C, the predicted ice nucleation temperature was higher than the observed one. The ice nucleation temperature depression during the cold acclimation period was 4.0–6.5 times (crown) and 5.0–9.0 times (leaf) greater than the predicted ice nucleation temperature depression due exclusively to osmotic potential (Franks 1985). The conclusion from these measurements was that during the acclimation process, the production of ice nucleation substances in both the leaf and the crown was suppressed, correlating therefore with the rapid up-regulation of genes encoding the major antifreeze (chitinases, glucanases, thaumatin-like proteins) and ice recrystallization inhibition proteins (Fig. 6, clusters 2 and 9; Fig. 5, cluster 1; Fig. S2, cluster 5). Genes encoding ice recrystallization inhibition proteins are strongly up-regulated in the wheat crown as well (Herman et al. 2006; Winfield et al. 2010). During the transition from 1PH to 2PH, the abundance of a number of proteins associated with antifreeze activity also rose in this organ (Herman et al. 2006).

Differentially expressed genes (DEGs)

As might have been predicted, the leaf and crown do share some aspects of the acclimation response. The first and transient common response (within 1 day of exposure to +3°C and -3°C) included the expression of genes involved in Ca²⁺ signalling (Ca²⁺-transporting ATPase, Ca²⁺ sensors (CBLs) and CBL-interacting protein kinases) and certain co-regulated genes (drought-related TFs, *LEAs*, *DHN*). The more delayed response (after 3 days of low temperatures) included genes involved in hormone (ABA, putrescine) signalling and osmotic adjustment (starch breakdown, proline biosynthesis), together with the expression of *COR* genes. These transcriptomic changes may be considered as a common (or basal) response to low temperature stress.

However, it has become clear that both these organs also display some specific features during response to low temperatures. Thus, in the leaf, the rapid transient response consisted of Ca²⁺ signalling via calmodulin (*CAM*), CAM-dependent kinases (*CPK*) and CAM-binding HSP (*lipase3*); some small *HSPs* and *CORs* were co-regulated. A rapid but more long-lasting response was shown by genes encoding chloroplast-localised proteins, a small HSP and HSPs 70 and 101, HSF and *DHN*. Additional classes of genes induced after 3 days following their initial partial suppression at 1 day included those encoding components of glycine betaine synthesis, as well as *P5CS*, *OSM34*, *LOS1* and 2. The product of *LOS1* is known to negatively regulate *CBFs* (Guo et al. 2002). Many of the *CBF* genes were not identified as DEGs because their expression tended to be very rapid (within a few hours of exposure to cold) and was typically no longer detectable after 24 h of stress exposure. However, a barley homologue of *TaCBF9* was clearly up-regulated in both the leaf and the crown after 1 day exposure to +3°C and -3°C.

The crown's rapid response to low temperature included some small-scale changes in the expression of genes involved in photosynthesis. These genes were more highly expressed in the leaf, but there, their expression was unaffected by cold stress. The surprising induction of genes related to photosynthesis in this non-photosynthetic organ has already been noted by Skinner (2009), who suggested a protective rather than a photosynthetic role for them. The more delayed response included genes responsive to ABA and auxin as well as some involved in nucleosome assembly. The local unwrapping of DNA is needed for RNA polymerase in order to transcribe the up-regulated genes as well as for the ability of repressors to bind to DNA and block the transcription of down-regulated genes (summarised by Kumar and Wigge 2010). In *A. thaliana*, the histone H2A.Z appears to wrap the DNA more tightly at

lower temperatures (12°C), but it changes to its more permissive canonical form H2A at higher temperatures which allows to transcribe the “warm transcriptome” (Kumar and Wigge 2010). Here, a number of histone genes, but especially *H2A* and *HTA11*, were transiently down-regulated and co-regulated with the expression of a histone-like TF (nuclear factor-Y; *At1g07980*) as a response to cold stress; note that the *AtHTA11* sequence shares high homology with various *H2A.Z* orthologues (March-Diaz and Reyes 2009). The down-regulation of *HTA11* barley homologue, which began after the first day at +3°C and peaked in the crown between days 3 and 7, could therefore have led to a transient release of the DNA from histone wrapping and a subsequent burst of transcription of the “cold transcriptome”. Correlating, temporally at least, with the highest number of crown-specific DEGs (identified from the sample taken after 3 days of cold stress) and the aspects of the crown’s physiology (freezing survival, osmotic potential, dry weight and melting temperature) were also at their most dynamic between days 3 and 7. Thus, our data imply involvement of nucleosome remodeling in cold stress response as a specific feature of crown tissues in winter barley.

Metabolic pathways potentially activated during cold acclimation

Genes involved in some of the pathways responsible for osmolyte production or secondary metabolism frequently behaved differently in the crown and leaf.

Proline and GABA metabolism There was a pronounced level of proline accumulation during 1PH in both the leaf and crown, with a particularly high expression of *P5CS* in the leaf (producing an FC of 26.5 after 1 day and 79.0 after 3 days). The GABA biosynthesis gene *GAD* was expressed in the leaf at the beginning of 1PH (FC of ~10.0) and 2PH. Data support the suggestion of Mazzucotelli et al. (2006) that proline, as opposed to GABA, is not accumulated at sub-zero temperatures. Although the major role of both proline and GABA is in osmoprotection during stress, other roles during abiotic stress have been suggested for both proline (Kaul et al. 2008) and GABA (Kang and Turano 2003).

Sucrose and starch metabolism There is clear evidence that symplastic and apoplastic sugar contributes directly to membrane stabilisation and that the increased content of the soluble sugars glucose, fructose, sucrose and raffinose is an important component of the hardening process (Hinch and Hagemann 2004; Livingston and Henson 1998; Livingston et al. 2006; Pennycooke et al. 2003; Valluru et al. 2008). However, they have also been implicated as signalling molecules (Iordachescu and Imai

2008; Koch 2004; Roitsch and Gonzalez 2004; Rolland et al. 2006; Sauer 2007; Tabaei-Aghdaei et al. 2003).

The expression profiles suggested that maltose and glucose were accumulated in the leaf and crown during both 1PH and 2PH, as a consequence of the up-regulation of genes encoding starch and sucrose degradation enzymes. While in the crown, there is evidence for sucrose transport, in the leaf both vacuolar (FC >100.0 after 1 day at +3°C) and apoplastic (FC 175.6 after 1 day at +3°C) invertases were up-regulated during 1 and 2PH; this activity implies the consumption of sucrose by the leaf. The apoplastic invertase was up-regulated in the crown only at sub-zero temperatures (and down-regulated after 3 days of CA). This observation suggests that the alteration of invertase activity in the crown during 2PH is a specific adaptive response which aids plant survival in the face of extreme cold (Livingston and Henson 1998). Both α - and β -amylase encoding genes were induced in the crown during 1PH and 2PH, and in leaf during 1PH; β -, but not α -amylase genes, were further up-regulated in the leaf during the transition to 2PH, consistent with the observations of Kocsy et al. (2010). Note that maltose, a product of starch hydrolysis driven by β -amylase, is believed act as a cryoprotectant, as well as representing a precursor for soluble sugar metabolism (Kaplan and Guy 2004; Kaplan et al. 2006).

Trehalose metabolism Trehalose-6-phosphate (T6P), the product of TPS activity, is an indicator of sucrose availability and an inhibitor of starch breakdown. The presence of trehalose (produced from T6P by TPP activity), and the absence of T6P have opposite effects on sugar signalling, starch breakdown, leaf development and flowering (Lunn et al. 2006; Lunn et al. 2010; Paul 2007). The expression patterns of *TPS* and *TPP* were consistent with the possible absence of T6P and induction of genes coding for starch breakdown enzymes in both crown and leaf. Both TPP isoforms expressed in barley are homologous to *AtTPPA*, which encodes a functional enzyme (Vogel et al. 1998) similar to OsTPP1 (Pramanik and Imai 2005). The transient expression of *TPP* induced by low temperature in both the leaf and the crown, together with the transient accumulation of trehalose at low temperatures in rice (Pramanik and Imai 2005), support the hypothesis that both trehalose and trehalose-6-phosphate act as signalling molecules rather than as osmolytes. The expression data show that T6P was down-regulated during both 1 and 2PH in both the leaf and the crown. During 1PH, the level of T6P may have been depressed by the down-regulation of *TPS*. On the other hand, during 2PH, its level was probably restricted by the action of *TPP*, which forms trehalose from T6P. The consequence of this reaction is an accumulation of TPS substrates (UDP-glucose and glucose-6-phosphate) during

1PH, and possibly also of trehalose during exposure to sub-zero temperatures.

Raffinose metabolism Although there is some evidence for raffinose accumulation as a response to cold stress and some confirmation of its role as an osmoprotectant (Liu et al. 2007; Salerno and Pontis 1989), there is little difference between the freezing tolerance of *A. thaliana* plants differing in their raffinose content (Zuther et al. 2004). Our results, however, suggest that raffinose is accumulated in the crown over the whole cold acclimation period, whereas in the leaves this only occurs in the early stages of exposure to both low and sub-zero temperature stress. Galactinol was probably accumulated during 1PH in the leaf and during both 1PH (the galactinol synthase FC was >10.0) and 2PH in the crown. Overall, the raffinose oligosaccharides appear to be important components of the cold acclimation process in winter barley. Significant increases, measured during 1PH, in the abundance of galactinol synthase transcript, have been noted in wheat (Winfield et al. 2010). Both galactinol and raffinose are believed to protect cells against oxidative damage (Nishizawa et al. 2008).

Lignin metabolism The cold-induced up-regulation of *PAL*, *4CL*, *HCT*, *CCR* and *CAD* in the leaf, and also of the gene encoding laccase in both the leaf and crown (Tables 1 and 2) suggests that polymerization of monolignols could be protective against damage caused by reactive oxygen species (Takahama and Oniki 1997) and/or by the presence of free phenols (Whetten and Sederoff 1995), given that the esterified forms of phenolic acids allow their transport into the plant vacuole (Dixon and Paiva 1995). On the other hand, the peroxidase genes presumably involved in lignin polymerization were down-regulated by low temperature. Because lignin (and its precursors) is an important component of the cell wall, any change in lignin content and/or composition is bound to have an impact on the physical properties of the cell wall. Such changes may perhaps help avoid the mechanical stress caused by the formation of ice at sub-zero temperatures. Unlike in the leaf, the expression during CA of genes involved in monolignol synthesis was either down-regulated or unaffected in the crown. Similar investigation of the response of the wheat leaf and crown to cold stress (Olenichenko and Zagoskina 2005) showed that lignin content was unaffected in the leaf and increased in the crown, while PAL activity was reduced in both the crown and the leaf. On the other hand, low temperature reportedly increases PAL activity in both the oilseed rape leaf (Solecka and Kacperska 1995) and the soybean root (Janas et al. 2000). Overall, the suggestion is that monolignols, rather than lignin polymers, are synthesised in the leaf of barley in response to

acclimation, while in the crown, lignin polymers are favoured over monolignols.

The induction of expression of genes responsible for sucrose degradation in the leaf, and the up-regulation of many of the enzymes participating in monolignol synthesis support the hypothesis proposed by Rogers et al. (2005) that lignin synthesis depends on the availability of hexose carbohydrates. However, in the crown, it was the expression of sucrose transporter genes, rather than of the sucrose degradation machinery, which was induced, and the lignin synthesis enzyme genes were largely down-regulated; this implies that the crown enhances the polymorphism of its lignin via methyltransferase activity, rather than via any promotion of lignin precursor synthesis.

Summary

This study has represented an attempt to focus on specific processes and genes involved in the response of the barley crown to cold stress, based on the recognition that the crown is key for the successful overwintering of cereals. The most noteworthy features of this response were the transient changes observed in the transcription of nucleosome assembly genes and especially in the expression of *H2A* and *HTA11* which have been implicated in temperature sensing in *A. thaliana* (Kumar and Wigge 2010).

Many of the genes involved in pathways responsible for osmolyte production and secondary metabolism had distinct expression profiles in the crown and leaf. A number of genes responsible for sucrose degradation were induced in the leaf during CA, whereas, in the crown, it was the expression of sucrose transporter genes, rather than of the sucrose degradation machinery, which was induced. The gene expression data implied that raffinose oligosaccharides are likely to be important in the winter barley CA response, especially in the crown. The idea that both trehalose and trehalose-6-phosphate act as signalling molecules rather than as osmolytes was also supported by the data. Monolignols, rather than lignin polymers, are synthesised in the leaf of barley during CA, while in the crown, lignin polymers are favoured over monolignols. In addition, the crown enhances the polymorphism of its lignin via methyltransferase activity, rather than via the promotion of lignin precursor synthesis.

A major direction of our research effort is to characterise the crown/leaf responses to cold in both spring and winter barley cultivars which contrast for their degree of freezing tolerance, because an understanding of the basis of this tolerance should help formulate strategies to improve the crop's winter hardiness.

Acknowledgment This research was funded by the Czech Republic National Agency for Agricultural Research (project no. QH 81287), by the Czech Ministry of Agriculture (project no. Mze0002700604), by the Ministry of Education, Youth and Sports (project no. OC09032) and by the Italian Ministry of Agriculture, Special Project ESPLORA.

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