cDNA-AFLP Analysis of Cold-Acclimated Wheat Plants Reveals Unique Transcript Profiles in Crown Tissues

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Abstract

Low temperature (LT) adversely affects the productivity of plants. Hence, improving the cold hardiness of crop plants is an important goal in agriculture. However, further understanding of LT tolerance mechanisms in plants is required to achieve this objective. In wheat, survival of crown tissues after exposure to below freezing temperatures during the winter determines successful crop stand establishment at the onset of spring season. Therefore, identification of differentially expressed genes in crown tissues of cold acclimated wheat plants is important as it can allow dissection of molecular mechanisms and biochemical pathways within these tissues. In this study, cDNA-AFLP global transcriptomic profiles of crown tissues cold acclimated at 6^{o}C for 0, 2, 14, 21, 35, 42, 56 and 70 days were compared among a cold hardy winter (*vrn-A1*) cv. Norstar, a tender spring habit (*Vrn-A1)* cv. Manitou and two reciprocal near-isogenic lines derived from these two parents differing at the vernalization locus. A total of 2061 differentially expressed transcript-derived fragments (TDFs) were identified using 37 pairs of standard AFLP primer combinations, 30 of which were considered unique due to their genotypic and temporal presence or absence. The remaining TDFs showed differential expression patterns in the four genotypes. Cluster analysis of the unique TDFs revealed influence of the genetic background on expression of these TDFs. BLAST searches of 240 sequenced TDFs showed that 87% of the TDFs had similarity to genes coding for products involved in known functions such as signal transduction, RNA processing and translation, transcription, flowering, cell wall synthesis, metabolism, and protein folding. Thirty-two TDFs did not show similarity to any known genes. Quantitative real-time PCR (QPCR) analyses of these unknown TDFs validated their differential expression patterns. Characterization of their biological function will contribute to an understanding of the role of these novel genes in LT tolerance in wheat. These results suggest that crown tissues undergo a complex adaptive process by changing the expression levels of several genes that determine the level of LT tolerance.

Introduction

Low temperature (LT) stress is a major factor limiting productivity and geographical distribution of plants throughout the world. This has led to significant interest in understanding the complex processes that allow plants to adapt to LT stress. Wheat can adapt to a wide range of climates due to winter and spring growth habit. Winter wheat is seeded in the fall, over-winters, resumes growth in spring and is harvested in early summer whereas spring wheat is planted in the spring

and harvested in late summer or early fall. Winter-habit wheat requires an extended exposure to low non-freezing temperatures to induce flowering, a process called vernalization. The winter and spring habit in wheat is determined by the vernalization (*VRN-A1*) locus, which is also known to influence the LT tolerance levels (Fowler *et al.*, 1996a, b). Winter wheat has several advantages over spring wheat such as higher yield, reduced soil erosion, better moisture utilization, less use of herbicides, disease avoidance and maturation in early summer thus avoiding late season frost (Fowler, 2002). In general, winter wheat possesses better protective mechanisms for winter survival compared to spring ones. However it is still subjected to winter kill, which leads to reduction in crop stand and therefore yield. More refined understanding of the molecular mechanisms underlying LT tolerance of winter wheat will enable development of varieties with enhanced LT tolerance.

In addition to the vernalization process, exposure to low non-freezing temperatures is also required for wheat plants to acquire freezing tolerance by a process called cold acclimation. Cold acclimation has been shown to confer much higher freezing tolerance to winter than spring cultivars (Ohno *et al.*, 2001; Limin and Fowler, 2002; Ganeshan *et al.*, 2008). Cold acclimation, results in various physiological and biochemical changes mainly derived from alterations in the expression of a number of cold-responsive genes in wheat (Fowler *et al.*, 1999; Gulick *et al.*, 2005). Efforts have been made to understand the molecular basis of cold response in wheat by identification of genes whose expression is correlated with the development of freezing tolerance during cold acclimation (Tsvetanov *et al.*, 2000; Ohno *et al.*, 2001; Ganeshan *et al.*, 2008; Christov *et al.*, 2008). However, comprehensive strategies are needed to identify global changes in gene expression during cold acclimation in wheat plants, which may be achieved through the use of genomics technologies. Global changes in gene expression during cold acclimation in plants from winter and spring wheat cultivars, at the transcriptome level, have been demonstrated using microarray (Gulick *et al.*, 2005; Monroy *et al.*, 2007). However, many more new candidate genes involved in cold tolerance remain to be identified. As a differential screening method, cDNA-AFLP is more stringent and reproducible than many other methods because it can amplify low-abundance transcripts allowing detection of rarely expressed genes and distinguishing between homologous genes (Ganeshan *et al.*, 2009). The principal advantage of cDNA-AFLP compared with microarrays is that new genes can be identified and assessed. Therefore to gain a better understanding of the molecular mechanisms underlying LT tolerance, we used cDNA-AFLP transcriptome profiling approach to identify differentially expressed transcripts in crown tissues of wheat plants cold-acclimated at 6^{o}C for an extended period. The genotypes used included a cold hardy winter (*vrn-A1)* cv. Norstar, a tender spring habit (*Vrn-A1)* cv. Manitou and two reciprocal near-isogenic lines for the vernalization locus derived from these two parents, producing a spring Norstar and a winter Manitou to further understand the influence of the *Vrn-A1* locus on LT tolerance. Furthermore elucidation of the molecular mechanisms underlying LT tolerance in wheat crown tissues is important since survival of these tissues represents successful crop stand establishment at the onset of spring season.

Materials and Methods

Genetic materials and LT acclimation

The wheat (*Triticum aestivum* L.) genotypes used in this study have previously been characterized for their responses to LT exposure (Limin and Fowler, 2002; Ganeshan *et al.*, 2008). Briefly, reciprocal near-isogenic lines (NILs) of winter wheat cultivar Norstar and spring wheat cultivar Manitou were developed such that the *Vrn-A1* allele of spring habit Manitou was

transferred to winter Norstar to produce a spring habit Norstar and *vrn-A1* allele of winter habit Norstar was transferred to spring Manitou to produce a winter habit Manitou. The four genotypes were cold-acclimated for 70 days as previously described (Limin and Fowler, 2002; Ganeshan *et al.*, 2008).

RNA preparation and cDNA synthesis

Total RNA from crown tissues was extracted using a modified Trizol™ (Invitrogen Inc., Burlington, Ontario, Canada) method (Ganeshan *et al.*, 2008) and cleaned using the Purelink Micro-to-Midi RNA clean-up kit (Invitrogen Inc., Burlington, Ontario, Canada) according to manufacturer's instructions. RNA quality was assessed on a Bioanalyzer 2100 (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada). For cDNA-AFLP analyses, mRNA isolation from purified total RNA was performed with the PolyATtract mRNA isolation kit (Promega Corporation, Madison, Wisconsin, USA) according to manufacturer's instructions. First strand cDNA synthesis was done on 1 µg of mRNA using 1 µg oligod$T_{(12\text{-}18)}$ and 200 U Superscript III (Invitrogen Inc., Burlington, Ontario, Canada) according to manufacturer's instructions. After treatment of the first strand cDNA with RNAse H, second strand cDNA synthesis was carried out with DNA polymerase I, T4 DNA ligase and deoxynucleotide triphosphates. The double stranded cDNA was purified using the Purelink PCR purification kit (Invitrogen Inc., Burlington, Ontario, Canada) according to manufacturer's instructions.

cDNA–AFLP

cDNA AFLP was carried out according to the specification for IRDye™ Fluorescent AFLP® Kit for Large Plant Genome (LI-COR, Lincoln, NE, USA). Briefly, 300 ng cDNA was digested with 1.25 U of EcoRI and MseI for 2 h at 37°C to generate small cDNA fragments. To inactivate the restriction enzymes, the mixture was incubated for 20 minutes at 80°C. Double-stranded EcoRI and MseI adapters were ligated to the ends of the digested cDNA fragments at 20°C for 2 h to generate templates for amplification. The pre-amplification reaction was carried out using 2.5 µL of 1/10 dilution of the template and EcoRI+A and MseI+C primers. A 1/1000 dilution of this reaction was used for selective amplification with 37 standard AFLP primers (Vos *et al.*, 1995). Amplification products were resolved on 6% denaturing polyacrylamide gel and visualized by silver staining. Differentially expressed TDFs were excised from the gel, re-amplified, sub-cloned and sequenced. The NCBI BLAST search tools were used for homology searches in the GenBank database.

Quantitative real-time PCR (QPCR) and data analyses

For QPCR analyses, first-strand cDNA was synthesized using 200 U Superscript III (Invitrogen Inc., Burlington, Ontario, Canada) from 5 µg of cleaned, DNAse-treated total RNA. The PCR set-up consisted of 3 µL of a 1/15 dilution of the cDNA, forward and reverse primers, 1x Maxima™ SYBR Green I qPCR Master Mix (Fermentas) in a 25 µl reaction volume. QPCR was performed in a MX3000P machine (Stratagene, Cedar Creek, Texas, USA). Amplification was carried out as follows: after the initial activation step at 95°C for 10 min, 40 cycles each of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The wheat *Ubiquitin* gene was used as reference gene. Threshold values (Ct) generated were used to calculate relative expression by the ΔΔCt method (Livak and Schmittgen, 2001). Dissociation curves were generated for each reaction to ensure specific amplification.

Results and Discussion

cDNA-AFLP Profiling

Using cDNA-AFLP (37 pairs of standard AFLP primer combinations), a total of 2061 differentially expressed TDFs were identified in crown tissues, 30 of which were classified on the basis of their genotypic and temporal presence or absence (unique TDFs) and the remaining based on differential expression patterns in the four genotypes. Of the 2061 TDFs showing differential expression patterns, 1827 showed up-regulation and 204 showed down-regulation (Table 1). Unique TDFs were further analyzed by cluster analysis using the NTSYS-PC software, Version 2.21c (Exeter Software, Setauket, NY, USA) based on the presence (1) or absence (0) of the unique TDFs. The generated dendrogram revealed two clusters (Fig. 1). One cluster included unique TDFs present in Norstar and spring Norstar genotypes, and the other cluster included unique TDFs present in Manitou and winter Manitou genotypes at different days of cold acclimation. These results suggest that the genetic background has a significant influence on the expression of these unique TDFs.

Table 1. Differentially Expressed Transcripts in Crown Tissues of Cold Acclimated Wheat Plants Using 37 AFLP Primer Combinations.

Differentially expressed TDFs	Number of TDFs
Up-regulated TDFs	1827
Down-regulated TDFs	204
Unique TDFs	30
Total differentially expressed TDFs	2061

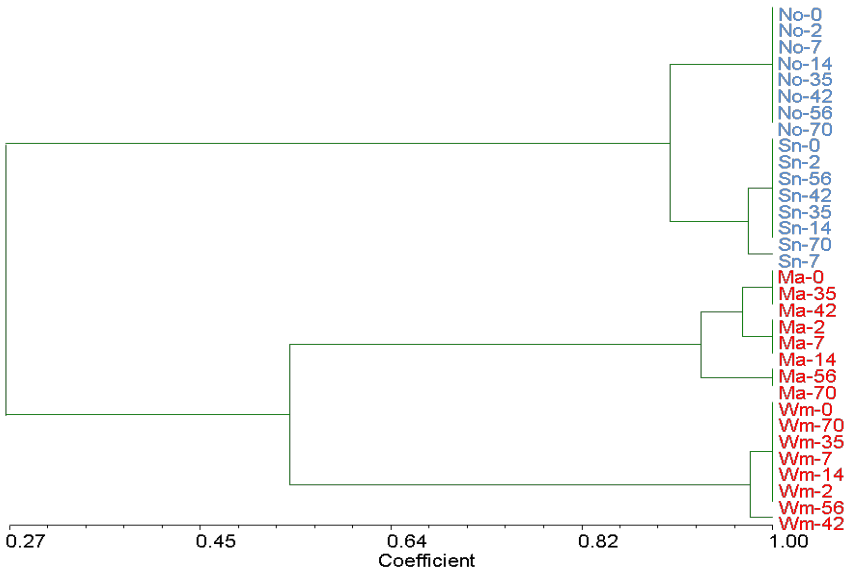

Fig.1. Cluster analysis of unique TDFs in crown tissues of wheat plants cold acclimated for 0, 2, 7, 14, 35, 42, 56 and 70 days at 6°C. No – winter Norstar; SN – spring Norstar; Ma – spring Manitou; WM – winter Manitou.

Sequencing and Functional Classification of TDFs

A total of 240 differentially expressed TDFs were cloned and sequenced. The NCBI BLAST search tools were used for homology searches of these sequences in the GenBank database. Of the 240 sequenced TDFs, 87% showed homology to genes or proteins of known function involved in transcription regulation (17%), signal transduction (17%), translation and RNA processing (20%), protein folding/unfolding (3%), flowering (3%) and metabolism (20%) (Fig. 2). Thirteen percent of TDFs did not show any significant homology to known genes or proteins and may represent novel LT-induced genes that have not been previously characterized.

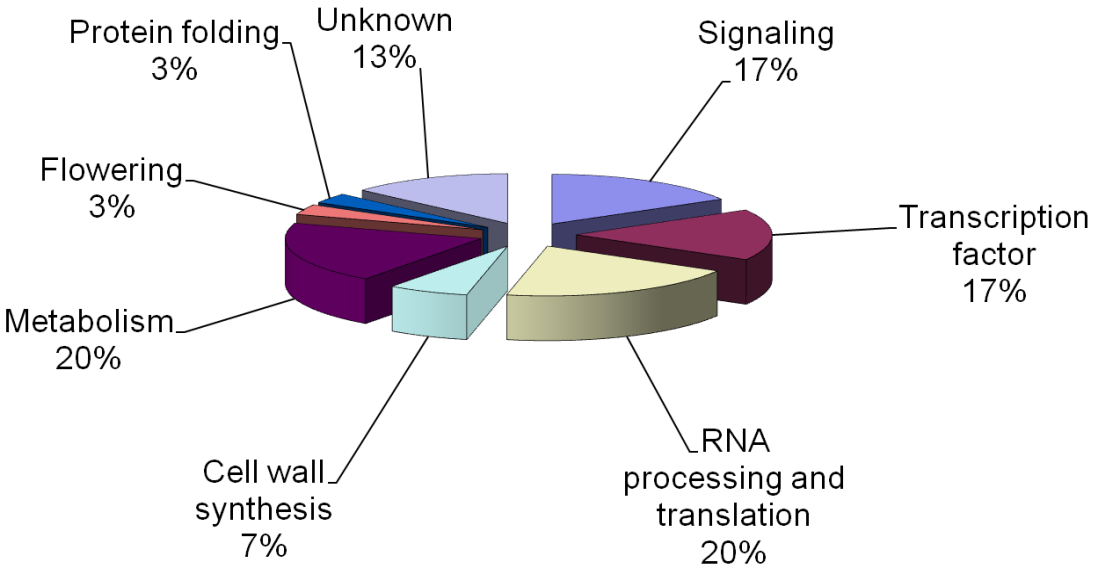

Fig.2. Functional classification of differentially expressed genes in crown tissues of cold acclimated wheat plants.

Validation of Expression Profiles of Unknown TDFs

QPCR analysis confirmed that the unknown TDFs were differentially expressed in cold acclimated crown tissues (Fig. 3 a-g). In Norstar, *UTDF3*, *UTDF5* and *UTDF8* showed highest expression after 2 days, while *UTDF7* showed highest expression after 21 days. The high expression after 2 days is consistent with previously reported expression for *COR* (cold regulated) genes (Ganeshan *et al.*, 2008). This is particularly consistent for *UTDF5* in all four genotypes, wherein Norstar and spring Norstar showed higher expression than Manitou and winter Manitou after 2 days of LT, reflecting the influence of the genetic background. For *UTDF2* and *UTDF6*, although expression in all four genotypes was high after 2 days, expression in Norstar was not the highest. However, expression of these two genes was maintained over long-term acclimation indicating involvement in maintenance of LT tolerance. Similarly, *UTDF1*, which was highly expressed in Norstar at 56 days of LT exposure, may be involved in maintenance of LT tolerance. Characterization of the biological function of these novel genes will contribute to an understanding of these previously uncharacterized genes in LT tolerance in wheat.

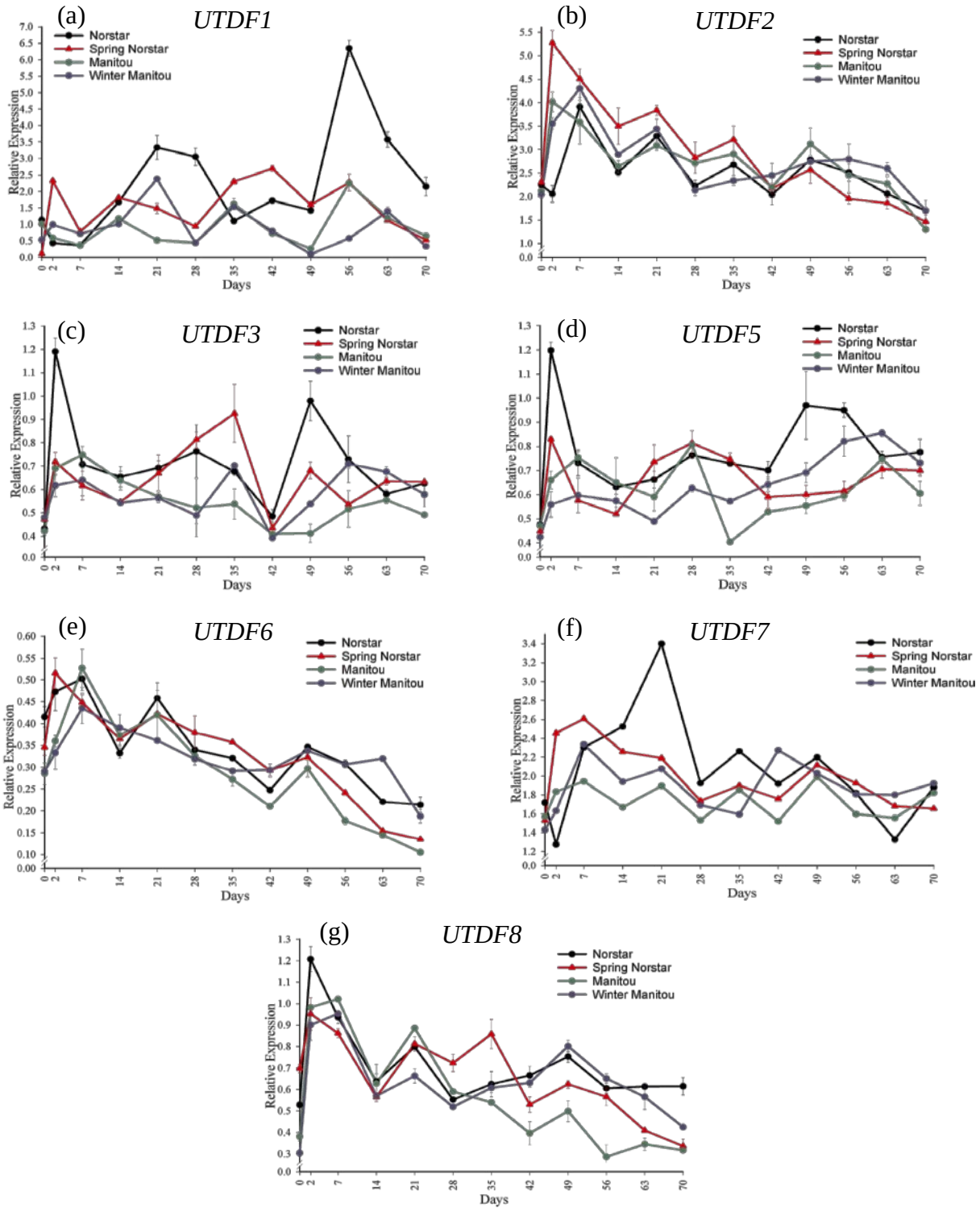

Fig.3. Relative expression patterns of (a) *UTDF1*, (b) *UTDF2*, (c) *UTDF3*, (d) *UTDF5*, (e) *UTDF6*, (f) *UTDF7*, (g) *UTDF8*, in crown tissues of wheat plants exposed to LT.

Conclusions

Crown tissues of wheat plants undergo a complex adaptive process during cold acclimation by changing the expression levels of several genes. cDNA-AFLP profiling is useful in identifying differentially expressed transcripts including novel transcripts in wheat plants. QPCR analyses of novel TDFs validated their differential expression patterns during cold acclimation in crown tissues. Characterization of the biological function of these novel genes will contribute to an understanding of the role of these novel genes in LT tolerance in wheat.

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