Transcript and hormone analyses reveal the involvement of ABA-signalling, hormone crosstalk and genotype-specific biological processes in cold-shock response in wheat

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Abstract

The effect of one-day cold-shock on the transcriptome and phytohormones (auxin, cytokinins, abscisic, jasmonic and salicylic acids) has been characterised in freezing-sensitive (Chinese Spring), highly freezing-tolerant (Cheyenne) and moderately freezing-tolerant (Chinese Spring substituted with Cheyenne's 5A chromosome) wheat genotypes. Altogether, 636 differentially expressed genes responding to cold-shock were identified. Defence genes encoding LEA proteins, dehydrins, chaperons and other temperature-stress responsive proteins were up-regulated in a genotype-independent manner. Abscisic acid was up-regulated by cold accompanied by adherent expression of its metabolic genes. Data revealed the involvement of particular routes within ABA-dependent signalling in response to cold-shock in the examined genotypes. Cold-shock affected gene expression along carbohydrate metabolic pathways. In photosynthesis, cold-shock changed the expression of a number of genes in the same way as it was previously reported for ABA. Overrepresentation analysis of the differentially expressed genes supported the ABA-signalling and carbohydrate metabolism results, and revealed some pronounced biological process GO categories associated with the cold-shock response of the genotypes. Protein network analysis indicated differences between the genotypes in the information flow along their signal perception and transduction, suggesting different biochemical and cellular strategies in their reaction to cold-shock.

Keywords

ABA-signalling; Carbon metabolism; Freezing-tolerance; Gene ontology; Plant hormones; Short-term cold-shock; *Triticum aestivum*

1. Introduction

Winter wheat varieties acquire their freezing-tolerance by exposure to decreasing temperature over a longer period [1]. The effects of this process, called cold-acclimation, on carbohydrates [2], amino acids and polyamines [3], the proteome [4,5], the phenolome [6],

phytohormones [7,8] and gene expression [9,10,11] have been studied in wheat. These reports suggested that re-programming of metabolism occurs during cold-acclimation leading to reduced growth and development, and activation of defence processes.

In addition to cold-hardening, the exposure of winter wheat to a prolonged cold period is also required for transition from the vegetative to the generative developmental stage, while spring wheat does not need such vernalization. At the end of the vegetative/generative transition, flower primordia are in the irreversible double-ridge phase and ready to flower. Since the reproductive meristems in the developing flower primordia are more sensitive to cold than vegetative meristems and organs [10], the optimal timing of the reproductive development by vernalization is important and ensures that flowers and seeds develop in spring/summer. The key factors in vernalization are the *VRN1* genes with different alleles [12], which were mapped to the long arm of Chr 5 [13]. The Cheyenne (Ch) winter variety contains three homeologous recessive (*vrn1*) alleles, while the spring wheat Chinese Spring (CS) carries one recessive allele on each chromosomes 5A and 5B and possesses a single vernalization-insensitive dominant allele *(Vrn-D1)* on Chr 5D. We note here that the CS(Ch5A) substitution line was used to map the *Vrn-A1* allele and the freezing tolerance 2 locus (*Fr-A2*) on the long arm of Chr 5A [14].

In wheat, single chromosomes can be transferred from one variety to another using cytogenetic chromosome manipulation techniques, and hence their genetics can be examined in a different and homogeneous genetic background [15]. Previous studies revealed that a substitution line constructed by replacing the 5A chromosomes of the freezing-sensitive CS variety with the corresponding chromosome pair from the freezing-tolerant winter wheat Cheyenne, had a much higher freezing-tolerance than the recipient CS variety [16,17] but had no change in its spring habit. The enhanced freezing-tolerance of the substitution line CS(Ch5A) was associated with the increased transcription of the Cheyenne *CBF* (C-repeat

binding factor) genes [18], which are clustered in the freezing tolerance locus (*FR2*) mapped to Chr 5A [19]. Of the *CBF* genes being partly responsible for the freezing-tolerance of cereals in the vegetative phase [20], *CBF14* was the most effective [21].

In nature, cold-acclimation and vernalization usually takes several weeks to months [22] and during this period plants can adapt to the gradually changing temperature. However, because of the recent climatic changes, plants can be exposed to very sudden weather changes. The average yearly number of extreme weather events, which have become more frequent and severe in the last few decades, has increased by 2.5-fold in the last 30 years [23]. Between 2000 and 2010, hydro-meteorological disasters caused 1070 billion USD losses worldwide, and in Europe alone the extreme climatic events (heat- and cold-waves, wildfire and drought) caused losses worth about seven billion USD [24]. Extreme weather has a large impact on economic systems including agriculture [25]. From a scientific point of view, extreme weather changes cause extreme stress to natural plant populations and crops. Amongst these extreme events, a number of cold waves and abnormally low temperature conditions have been reported [24].

A rapid drop in temperature is equivalent to a cold-shock for young wheat plantlets, which are in their early vegetative phase (both winter and spring varieties) or were not cold-acclimated yet (winter varieties). In cold-shocked wheat, a burst of gene activity was detected during the first day, and about 90% of all responsive genes were activated [9]. After 6 hours, the induction response was very similar in spring and winter wheat cultivars, but after 24 hours the transcript level of many transcription factors became significantly higher in winter than in a spring cultivars [9]. Similarly to this observation, our previous transcriptome analysis revealed the greatest changes in gene expression during the first day of a three weeks hardening period in wheat shoots [10]. In parallel to this phenomenon, the most rapid changes in freezing-tolerance occurred during the initial stages of acclimation in wheat [26,27].

The aim of this study was to compare the effect of one-day cold-shock on gene expression and the levels of different hormones in a model system of freezing-tolerant and freezing-sensitive genotypes in their early vegetative growth phase, and to integrate the obtained data. We were also interested in identifying possible key factors and system differences between tolerant and sensitive wheat genotypes in response to cold shock. Identifying possible differences caused by the substitution of Chr 5A of a winter wheat variety into a spring wheat was in our interest too.

2. Materials and Methods

2.1. Plant material

Seeds of the CS and Ch varieties, and the CS(Ch5A) substitution line were germinated on wet filter paper in Petri dishes (1 day at 25°C, 3 days at 4°C and 2 days at 25°C). Seedlings were then grown in half-strength modified Hoagland solution under 16 hours illumination at 270 µmol m^{-2} s^{-1}, 20/15 °C day/night temperature and 70-75% relative humidity in a growth chamber (Conviron PGV-15; Controlled Environments Ltd., Winnipeg, Canada) for ten days. Half of the plants were then kept for one day at 4°C for cold-shock, while the other half of the plants were kept for a further day at the control temperature. Illumination and humidity conditions for both groups during the additional day were the same that were used in the ten days growing period. No vernalization treatment was carried out for the plantlets. The ten days old plantlets subjected to cold-shock had two fully developed leaves and the presence of the single ridge shoot apex and the absence of the tillers indicated that the plants were in their vegetative phase. The shocked plants did not show any cold-related symptoms, such as senescence or retarded growth, after one day chilling. The shoots of the plantlets were collected for the microarray and hormone studies.

2.2. Microarray experiments

RNA was isolated in two biological replicates using a standard TRI reagent (Sigma-Aldrich, Budapest, Hungary) method according to the instruction of the manufacturer and applying DNase I (Promega, Madison, WI, USA) treatment. Preparation of Cy5- and Cy3-labelled cDNA using RNA isolated from the control and cold-shocked samples, respectively, and microarray hybridisation to a stress-specific 15k wheat oligonucleotide microarray [28] were performed as described [29]. An Agilent scanner (Agilent, Santa Clara, CA, USA) was employed for microarray scanning and data collection.

To validate microarray data, the expression of seven genes (about 1% of all differentially regulated genes) was investigated by qRT-PCR in three biological replicates. RNA was transcribed into cDNA using M-MLV Reverse Transcriptase and Oligo(dT) 18 primer (Thermo Scientific/Life Technologies, Budapest, Hungary) and amplification was performed using the KAPA SYBR® FAST qPCR kit (Kapa Biosystems, London, UK) and a CFX96 thermo-cycler (Bio-Rad, Budapest, Hungary) with the following cycle: denaturation at 95 ºC for 3:00 min followed by 40 cycles of 5 s at 95 ºC and 30 s at 60 ºC. The primer sequences are shown in Supplementary Table S1. The melting curve was recorded between 60 and 95 ºC in 0.5 ºC increments. The relative quantities of the individual transcripts were calculated by the ΔΔCt method using a phosphogluconate-dehydrogenase (UniGene ID: Ta30797) housekeeping gene for normalization [30]. The ratio between the treatment and control data and their Student's *t*-test were calculated in Excel.

2.3. Hormone analysis

Extraction and analysis of plant hormones were performed according to established methods [31,32] in three biological replicates. Briefly, samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v). The following labelled internal standards (10 pmol per sample) were added: $^{2}H_{6}$-ABA, $^{2}H_{3}$-PA, $^{13}C_{6}$-IAA, $^{2}H_{2}$-OxIAA, $^{2}H_{5}$-$^{15}N_{1}$-IAA-Asp**,** $^{2}H_{4}$-SA, $^{2}H_{5}$-JA, $^{2}H_{5}$-*trans*Z, $^{2}H_{5}$-*trans*ZR, $^{2}H_{5}$-*trans*Z7G, $^{2}H_{5}$-*trans*Z9G, $^{2}H_{5}$-

*trans*ZOG, $^{2}H_5$-*trans*ZROG, $^{2}H_5$-*trans*ZRMP, $^{2}H_3$-DHZ, $^{2}H_3$-DHZR, $^{2}H_3$-DHZ9G, $^{2}H_6$-iP, $^{2}H_6$-iPR, $^{2}H_6$-iP7G, $^{2}H_6$-iP9G, $^{2}H_6$-iPRMP (Olchemim, Olomouc, Czech Republic). Extracts were purified using an SPE-C18 column (SepPak-C18, Waters, Milford, MA, USA) and separated on a reverse phase-cation exchange SPE column (Oasis-MCX, Waters, Milford, MA, USA). The hormone fractions eluted with methanol or with 0.35 M NH_4OH in 70% methanol were separated by HPLC (Ultimate 3000, Dionex/Thermo Fisher Scientific, Vienna, Austria) and hormones were quantified using a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

2.4. Data analyses

Microarray data for all genotypes were analysed pairwise between the control and cold-shocked samples. First, within-array-normalised data obtained from the Agilent scanner were normalised between-arrays using the quantile-normalisation method [33] in Excel. Quantile-normalised data were then log2 transformed and analysed in the Multiple Experiment Viewer (MeV) software of the TM4 Microarray Software Suite [34]. Since only two biological replicates was performed, the robust Rank Product method [35] was used for data analysis applying two-class unpaired calculation (group1, cold-treated; group2, control) in which the number of permutations and the critical *P*-value were set to 100 and < 0.01, respectively. Using this statistical method, only that genes, whose expression was statistically different ($P < 0.01$) between the cold-shocked and control samples and displayed the same expression trend (either up- or down-regulated) in both biological replicates were represented in the output file by a single fold-change value. Only those genes, which had at least 3-fold expression difference, i.e. their log2 ratio was larger than 1.585 (up-regulation in the cold-treated plants) or less than -1.585 (down-regulation in the cold-treated plants) were analysed further, and are described as the 636 differentially expressed genes throughout this work. Because of the single fold-change value of these genes, no standard deviation for the

two biological replicates is applicable. In the manufacturing process of the microarray, some probes representing the same gene were spotted in replicates. In addition, the updated annotation of the microarray probes, as described below, revealed that certain different oligonucleotide probes on the array represent the same gene. Therefore, the expression ratios for such genes were averaged within and between the biological replicates.

Annotation of the differentially expressed genes represented by the oligonucleotide probes on the microarray was updated as follows. The *T. aestivum* TAGI release 12 EST library was downloaded from the TGI database [36]. Blast search was performed using the sequence of each oligonucleotide probe representing the differentially expressed genes, and ESTs with 100 % match were selected. Then, for each probe, either the EST whose TC identification number had a match in the database of the software MapMan [37] or, if no such match was found, the longest EST was selected. Using those ESTs, a blastx search was performed against the NCBI protein database and the protein with the best E-value (threshold $<10^{-5}$) was annotated to each probe.

A. thaliana and *O. sativa japonica* orthologues of the wheat genes were retrieved from the UniProt database. For the over-representation analysis, the Biological Process GO terms for the differentially regulated genes were retrieved by the GORetriever and GOSlimViewer modules of the AgBase functional genomics resource [38] using the UniProt IDs of the encoded proteins. Over-representation analysis was performed using agriGO [39] with hypergeometric statistical test and Hochberg False Discovery Rate (FDR) correction, in which the significance level was set to $P < 0.01$. For functional annotation of the genes, the software MapMan [37] and the KEGG database were used. Predicted protein-protein and protein-hormone connections were retrieved using the STRING [40] and STITCH [41] tools using default settings, and were visualised using the software Cytoscape (http://cytoscape.org/).

Hormone concentration data were analysed by the Kruskal-Wallis test using the R environment (http://www.r-project.org/) in order to identify significant differences between the cold-treated and control samples. Data were averaged and the cold-treated/control ratio was calculated and transformed into log2 values.

Microarray data were deposited to ArrayExpress under accession number E-MTAB-3937.

3. Results

In this study we examined the effect of one day cold-chock on the chromosome 5A donor winter wheat Cheyenne (Ch), the recipient spring wheat Chinese Spring (CS) variety, which was widely used as a model to create different cytogenetic stocks including single chromosome substitutions [42], and the CS(Ch5A) substitution line carrying Chr 5A from Ch in a CS background [20]. It was determined previously [21,43] that the three genotypes display marked difference in their freezing-tolerance (Supplementary Fig. S1).

Similarly to the cold-shock treatment described by Monroy et al. [9], we transferred the plants rapidly from 20 to 4 ºC. According to our meteorological data survey, very similar temperature changes (on average 20.9 to 6.2 ºC and 21.1 to 5.5 ºC, respectively) occured under field conditions on 1,780 days in total in the October-November and March-April sowing/early growth periods for wheat in Hungary in the last 110 years (Supplementary Fig. S2). This indicates that temperature conditions were quite realistically chosen for the current study. Regarding light conditions, the number of average sunshine hours was slightly higher (6.4 versus 5.1 hours) in the March-April than in the October-November period in the surveyed years, while the photoperiod in Hungary is between short and long day conditions (Supplementary Fig. S2). We, therefore, applied the 16/8 hours long photoperiod in our model study, similar to the experimental system used by others [9].

The applied cold-shock induced expression of *CBF14*, a biomarker gene for cold-treatment of wheat [21], in all three genotypes in proportion with their freezing-sensitivity/tolerance, although its basal expression in the control plants grown at 20 ^{o}C was higher in the freezing-tolerant Ch and CS(Ch5A) than in the freezing-sensitive CS genotype (Supplementary Fig. S1). The *CBF14* expression data confirmed the freezing-tolerance level of the genotypes and indicated the appropriateness of the cold-shock experiment.

3.1. Transcriptome changes in response to cold-shock

In total, 636 genes with significantly different expression ($P < 0.01$) between the cold-shocked and control samples and with a minimum of 3-fold treated/control expression ratio (larger than 1.585 or lower than -1.585 in log2 value) were identified (Supplementary Table S2). Slightly more up- than down-regulated genes were identified in each genotype (Fig. 1).

For the technical validation of the microarray results, the expression ratio of seven genes (about 1% of the differentially regulated genes) was determined by qRT-PCR. A strong positive correlation ($R^2 = 0.8297$) was found between the microarray and the qRT-PCR expression ratios, confirming the reliability of the microarray results.

Both genotype-specific and common genes were found amongst the 636 differentially expressed genes (Fig. 2). The common genes between the Ch and CS(Ch5A) genotypes might be due to the presence of the Cheyenne 5A chromosomes in both of them. On the other hand, the genes that were only expressed differentially in either CS or CS(Ch5A), might be harboured by their genotype-specific 5A chromosomes. We, therefore, performed a Blast search for the 636 genes in the available wheat chromosome survey sequence database (https://urgi.versailles.inra.fr/blast/blast.php) in order to pick up any 5A chromosome-specific genes. Although we have identified 44 genes that had a hit to Chr 5, of which seven were

common between CS(Ch5A) and Ch (Supplementary Table S3), none of the 636 genes were exclusively located to Chr 5A (data not shown).

The differentially expressed genes were annotated into functional groups using the MapMan software [37] and manual curation. Of the 636 genes, 509 could be assigned into 28 functional groups, whereas 127 genes were in the unknown/not assigned/miscellaneous categories (Supplementary Table S2). Functional groups with more than ten annotated genes were Amino acid metabolism, Cell, Cell wall, Development, DNA, Hormone metabolism, Lipid metabolism, Major CHO metabolism, Photosynthesis, Protein, Redox, RNA, Secondary metabolism, Signalling, Stress and Transport (Fig. 3). Using their *Arabidopsis thaliana* and *Oryza sativa japonica* orthologues, 243 differentially regulated genes could be annotated to KEGG database pathways (Supplementary Table S2). Some of these groups are described in more detail in the forthcoming sections.

3.1.1. Defence genes

A number of genes encoding proteins, which defend cells against the primary and secondary effects of cold-shock, for example osmotic (dehydration) and oxidative stresses, were identified in this study as cold-responsive.

Late embryogenesis abundant (LEA) proteins and their dehydrin subfamily [44] protect cells against cold-mediated dehydration. The *LEA14-A* gene (WOC_07289) and the *DHN3* (WOC_14245) and *COR410* (WOC_01768) dehydrin genes were up-regulated in each genotypes by cold, another LEA gene (WOC_08160) was up-regulated in both tolerant genotypes, and finally the *DHN4* (WOC_09515) dehydrin gene was up-regulated in the tolerant Ch variety (Supplementary Table S4).

Six differentially regulated genes encoding chaperones [45], which aid protein folding, were identified. Four and one of these genes were uniformly up- and down-regulated in all of the genotypes, respectively, while one gene was only up-regulated in the sensitive CS variety

(Supplementary Table S4). Cold-regulated plasma membrane proteins, heat- and cold-shock proteins, low-temperature-induced proteins, heat-stress transcription factors and a salt-stress-induced protein were also identified, of which only one, a low-temperature-induced protein gene was down-regulated in each genotype, while all other genes encoding these defence proteins were up-regulated variously amongst the genotypes (Supplementary Table S4).

The majority of the eleven genes encoding proteins involved in the biosynthesis of compounds such as chitin, lignin, cutin and wax, which form physical barriers against different stresses, were particularly up-regulated in the tolerant genotypes, although some of them also displayed the same expression in variety CS. (Supplementary Table S4). Thirteen other genes, which were annotated into the Stress MapMan group and might have a role in defence, were also identified as cold-responsive (Supplementary Table S4).

Thirteen differentially regulated genes were annotated to the Redox MapMan functional category, and six of these genes had a definite position in the glutathione metabolism pathways (Supplementary Table S5). Three of these genes were up-regulated uniformly in every genotypes, one was up-regulated in the tolerant genotypes, and two were up-regulated in genotype-unspecific fashion. The proteins encoded by these genes defend cells against oxidative stress and maintain cellular homeostasis [46].

3.1.2. Genes in carbohydrate metabolism

Plants assure their energy needs and metabolites by photosynthesis and successive pathways, in which a number of genes were identified as cold-responsive in every genotype (Fig. 4 and Supplementary Table S6). Antenna protein genes were mainly down-regulated (in 28 cases out of the 33 genotype/gene combinations; in five cases no change was detected), while photosynthesis-associated genes displayed a mixture of up- and down-regulation amongst the genotypes. In C4 carbon fixation, the *NADP-ME1* gene (WOC_07957), encoding

a NADP-dependent malate-dehydrogenase, was up-regulated in the highly freezing-tolerant Ch variety. In the subsequent Calvin-cycle, *RuBisCO* small and large chain genes were down-regulated in the tolerant genotypes, but their expression did not change in CS. Genes in the downstream Glycolysis/Gluconeogenesis pathway were up-regulated amongst the three genotypes. Finally, α-D-glucose biosynthesis and catabolism genes displayed up- and down-regulation, respectively (Fig. 4).

3.2. Hormones and hormone metabolism genes

Regarding hormones, the levels of abscisic acid, cytokinins, auxins, salicylic acid, jasmonates and their metabolites (34 compounds in total, Supplementary Table S7) were determined in the three wheat genotypes in response to one-day cold-shock. Statistical analysis highlighted a number of compounds the levels of which were significantly changed in the cold-treated plants compared to the controls (Supplementary Table S8). In parallel with this, 45 differentially regulated genes were annotated to the MapMan hormone metabolism group (Supplementary Table S9), of which 37 were mapped to pathways in plant hormone biosynthesis (KEGG:01070). The metabolic genes of those hormones, which were not determined in this study, are shown in Supplementary Table S9, but are not described in details.

In ABA biosynthesis, a carotenoid cleavage dioxygenase (*CCD4*, WOC_02449) and a beta-carotene-3-hydroxylase (*BETA-OHASE*, WOC_04922) were uniformly down- and up-regulated by cold-shock, respectively, in every genotype (Fig. 5 and Supplementary Table S9), implying that conversion of β-carotene is shifted from strigol to ABA synthesis. Furthermore, a zeaxanthin epoxidase (*ZEP*, WOC_11286) and the rate-limiting 9-cis-epoxycarotenoid dioxygenase (*NCED1*, not on the array) were up-regulated in CS(Ch5A) and Ch (Fig. 5 and Supplementary Fig. S3) suggesting that ABA synthesis is enhanced by cold-shock in the tolerant genotypes. Accordingly, we have found that cold-shock

up-regulated the level of ABA (C06082) in all of the genotypes, although the change was statistically significant only in the highly freezing-tolerant Cheyenne (Fig. 6). The cytochrome P450 8'-hydroxylase gene (*CYP707A2*, WOC_13199), catalysing the first step of ABA catabolism toward PA and DPA [47], was down-regulated on the array in every genotype (and confirmed by qRT-PCR in lines CS and Ch, Supplementary Fig. S3). In parallel, the level of the biologically inactive 8'-hydroxy abscisic acid (C15514), and the downstream ABA catabolites, phaseic acid (PA, C09707), dihydrophaseic acid (DPA, C15971) and neophaseic acid (neo-PA, 91820491), decreased significantly in each genotype (Supplementary Table S8).

Cold-shock significantly decreased and increased the total level of active cytokinins (*trans*-zeatin, C00371; isopentenyl adenine, C04083; dihydrozeatin, C02029; and *cis*-zeatin, C15545) in the tolerant Cheyenne and the sensitive Chinese Spring varieties, respectively, while no significant change has been detected in the substitution line CS(Ch5A) (Fig. 6). This is consistent with previous results obtained with other spring and winter wheat varieties [7,8].

Three genes controlling the metabolism of active cytokinins were expressed differentially. *CKX7* (WOC_12878), which encodes cytokinin dehydrogenase, the key cytokinin degrading enzyme [48], was down-regulated in variety CS on the array (Supplementary Table S9). S-adenosylmethionine decarboxylases (SAMDCs) are also connected with the metabolism of active cytokinins (Supplementary Fig. S4). The *SAMDC1* (WOC_01221) and *SAMDC2* (WOC_13463) genes were up-regulated by cold in the tolerant and in every genotype, respectively. Up-regulation of the *SAMDC1* genes was confirmed by qRT-PCR (Supplementary Fig. S3). Gene *IPT8* (it was not present on the array), encoding an isopentenyltransferase, which provides isopentenyl-adenosine-phosphates for the biosynthesis of active cytokinins, thus controlling the inflow (Supplementary Fig. S4), was also identified by qRT-PCR in the examined genotypes (Supplementary Fig. S3). Although the catalysing

enzyme and metabolite levels might not be directly proportional to the expression level of the genes encoding the corresponding enzymes, we predict that the differential expression of these genes might explain the observed changes in the active cytokinin levels.

The level of the growth promoting hormone, indole-3-acetic acid (IAA, C00954), was significantly up-regulated in the Ch variety (Fig. 6), while two IAA metabolites, indole-3-acetic acid-aspartate (IAA-Asp) and oxo-indole-3-acetic acid (OxIAA) were up- and down-regulated in CS and CS(Ch5A), respectively (Supplementary Table S8). Changes in the pool of auxin metabolites were accompanied with the change of expression of two metabolic genes. Down-regulation of an IAA-amino acid hydrolase (*ILL5*, WOC_09197) might be related to the up-regulation of IAA-Asp in CS, while in the freezing-tolerant genotypes, down-regulation of a indole-3-glycerol phosphate synthase gene (*AT2G04400*, WOC_01871) was observed (Supplementary Table S9).

Salicylic acid (SA, C00805) and jasmonic acid (JA, C08491), which affect diverse growth, development and stress-response processes in plants [49,50], were regulated in the opposite fashion in the freezing-sensitive CS variety, i.e. SA was up- while JA and jasmonic acid-isoleucine (JA-Ile, 44123531) were down-regulated. In contrast, all three compounds were down-regulated in both freezing-tolerant genotypes (Fig. 6 and Supplementary Table S8), suggesting that the SA-JA antagonism was absent in the freezing-tolerant genotypes, which might contribute to their tolerant character.

More differentially regulated SA and JA biosynthetic genes were identified in Ch and CS(Ch5A) than in CS (Supplementary Table S9), suggesting that the modulation of biosynthesis of the two hormones might be a tolerant genotype-specific response. However, these genes displayed a mixture of various up- and down-regulation (Supplementary Table S9), and, therefore, no sound correlation between hormone levels and gene expression could be established.

3.3. Genes in ABA signalling

The results described in section *3.2.* indicated that the ratio of ABA and its metabolites might be increased in response to cold-shock in each genotype. We postulated, therefore, that, in concordance with previous suggestions [47], the ABA-dependent regulatory cascade [51] might have a role in cold-signalling in the examined wheat genotypes. In that cascade, a complex formed by ABA and the PYR or PYL ABA-receptor protein inhibits type-2C protein phosphatases [52]. PP2Cs, therefore, cannot dephosphorylate downstream SnRK2 kinases [53], thus they remain active and phosphorylate the LHY and MYB transcription factors [54]. Our results revealed that, (i) the ABA receptor protein genes *PYR1* (WOC_13577) and *PYL5* (WOC_13517) were down-regulated in each genotypes, (ii) type-2C protein phosphatase genes (*PP2C32*, WOC_10489; *PP2C50*, WOC_14117; *PP2C6/HAB2*, WOC_13859; *PP2C8*, WOC_09183; *PP2C9*, WOC_08078 and *BIPP2C1*, WOC_13019), with one exception (*BIPP2C1*), displayed mixed up-regulation in the genotypes, (iii) of the two regulated SnRK2-type serine/threonine-protein kinase genes, *SAPK1* (WOC_00163) was up-regulated in each genotypes, while *SAPK2* (WOC_05902) was down-regulated in Ch, and (iv) the *LHY* (WOC_05246) and *MYB59* (WOC_05262) transcription factor genes were up-regulated in every genotype and in variety Ch, respectively, (Fig. 5). Consequently, we propose that the ABA-dependent PYR1/PYL5 - PP2C - SnRK2 - LHY signalling route is very likely involved in cold-shock response in all genotypes.

Expression of the genes in ABA-signalling was tested by qRT-PCR. In 21 case out of the 36 gene/genotype combinations, the expression trend was the same for the array and qRT-PCR; in eleven cases, where the microarray did not gave any result above the set threshold, qRT-PCR revealed the expression and the two tests gave adverse result only in four cases (Supplementary Fig. S3).

3.4. System differences

The gene expression and hormone result described above revealed no major differences between the freezing-sensitive and -tolerant genotypes, which responded similarly to cold-shock by various, mixed and inconsistent expression of similar genes, gene-types or member of gene families. Thus, it seemed that gene expression itself is not sufficient to typify freezing-sensitive and -tolerant lines and no particular genes can be pinpointed to be responsible for their phenotypes. This prompted us to examine our results at a higher, system level, for which we used two approaches.

First, the Biological Process (BP) Gene Ontology (GO) category for the differentially regulated genes was determined and an over-representation analysis (ORA) was performed. Comparing the over-represented BP categories obtained for the genes expressed merely in each genotypes, for the common genes between the tolerant genotypes and for the common genes between the three genotypes (Fig. 2), revealed several categories that were either specific for or common between the genotypes (Supplementary Table S10). In good agreement with and in support of the above proposed general phenomenon of ABA-dependent signalling (section *3.3.*), the "abscisic acid mediated signalling pathway" BP category was over-represented amongst the differentially expressed genes that were common between the freezing-sensitive and -tolerant genotypes (Supplementary Table S10). Sugar-related BP categories were over-represented in the freezing-tolerant Ch variety. BP categories related to homeostasis was characteristic for each genotype in concordance with the genotype-independent up-regulation of genes in the Redox functional group (Supplementary Table S5).

Second, the known and predicted connections between the proteins encoded by the differentially regulated genes (Supplementary Table S2) and between the proteins and the measured hormones were mined for in the STRING 10 and the STITCH 4.0 databases [40,41], respectively. Since the annotation of *T. aestivum* genes and proteins in databases is

very incomplete, the *A. thaliana* orthologues of the proteins encoded by the differentially regulated genes were used in the search. Of the 636 genes, 490 had an *A. thaliana* orthologue, and of these 488 were found in the databases. Regarding hormones, only those 21 were used as input, which have a compound ID (Supplementary Table S7). The cumulative results conferred 1555 interactions (Supplementary Table S11), involving 422 proteins and nine hormones (Supplementary Table S12).

It is beyond the scope of this report to analyse all interactions in details, and therefore we only highlight a few of them. PP2C-type phosphatases, PP2C32, PP2C50 and PP2C6, which participate in ABA-dependent signalling (Fig. 5), were amongst the ten top interacting proteins with 41, 44 and 36 interactions, respectively (Supplementary Tables S11 and S12). PP2C32 have interaction with proteins in the jasmonic acid (MYC2 and JAZ1) and ethylene (ERF2) signal transduction pathways indicating the role of dephosphorylation in the signal transduction of these two hormones and possible crosstalk between the ABA, JA and ethylene signalling pathways. In addition to PP2C32, the MYC2 transcription factor, which was considered as a "master regulator" [55], interacts with the JAZ1 and JAZ6 proteins of jasmonate signalling (encoded by the *TIFY10A*, WOC_08182 and *TIFY11B*, WOC_14532 genes, respectively). Furthermore, MYC2 interacts with ERF2 (*ERF2*, WOC_08993) in ethylene signalling and with WRKY70 (*WRKY70*, WOC_06485), which was allocated to JA/SA crosstalk [56]. It was reported recently that ABA enhances the interaction between the PYL6 ABA-receptor and MYC2, indicating a direct link between ABA and JA signalling [57]. The *PYL5* gene, which is homologous to *PYL6* and participates in ABA signalling (Fig. 5) was regulated by cold in all three genotypes and might also bind to MYC2 as PYL6 does. It is worth to mention the presence of numerous connections between WRKY transcription factors and proteins in hormone signal transduction pathways (Supplementary Table S11), which in very good agreement with the proposed interacting [58] and network-forming [59]

nature of WRKY factors in biotic and abiotic stresses [11,60]. It is also interesting to note that, in contrast to the usual up-regulation of WRKY factors by cold, five out of the six *WRKY* genes were down-regulated in our system, which is, however, similar to that was observed in rice [61].

Interactions between proteins, of which genes were uniformly expressed in both freezing-sensitive and -tolerant genotypes, formed a fundamental network (Supplementary Fig. S5). Superimposing the genotype-specific interactions on that network revealed some marked differences between the genotypes. In the freezing-sensitive CS variety, additional interactions between Ca-signalling proteins and translation were observed (Supplementary Fig. S5). In contrast, in the freezing-tolerant Ch variety numerous connections were observed between receptor kinases and translation, between ABA-signalling and receptor kinases/wall-associated receptor kinases, and connections between ABA-signalling and translation are also appeared (Supplementary Fig. S5). The visual appearance of line CS(Ch5A)-specific interactions clearly seemed to be an intermediate between the CS and Ch varieties (Supplementary Fig. S5), which is in good agreement with its freezing-tolerance degree fallen between CS and Ch. In this substitution line, less connections between receptor kinases and translation were present than in Ch, but it had more interactions between ABA-signalling and receptor kinases than either CS or Ch (Supplementary Fig. S5). Compared to the genotype-unspecific response, CS and Ch had additional connections between the regulated genes and transcription and translation indicating the enhanced role of both type of regulations in stress response, while in line CS(Ch5A) only translational regulation seemed to be enhanced (Supplementary Fig. S5).

4. Discussion

4.1. General transcriptome changes in response to cold-shock

In this report we described the effects of one day cold-shock on the transcriptome and different phytohormones of freezing-sensitive and -tolerant wheat genotypes. Regarding transcriptome changes, we have identified 636 differentially expressed genes in total using an oligonucleotide-based microarray [28], and revealed that 2.54%, 2.63% and 2.89% of the genes on the array displayed more than 3-fold change after one day cold-shock in genotypes CS, CS(Ch5A) and Ch, respectively. In similar studies, employing a cDNA-based wheat microarray or the Affymetrix microarray platform, about 8 and 3% of the examined genes responded, respectively [9,11]. As far as we concern, the different microarray platforms and genotypes used in those and our study should explain such moderate differences.

The cold-responsive genes identified in this study belonged to those broad functional categories, such as defence, metabolisms, regulatory processes and a number of other functions (Fig. 3 and Supplemental Table S2) that were also reported in other similar studies [9,10,11].

4.2. ABA-related responses to cold-shock

ABA regulates a number of physiological and developmental processes in plants and it is also considered as a major stress factor since abiotic stresses up-regulate its synthesis [62]. A fine balance between ABA biosynthesis and metabolism is important for the accurate cellular level of ABA and its metabolites [63]. Regarding this, we have found that ABA biosynthetic genes were up-regulated in either all or in the freezing tolerant genotypes, which is in good agreement with the observation that ABA biosynthesis genes are transcriptionally up-regulated under abiotic stress [64]. On the other hand, the gene encoding the enzyme catalysing the first step in ABA catabolism was down-regulated in each genotype. In parallel with these altered genes expressions, we have observed the up-regulation of ABA by cold-shock in every genotype, although the change was only significant in variety Ch. ABA

catabolites, however, were significantly down-regulated in all of the genotypes. Thus we predicted that the ratio of ABA/ABA metabolites increased in each genotypes.

A number of our observations are in good agreement with the roles of ABA in cellular physiology. One such role is the impact of ABA on photosynthesis/carbohydrate metabolism. We have identified several up-regulated *PP2C* and a *SnRK* gene in the ABA signalling pathway (Fig. 5). The encoded proteins mediate stomatal closure in an ABA-dependent fashion [65,66], which influence gas exchange. ABA also effects photosyntesis by a number of potential mechanisms [67]. For example, exogenous ABA inhibited photosynthesis and carbon assimilation [68], and decreased expression of the ligh-harvesting chlorophyll-binding (antenna) protein genes in a number of plant sepecies [69]. In concordance with this, we observed the parellel down-regulation of the *LHCA*, *LHCB* and *CAB* genes and the up-regulation of ABA in every genotype (Fig. 4 and Supplementary Tables S6, S8). We have also obserbed that the *RuBisCO* large and small subunit genes were down-regulated in the tolerant genotypes (Fig. 4 and Supplementary Table S6), which might be mediated via a feedback regulatory mechanism by the RuBisCO protein, to which ABA binds and affects its activity [68]. Along the downstream carbohydrate metabolic pathways (Fig. 4), differential gene expression might compensate the ABA-mediated down-regulation of photosynthesis to ensure the appropriate level of compounds for the subsequent metabolic step. We predict that the net outcome of this entire process (Fig. 4) can be an increased level of D-glucose in order to maintain glucose homeostasis. This prediction is supported by a study [70] demonstrating that the level of glucose was elevated by cold-stress in the freezing-tolerant CS(Ch5A) but not in a freezing-sensitive CS genotype (line Ch was not examined in that study). The importance of glucose homeostasis in cold-shock response is further supported by the result that monosaccharide (glucose/hexose) metabolism-related BP categories were overrepresented amongst the genes differentially expressed in variety Ch (Supplementary Table S10). These

results indicate that the proposed interaction between sugar and ABA signalling [67] might exist in wheat too. This process can be similar to Arabidopsis, in which the interaction of the two signalling pathways resulted in stress-tolerance [71], although in wheat this might happen by compensating the ABA-mediated down-regulation of photosynthesis via up-regulation of glucose biosynthesis (Fig. 4). We note here, that active cytokinins were down-regulated in variety Ch, which is required to suppress growth in order to reallocate energy sources for efficient defence [7,8,72].

The ABA-PP2C/SnRK-mediated stomatal closure can mimic drought-stress [60]. This together with down-regulated aquaporin genes (Fig. 5) that encode membrane proteins, which are expressed under hormonal control and facilitate the transport of water [73], can result in a net water deficit in the cold-shocked wheat genotypes, which then can promote ABA-dependent signalling. Water deficit and ABA were described to up-regulate the dehydration-defending LEA/dehydrins genes [74], and our results (section *3.1.1.*) are in agreement with this.

4.3. System differences between sensitive and tolerant genotypes

It is an intriguing question that what makes the difference between freezing-sensitive and -tolerant wheat genotypes [11], in particular in their response to cold-shock. Our transcriptome and hormone results did not revealed much difference between them, and therefore we investigated the genotypes as complex system using overrepresentation and protein-network studies. Comparing the results of these analysis with the hormone and gene expression results provided some insight into the nature of the different genotypes and here we highlight these similarities and differences.

The hormone, gene expression and overrepresentation results indicated that ABA-dependent signalling is important in cold-shock response in all genotypes. However, examining gene expression in the protein interaction subnetwork comprised of the

ABA-signalling and hormone crosstalk proteins (Supplementary Fig. S6) suggested that in every genotype the PYR1/PYL5 - PP2C6 - SAPK1 - LHY - CDF1 signalling route might be utilised, while in the freezing-tolerant genotypes the PYR1/PYL5 - PP2C50 - SAPK1 - MYB59 - E2FA/GATA4 route might be additionally engaged too (Supplementary Fig. S6). This suggested role of ABA-dependent signalling pathway in cold shock response in wheat is in good agreement with the observation that exogenous ABA increased freezing tolerance of wheat seedlings [72]. Crosstalk might occur between ABA and JA/SA/ethylene signalling in cold-shocked wheat via the MYC2, WRKY70, WRKY46 and WRKY11 proteins, which might act as a switch between hormone signalling routes [55,75,76,77].

Despite of the complex gene expression patterns in the carbohydrate metabolic pathways displayed by each genotype, glucose homeostasis might be more important in the highly freezing-tolerant Ch variety as indicated by overrepresentation of glucose/hexose metabolism BP categories amongst the genes specifically expressed in this genotype (Supplementary Table S10). Examining the line-specific over-represented BP terms, it seemed to be plausible that maintaining homeostasis is important in every genotype, but in addition to that, in the freezing-sensitive CS variety maintaining the structure of cellular components and ensuring the progenies, while in the freezing-tolerant Ch and CS(Ch5A) genotypes the protection of cellular biochemistry is preferred (Supplementary Table S10).

Similarly to another study [9], our transcriptome analysis revealed that dozens of different regulatory genes belonging to different functional families, such as Ca signalling, kinases, post-translational protein modification, protein degradation, receptor kinases, translational and transcriptional proteins and transcription factors, displayed complex expression patterns in the genotypes. Inspection of the interactions between proteins encoded by such regulatory genes that were expressed in a genotype-specific fashion, revealed that

certain differences between the information-flow in cold-signal perception and transduction of the freezing-sensitive and -tolerant genotypes (Fig. 7) is plausible, which might be one reason behind their distinct phenotypes.

Low and sub-zero temperatures make wheat production limited in many geographical locations or can cause major damages and yield loss. Since our metrological data survey showed, in spite of the generally considered global warming, that the number of days with sub-zero temperatures were on the rise in the last 100 years (Supplementary Fig. S7), the development of new wheat varieties with enhanced cold/frost-tolerance and the determination of molecular/biomarkers with the aid of genes expression and hormonal studies would be desirable for wheat-producers worldwide. However, care should be taken since genome modifications either by traditional breeding or molecular methods, similar to the targeted stress itself, can result in large scale systemic alterations in the modified organism thus causing unwanted intermediate- and end-phenotypes.

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Figure legends

Fig. 1. The number of differentially expressed genes in wheat genotypes in response to one-day cold-shock.

CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne.

Fig. 2. Venn diagram of the differentially expressed genes in wheat genotypes.

CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne.

Fig. 3. MapMan annotation of the differentially expressed cold-responsive genes.

The MapMan main bin name, the number and percentage of the genes are shown only for those categories into which ten or more genes were annotated.

Fig. 4. Simplified overview of major carbohydrate metabolism pathways with gene expression data in cold-shocked wheat.

Genes are labelled by their UniProt gene names (Supplementary Table S2). Triplets of coloured squares from left to right show gene expression for lines CS, CS(Ch5A) and Ch.

Down- and up-regulation is indicated by ■ and ■, respectively. ■ indicates that the Rank Product statistical analysis did not return an expression ratio for the gene. ➡ and ➡ indicate the flow of metabolites with possible elevated and reduced level, respectively, and ➡ indicate the flow of compounds. 3-PG, 3-phosphoglycerate (C00197); α-DG6P, α-D-glucose-6-phosphate (C00668); α-DG1P, α-D-glucose-1-phosphate (C00103); DG, α- and β-D-glucose (C00221 and C00267). For detailed explanation, refer to the main text.

Fig. 5. Gene expression in ABA-metabolism and -signalling in cold-shocked wheat. ●, represent metabolites: β-Ca, β-carotene (C02094); STI, strigol (C09190); ZX, zeaxanthin (C06098); VX, violaxanthin (C08614); XA, xanthoxin (C13453); ABA, abscisic acid (C06082); 8HA, 8'-hydroxy abscisic acid (C15514); PA; phaseic acid (C09707); DPA, Dihydrophaseic acid (C15971). For metabolites, triplets of circles represent their regulation by cold-shock in genotypes CS, CS(Ch5A) and Ch (from left to right): ●, up-regulation; ●, down-regulation; ● with +, statistically not significant up-regulation. Genes shown for ABA-metabolism: *CCD4*, carotenoid cleavage dioxygenase 4 (WOC_02449); *BETA-OHASE*, Beta-carotene 3-hydroxylase 1 (WOC_04922); *ZEP*, Zeaxanthin epoxidase (WOC_11286); *NCED1*, 9-cis-epoxycarotenoid dioxygenase (asterisk indicates that this gene was not represented on the array); *CYP707A2*, Abscisic acid 8'-hydroxylase 2 (WOC_13199). In ABA-signalling, ●, represent proteins. Their genes are: the abscisic acid receptors *PYR1* (WOC_13577) and *PYL5* (WOC_13517); the type 2C protein phosphatases *PP2C32* (WOC_10489), *PP2C50* (WOC_14117), *PP2C6/HAB2* (WOC_13859), *PP2C8* (WOC_09183), *PP2C9* (WOC_08078) and *BIPP2C1* (WOC_13019); the SnRK-type serine/threonine-protein kinases *SAPK1* (WOC_00163) and *SAPK2* (WOC_05902); and the MYB transcription factors *LHY* (WOC_05246) and *MYB59* (WOC_05262). For all genes, triplets of squares represent their regulation by cold-shock in lines CS, CS(Ch5A) and Ch

(from left to right): ■, up-regulation; ■, down-regulation; ■ indicates that the Rank Product statistical analysis did not return an expression ratio for the gene in a particular line. T-shaped blue lines represent suppression/inhibition; -P, dephosphorylation, +P, phosphorylation.

Fig. 6. The effect of one-day cold-shock on hormone levels.

Hormone concentrations are expressed as pmol/g fresh weight (FW). CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne. act CK, active cytokinins; IAA, indole-3-acetic acid (C00954); JA, jasmonic acid (C08491); SA, salicylic acid (C00805); ABA, abscisic acid (C06082); DPA, dihydrophaseic acid (C15971); PA, Phaseic acid (C09707); ABA-GE, Abscisic acid-glucose ester (C15970). Mean and standard deviation values of three biological replicates are shown by bars and error bars, respectively. Cold-shock/control ratios are presented in Supplementary Table S8. Asterisk indicate statistically significant differences between cold-shock and control values ($P < 0.05$) determined by Kruskal-Wallis test.

Fig. 7. Information-flow between elements of cold-shock response in wheat.

Panels G, CS, CS(Ch5A) and Ch shows general and genotype responses, respectively. Grey and thicker arrows indicate appearing and enhanced information-flows, respectively, compared to the general response. CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne. Ca, Ca-signalling proteins; K, kinases; RK, receptor kinases; WRK, wall-associated receptor kinases; ABA-S, ABA-signalling proteins; TR, transcription; TL, translation.

Supplementary material

Fig. S1. Freezing tolerance and the expression of *CBF14* in wheat genotypes.

CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne. Bars represent the fold-change induction (in log2 values) of

expression of the *CBF14* gene relative to the expression (=1, log2=0) in line CS at 20 oC. Diamonds represent the survival rate of the genotypes in a freezing test [adapted from ref. 43].

Fig. S2. 24 hours temperature data at 47° 31' North - 21° 37' East in Hungary.

24 hours maximum (red line) and minimum (blue line) temperatures and their difference (grey line) in March-April (A) and October-November (B) between 1901 and 2010. Daily sunshine hours (orange line) in March-April (C) and October-November (D) between 1920 and 2010. Black lines show averages for the data sets. Panel E shows the average time for sunrise, sunset and the number of light hours for the given months.

Fig. S3. Array and qRT-PCR expression ratios of selected genes.

Log2 value fold change of genes in active cytokinin metabolism (a), abscisic acid metabolism (b) and abscisic acid signalling (c, d and e). For array data, bars represent the expression ratio obtained by the Rank Product method [35], which always produce only a single value for any replicates, therefore no SD is applicable. In the Rank Product method settings, the significance threshold was adjusted to $P < 0.01$ level, thus all array data represent statistically significant difference between the treated and control samples at that level, and therefore significance levels are not shown for clarity. For qRT-PCR data, bars and error bars represent the mean and SD values calculated from three biological replicates. One and two asterisks indicate statistically significant difference between the treated and control samples at $P < 0.05$ and $P < 0.01$ levels, respectively. The genes in this figure are: S-adenosylmethionine decarboxylase (SAMDC1, WOC_01221); isopentenyltransferase 8 (*IPT8*, not on the array); Two-component response regulators (*ARR6*, WOC_01876 and *ARR18*, not on the array); 9-cis-epoxycarotenoid dioxygenase (*NCED1*, not on the array); zeaxanthin epoxidase (*ZEP*, WOC_11286); abscisic acid 8'-hydroxylase (*CYP707A2*, WOC_13199); ABSCISIC ACID-INSENSITIVE 5-like protein 2 (*DPBF3*, WOC_02474); ABA receptor proteins (*PYR1*, WOC_13577 and *PYL5*, WOC_13517); type-2C protein phosphatases (*PP2C32*,

WOC_10489; *PP2C50*, WOC_14117; *PP2C6/HAB2*, WOC_13859; *PP2C8*, WOC_09183; *PP2C9*, WOC_08078 and *BIPP2C1*, WOC_13019); SnRK2-type serine/threonine-protein kinases (*SAPK1*, WOC_00163 and *SAPK2*, WOC_05902) and MYB transcription factors *LHY* (WOC_05246) and *MYB59* (WOC_05262).

Fig. S4. Simplified cartoon of active cytokinin metabolism.

Circles represent active cytokinins with their name and compound ID are shown. Sections from top left are: CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne. Numbers in the sections are the cold-shock/control ratios of hormone concentrations; yellow and blue colours represent statistically significant up- and down-regulation, respectively; grey colour represent statistically not significant changes (Supplementary Table S7). Coloured squares represent gene expression ratios in the lines, from left to right: CS, Chinese Spring; CS(Ch5A), Chinese Spring substitution line with Cheyenne 5A chromosome Ch, Cheyenne. Green, up-regulation; red, down-regulation. The genes is this figure are: isopentenyltransferase 8 (*IPT8*, not on the array); S-adenosylmethionine decarboxylase (SAMDC1, WOC_01221 and *SAMDC2*, WOC_13463) and cytokinin dehydrogenase 7 (*CKX7*, WOC_12878).

Fig. S5. Connections between proteins encoded by cold-regulated genes in wheat.

Panel G shows the connections of proteins encoded by uniformly regulated genes in all three genotypes (darker nodes). Panels CS, CS(Ch5A) and Ch show connections between genotype-specific proteins (blue edges) overlaid on panel G. In the bottom three panels, small green and red circles indicate up- and down-regulated genes. Ca, Ca-signalling proteins; K, kinases; RK, receptor kinases; WRK, wall-associated receptor kinases; ABA-S, ABA-signalling proteins; TR, transcription; TL, translation.

Fig. S6. Protein interactions in hormone signalling.

Triangles and diamonds shaped nodes indicate up- and down-regulation of the encoding genes, respectively. Border colour indicates differential expression in: —, both freezing-sensitive and -tolerant lines; —, freezing-tolerant line(s); —, freezing-sensitive line. — and — edges indicate putative general and freezing-tolerant line-specific ABA-signalling routes, respectively. Dashed grey edge indicate interaction described by Aleman et al. [57]. Solid grey edges indicate known and predicted interactions in the STRING database [40].

Fig. S7. The number of days with below -4 °C temperature in the first and last three decades of the last 110 years.

Table S1. Primers used for qRT-PCR validation of selected genes.

Table S2. Differentially expressed genes in wheat genotypes in response to one day cold-shock.

Table S3. Common genes between genotypes CS(Ch5A) and Ch mapped on Chr 5.

Table S4. Cold-responsive defence genes.

Table S5. Cold-responsive redox genes.

Genes associated with glutathione metabolism are in boldface.

Table S6. Cold-responsive carbohydrate metabolism genes.

Table S7. Hormones measured in this study.

[1]KEGG (Cxxxxx) or PubChem compound ID.

Table S8. Cold-shock/control ratio (log2 value) of hormones levels in wheat genotypes.

[1]Green, hormones annotated to KEGG pathways; [2]KEGG (Cxxxxx) or PubChem compound ID; [3]Yellow and blue, statistically significant up- or down-regulation, respectively; Grey, no significant difference; Red, not detected.

Table S9. Cold-responsive hormone metabolism genes.

Genes associated with ABA metabolism are in boldface.

Table S10. Genotype-specific Biological Process GO terms for the differentially regulated genes.

Table S11. Connections between proteins encoded by differentially regulated genes.

Table S12. Proteins with network connections.