Expression Analysis of Low Temperature-Induced Genes in Wheat

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Key Words: cold-regulated genes, expression profiling, winter wheat

Abstract

Wheat (*Triticum aestivum* L.) is a widely adapted, economically important crop exhibiting winter, spring and intermediate growth habits. Winter wheat is seeded in the fall, over-winters, resumes growth in spring and is harvested in early summer. It also requires a period of low temperature (LT) exposure, experienced during the fall, to switch from the vegetative to reproductive phase in spring, a process known as vernalization. Low temperature also allows the wheat plant to cold-acclimate to withstand freezing winter temperatures. There has always been an interest to grow winter wheat because of its yield advantage over spring wheat. However, LT tolerance needs to be improved to prevent winter kill and maximize its yield potential. To achieve this more detailed understanding of molecular mechanisms underlying LT tolerance is required. Thus, objectives of this study were to determine the expression of a LT-induced gene and cDNA-AFLP profile in leaf and crown tissues of LT-exposed wheat plants. Survival of crown tissues after exposure to sub-zero temperatures is an indication of the level of LT tolerance of a cultivar. Thus, pattern and levels of expression of LT-induced genes and identification of LT-induced transcripts in this tissue will add to understanding of LT tolerance. Genotypes used in this study included a winter hardy cultivar, Norstar, a tender spring cultivar, Manitou and two-near-isogenic lines with the *Vrn-A1* (spring Norstar) and *vrn-A1* (winter Manitou) alleles of Manitou and Norstar, respectively. The dominant *Vrn-A1* locus confers spring habit and therefore no requirement for vernalization. Quantitative real-time polymerase chain reaction (QPCR) for the cold-regulated gene, *Wcor410*, indicated that in leaf tissue the Vrn-A1 locus determined level of expression, being higher in the lines having the recessive *vrn-A1* allele compared to the dominant *Vrn-A1* allele lines. In the crown tissue, the Norstar genetic background led to the higher level of expression than in the Manitou background. cDNA-AFLP analysis also exhibited variable profiles between the two tissues.

Introduction

Wheat (*Triticum aestivum* L.) has a widely adapted growth habit, including the spring, intermediate (facultative) and winter types. Winter wheat is higher yielding than spring wheat and therefore returns on production of winter wheat are more significant. Furthermore winter wheat is capable of better utilizing moisture, reduces soil erosion, competes better with weeds thereby reducing use of herbicides, matures early in summer avoiding late season frost and also avoids diseases (Fowler, 2002). However one of the deterrents to growing winter wheat is winter kill, which leads to reduction in crop stand and therefore yield. Therefore enhancing the low temperature (LT) tolerance of winter wheat will allow for more extensive cultivation. Over the years emphasis has been placed on management practices to maximize winter wheat yield and minimize damage resulting from LT exposure (Entz and Fowler, 1991). However these

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management practices only allow for some limited gains in winter wheat production. Further benefits to enhancing winter wheat production and reducing winter kill is likely to come from a better understanding of the LT tolerance mechanisms at the molecular level, and thereby allow for development of breeding approaches maximizing LT tolerance potential in new varieties.

Winter wheat is seeded in the fall, over-winters, resumes growth in spring and is harvested in early summer. The low temperature experienced during the fall allows the winter wheat plant to cold acclimate, a process during which winter wheat acquires freezing tolerance to withstand the onset of sub-zero temperatures. During this process of cold acclimation a series of physiological and biochemical changes occur in the plant (Fowler *et al.*, 1999) and constitute concerted interactions among networks and pathways at the molecular level (e.g., Fowler *et al.*, 2001; Mahfoozi *et al.*, 2001; Fowler and Limin, 2004; Chinnusamy *et al.*, 2007). In recent years the involvement of a number of genes of these networks and pathways has been implicated in the development of LT tolerance. The induction of the cold-regulated (*COR*) genes upon exposure to low non-freezing temperatures, for example, has been attributed to LT tolerance potential (Thomashow, 1999; Thomashow, 2001). Similarly, the genes coding for the CBF transcription factors have been shown to be up-regulated in response to LT exposure (Thomashow *et al.*, 2001; Van Buskirk and Thomashow, 2006; Galiba *et al.*, 2009).

In addition to the LT acclimation process during the fall season, this period of LT also fulfills the requirement of the winter wheat plant to switch from vegetative to reproductive phase in the spring, a process known as vernalization. The winter and spring habit in wheat is determined by the recessive *vrn-A1* locus and the dominant *Vrn-A1* locus, respectively, and is also known to influence the LT tolerance levels (Sutka *et al.*, 1999; Sutka, 2001). The vernalization locus has also been tightly linked to frost tolerance genes (Sutka and Snape, 1989; Galiba *et al.*, 1995; Storlie *et al.*, 1998; Limin and Fowler, 2002; Koemel *et al.*, 2004; Prásil *et al.*, 2005; Baga *et al.*, 2007). It is therefore important to understand the expression of *COR* genes and other LT-induced transcripts with regards to the wheat growth habit. To this end, near-isogenic lines (NILs) for the Vrn-A1 locus have been developed using the spring habit cv. Manitou and the winter habit cv. Norstar (Limin and Fowler, 2002). Thus the objectives of this study were to determine the quantitative expression of a LT-induced gene, *Wcor410*, and the cDNA-AFLP profiles in wheat leaf and crown tissues exposed to LT. Generally expression of LT-induced genes has been assessed in leaf tissues. However, the crown, which represents the survival of the wheat plant at the on set of spring, is likely to have patterns of expression of the LT-induced genes different from the ones in the leaf tissues. It was recently shown that was indeed the case when comparing the expression patterns of some *COR* genes in these tissues from wheat plants cold acclimated for 0 to 98 days (Ganeshan *et al.*, 2008).

Materials and Methods

Genetic materials and LT acclimation

The genotypes used in this study have previously been studied for their responses to LT exposure (Limin and Fowler, 2002; Ganeshan *et al.*, 2008). Briefly, reciprocal backcrosses between the winter habit Norstar and the spring habit Manitou were performed to produce near-isogenic lines (NILs) with the dominant *Vrn-A1* locus from Manitou in the Norstar genetic background and the recessive *vrn-A1* locus from winter Norstar in the Manitou genetic background. Thus the NILs

represented a spring Norstar and a winter Manitou, with altered vernalization requirements compared to their respective parents. The four genotypes were cold-acclimated for 70 days as previously described (Limin and Fowler, 2002; Fowler and Limin, 2004; Ganeshan *et al.*, 2008).

RNA Extraction and cDNA Synthesis

Total RNA from frozen leaf and crown tissues was extracted using a modified Trizol^{TM} (Invitrogen, Inc., Burlington, Ontario, Canada) method (Ganeshan *et al.*, 2008), quantified 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 real-time PCR 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, 1X Quantitect$^{®}$ SYBR$^{®}$ Green I Master Mix and 30 nM ROX dye (Qiagen, Inc., Mississauga, Ontario, Canada) in a 25 µL reaction volume with appropriate primers for the *COR* genes. The *Ubiquitin* gene was used as reference gene.

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 oligodT$_{(12-18)}$ 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 Digest, Ligation and PCR

cDNA was quantified and 1 µg was digested with 5U of EcoRI and MseI. EcoRI and MseI adapters were ligated to the digested cDNA and 10 µL of the ligation mix was used for pre-selective amplification using EcoRI+A primer (5'-GACTGCGTACCAATTCA-3') and MseI+C primer (5'-GATGAGTCCTGAGTAAC-3'). A 1/10 dilution of the pre-selective reaction was used for selective amplification. Standard EcoRI and MseI AFLP primers were used for AFLP analysis (Vos *et al.*, 1995). Selective amplification products were resolved on a 6% denaturing polyacrylamide gel and visualized by silver staining (Båga *et al.*, 2007). Transcript-derived fragments (TDFs) of interest were excised from the gel, re-amplified, sub-cloned and DNA sequenced.

Results and Discussion

Wcor410 Expression in Leaf and Crown Tissues

The expression patterns of *COR* genes in leaf and crown tissues from wheat plants exposed to LT is important to gain better insight on the perception of LT in these two tissues. Our study reveals that while in the leaf tissues the Vrn-A1 locus determines expression of the *COR* gene, in crown tissues the genetic background influences its expression (Figure 1). Thus winter Norstar and

winter Manitou, both possessing the recessive *vrn-A1* allele showed highest expression after two days of exposure to LT compared to the spring Manitou and spring Norstar.
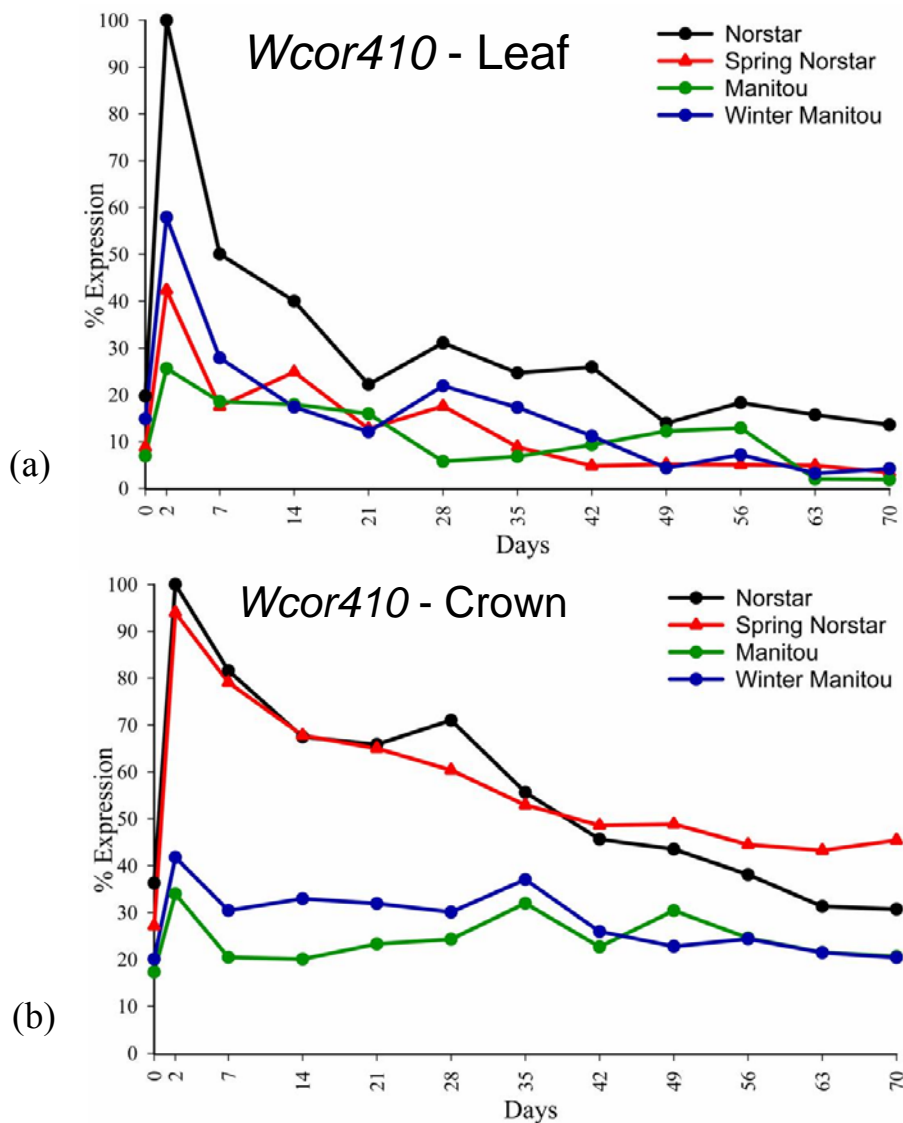

Figure 1. Relative expression patterns of *Wcor410* in leaf and crown tissues of spring and winter wheat plants exposed to LT. (a) Expression in leaf tissues. (b) Expression in crown tissues. (Adapted from Ganeshan et al., 2008)

The peak in expression observed at two days indicates that the *COR* gene was a rapidly induced upon exposure of the plant to LT as previously reported (Ganeshan *et al.*, 2008). Studies have also shown the influence of the circadian clock on LT responses in *Arabidopsis* (Michael *et al.*, 2003). Therefore the expression of the *Wcor410* gene was studied in winter Norstar and spring Manitou exposed to LT over three days and sampling done at various time intervals over each of 24 h periods. Again, it was observed that there was a difference in expression pattern of *Wcor410* in leaf and crown tissues, with a circadian influence present in the leaf and absent in the

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crown tissues (Figure 2). In the crown, the LT exposure was solely responsible for the increase in the expression of the *Wcor410*. In both tissues, there is a coincident peak at 2 d, as observed earlier (Figure 1). This difference in expression between these two tissues is likely due to influence of light on the leaves, since the crown tissue is below ground.
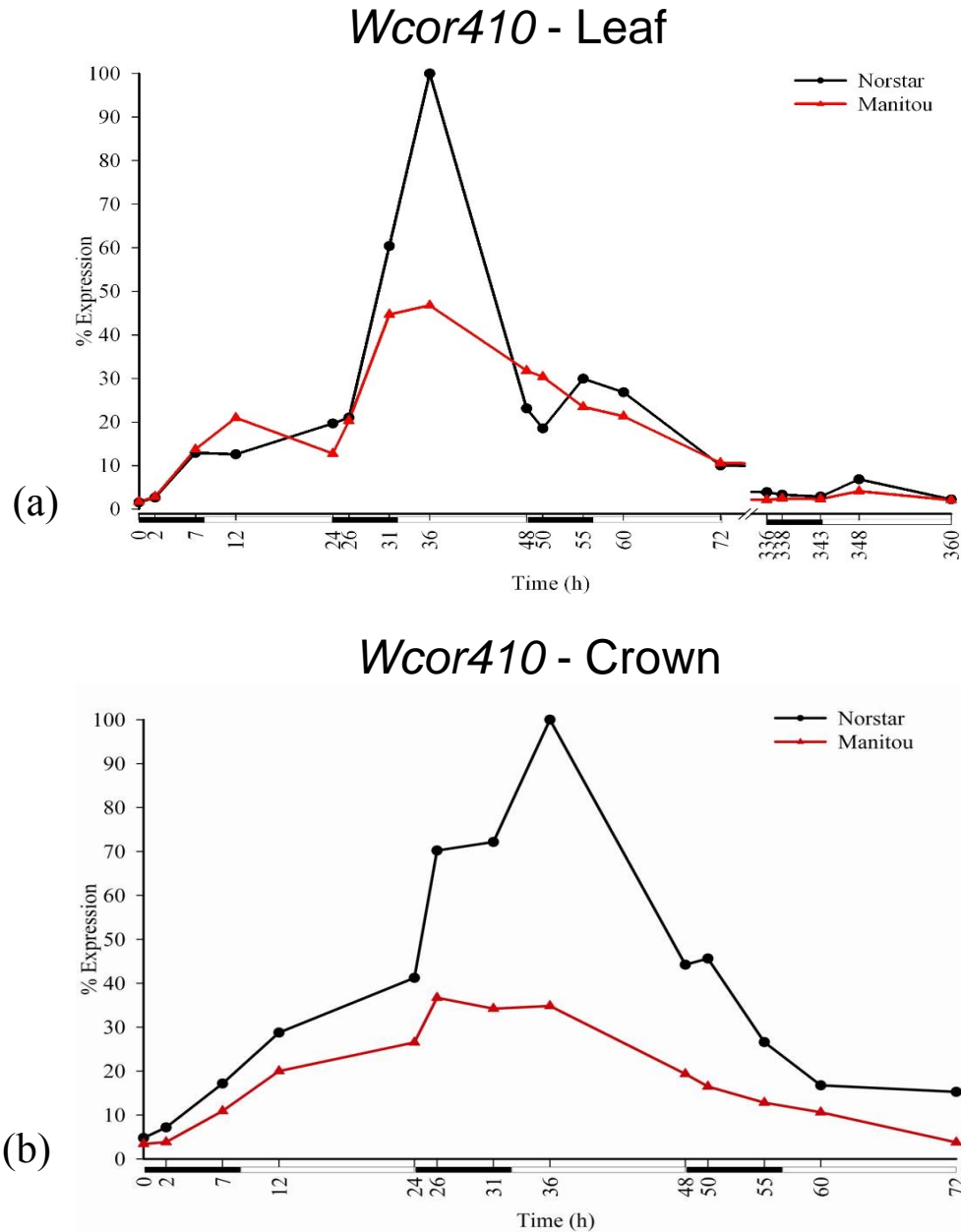
Wcor410 - Leaf

(a)

Wcor410 - Crown

(b)

Figure 2. Expression pattern of *Wcor410* in leaf and crown tissues of winter and spring wheat genotypes over three- day exposure to LT. (a) Expression in leaf tissues. (b) Expression in crown tissues. Black and white rectangles below x-axes indicate dark (8h) and light (16h) cycles, respectively.

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cDNA-AFLP Profiling

The cDNA-AFLP profiling is in principle, similar to conventional AFLP. Messenger RNA is reverse-transcribed into cDNA for AFLP analysis. Thus, differentially expressed transcripts can be detected from LT-exposed wheat plants. With regards to LT-induced gene expression, cDNA-AFLP can allow identification of transcripts expressed in temporal, spatial, developmental and sensorial conjectures. Previous studies employing cDNA-AFLP showed that wheat plants exposed to a LT had differentially expressed transcripts, some absent prior to exposure to LT (Figure 3) (Ganeshan *et al.*, 2007). Since differentially expressed transcripts were detected in short duration LT exposure, informative transcript profiles were expected in long duration LT exposed plants.
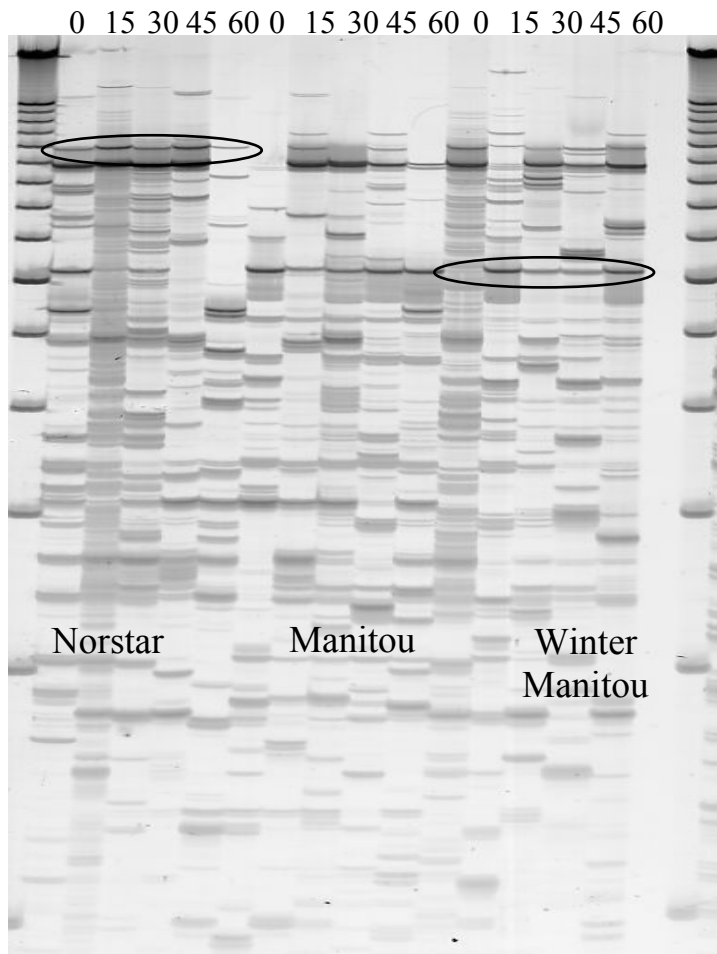

Figure 3. cDNA-AFLP profile of leaf tissues from wheat plants exposed to LT from 0 to 60 min. Differentially expressed TDFs are circled.

Similar to the QPCR expression analysis in leaf and crown tissues, the expression profiles of long-term LT-acclimated wheat plants were different for the leaf and crown tissues (Figure 4). Several of the TDFs present in the crown tissue profile were not detected in the leaf tissue profile (Figure 4). After screening three primer combinations, 144 TDFs were identified from the crown

tissue profiles (Table 1). Eight of these TDFs have been sequenced and one of them has shown 90% identity to a rice enolase gene. It has been reported that this gene acts downstream of the *CBF* genes as a transcriptional activator of cold-regulated genes in *Arabidopsis* (Lee *et al.*, 2002). In rice roots enolase has been found to be up-regulated in response to exposure at $10^{o}C$ (Lee *et al.*, 2009). Profiles from leaf tissues showed 644 TDFs after screening 64 primer combinations (Table 2). From these preliminary data, it is likely that more informative TDFs will be identified from crown tissue profiles than from leaf tissue profiles. Sequence information from the TDFs from the leaf profiles indicated only 18% were related to stress tolerance (Young *et al.*, 2008) and surprisingly none were related to LT tolerance. This was probably due to the PCR having reached saturation after 35 cycles would have precluded identification of differentially expressed LT-related transcripts, since these would have appeared as constitutively expressed transcripts of similar intensities on the DNA sequencing gels. This can be prevented by analyzing transcript profiles after 25-30 cycles.
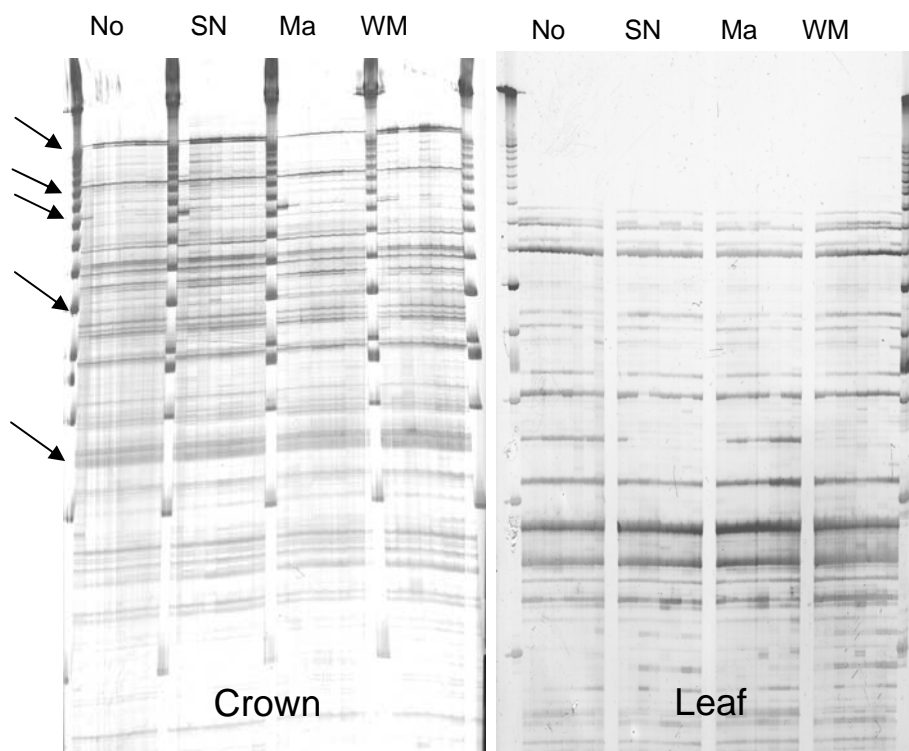

Figure 4. cDNA-AFLP profiles from LT-exposed leaf and crown tissue. Arrows indicates transcripts unique to crown tissues. No – winter Norstar; SN – spring Norstar; Ma – spring Manitou; WM – winter Manitou

Table 1. Differentially expressed transcripts from crown tissues after screening three primer combinations. No – winter Norstar; SN – spring Norstar; Ma – spring Manitou; WM – winter Manitou

Genotypes	No	SN	Ma	WM
Up-regulated TDFs	2	31	27	31
Down-regulated TDFs	5	5	3	4
Unique TDFs	1	3	3	3
Total TDFs (144)	34	39	33	38

Table 2. Differentially expressed transcripts from leaf tissues after screening 64 primer combinations. No – winter Norstar; SN – spring Norstar; Ma – spring Manitou; WM – winter Manitou

Genotypes	No	SN	Ma	WM
Up-regulated TDFs	151	135	128	129
Down-regulated TDFs	18	33	28	16
Unique TDFs	4	0	2	0
Total TDFs (644)	173	168	158	145

Conclusions

LT tolerance in wheat is a complex quantitative trait and dissecting the underlying molecular mechanisms will enable development of winter wheat varieties with enhanced LT tolerance. The present study has indicated that tissue selection and sampling for LT tolerance experiments is important. The differential expression observed in leaf and crown tissues in the QPCR and the cDNA-AFLP profiling attest to the need for such a selection. Similarly, the time of sampling needs to be consistent as the circadian influence also affects the expression f the LT-induced genes in these two tissues.

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