cDNA-AFLP profiling of low-temperature-induced transcripts in winter and spring wheats

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ABSTRACT

The objective of this study was to determine temporal expression profiles of transcripts during cold-acclimation of wheat plants using cDNA-AFLP. A winter hardy cultivar, Norstar, a tender spring cultivar, Manitou and two near-isogenic lines (NILs) with the *Vrn-A1* (spring Norstar) and *vrn-A1* (winter Manitou) alleles of Manitou and Norstar were used in these studies Plants were grown at 6^{o}C and leaves were collected at specific time intervals during early stages (0, 2, 14, 21, 35, 42, 56 and 70 days) of cold acclimation. Total RNA was extracted, from which mRNA was isolated and reverse transcribed prior to making double stranded cDNA. cDNA was digested with EcoRI and MseI, and after ligation of EcoRI and MseI adaptors, pre-amplification was performed. After selective amplification, PCR products were resolved on a PAGE gel. Differentially expressed transcripts were excised, re-amplified and sub-cloned for sequencing. After screening 64 primer combinations, 548 transcript-derived fragments (TDFs) were identified. One hundred and seventy-two of these TDFs have been sequenced and annotated. BLASTX search results indicate that about 27% of the TDFs code for unknown proteins which may be of interest for further studies.

INTRODUCTION

Transcriptome profiling has become an integral part of functional genomics studies for gene expression and discovery in various biological systems, particularly with regard to spatially, temporally and developmentally controlled cascades of genes. Different profiling methodologies have their own merits and demerits. Most approaches attempt to obtain a snapshot of global gene expression patterns within specific cell types, tissues, organs or treatments.

Since the discovery of low temperature (LT)-induced gene expression changes in spinach (Guy et al., 1985), understanding of the LT tolerance pathway has progressed significantly, especially with the elucidation of the CBF/DREB pathway during cold acclimation in *Arabidopsis* (Thomashow et al., 2001; Thomashow 2001). In cereals, extensive research has been conducted to dissect the LT acclimation/tolerance pathways. However, understanding LT tolerance in cereals has been more challenging, primarily due to complex genome organization, gene networks and interactions. Many important LT-induced genes have been cloned, but most of the upstream and downstream events due to LT exposure are yet to be fully elucidated. The objective of this study was to use the cDNA-AFLP technique to study the expression profiles of wheat plants under extended LT exposure. The wheat genotypes used included a winter wheat cv. Norstar, a spring wheat cv. Manitou and near-isogenic lines spring Norstar and winter Manitou (Limin and Fowler 2002). Since the chromosome segment determining winter/spring habit is the only difference among the parental line and its corresponding NIL, differentially expressed transcripts from cDNA-AFLP profiles will give an indication of LT response patterns affected by the *Vrn-A1* locus and/or its interactions with other loci/regions. Furthermore, the cDNA-AFLP can potentially lead to gene discovery, since no *a priori* sequence knowledge of the amplification profiles are known.

The cDNA-AFLP technique is in principle, similar to the conventional AFLP except that messenger RNA is the starting material and is reverse transcribed into cDNA for AFLP analysis. Thus, differentially expressed transcripts can be detected from the variously treated wheat plants. In terms of LT gene expression, cDNA-AFLP would allow identification of differentially expressed transcripts as a function of time, temperature and developmental stage. In previous studies using the cDNA-AFLP technique, winter wheat plants exposed to a LT treatment for 0, 15, 30, 45 and 60 minutes showed differentially expressed transcripts, some absent prior to exposure to LT treatments (Ganeshan et al., 2007). The increase or decrease in the intensity of some of the transcripts also enabled a quantitative cDNA-AFLP approach to be used, as demonstrated for cell cycle-modulated genes in tobacco (Breyne et al., 2003). Since differentially expressed transcripts were detected in short duration cold treatments, informative transcript profiles are also expected in experimental genetic stocks used in this study. Using cloned LT-responsive genes as probes, we have also shown that products from the transcript profiles corresponded to coding sequences (Ganeshan et al., 2007).

MATERIALS AND METHODS

Genetic stocks and cold acclimation

The genotypes used in this study have previously been characterized for their responses to LT exposure (Limin and Fowler 2002). Briefly, the winter habit Norstar and

the spring habit Manitou were used in reciprocal backcrosses in order to produce near-isogenic lines, such that the dominant *Vrn-A1* locus from Manitou was transferred to Norstar to produce a spring Norstar and the recessive *vrn-A1* locus from Norstar was transferred to Manitou to produce winter Manitou.

The four genotypes used in this study were cold acclimated as previously described (Limin and Fowler 2002; Fowler and Limin 2004; Ganeshan et al., 2008). Briefly, seedlings grown hydroponically in continuously aerated half-strength Hoagland's solution at 20°C up to 2-3 leaf stage (about 15 days old), were transferred to a growth chamber at 6°C under a 16 h photoperiod and 250 μmol m^{-2} s^{-1} PPFD. Leaves were collected at intervals of time (0, 2, 14, 21, 35, 42, 56, 70 days) from the plants and frozen at -80°C for total RNA extraction. LT_{50} values (temperature at which 50% of the plants are killed) have also been determined at each time interval as previously reported (Ganeshan et al., 2008).

Total RNA from frozen leaf tissues was extracted using a modified Trizol™ (Invitrogen, Inc., Burlington, Ontario, Canada) method (Ganeshan et al., 2008). Total RNA was 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 checked on a Bioanalyzer 2100 (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada). mRNA was isolated from the cleaned total RNA using the PolyATtract mRNA isolation kit (Promega Corporation, Madison, Wisconsin, USA) according to manufacturer's instructions. First strand cDNA was synthesized from 1 μg of mRNA using 1 μg $oligodT_{(12-18)}$ and Superscript III (Invitrogen, Inc., Burlington, Ontario, Canada) according to manufacturer's instructions. Second strand cDNA was synthesized, after treatment of first strand cDNA with RNAse H, using DNA polymerase I, T4 DNA ligase and deoxynucleotide triphosphates.

The double stranded cDNA was cleaned using the Purelink PCR purification kit (Invitrogen, Inc., Burlington, Ontario, Canada) according to manufacturer's instructions. cDNA was quantified and 1 μg was digested with 5U of EcoRI and MseI. EcoRI and MseI adapters were then ligated to the digested cDNA. Ten uL of the ligation mix was used for pre-selective amplification using EcoRI+A primer (5'-GACTGCGTACCAATTCA-3') and MseI primer (5'-GATGAGTCCTGAGTAAC-3'). A 1/10 dilution of the pre-selective reaction was used for selective amplification. Standard EcoRI and MseI AFLP primers as originally reported were used with three selective nucleotides (Vos et al., 1995). Selective amplification products were resolved on a 6% denaturing polyacrylamide gel and visualized by silver staining (Baga et al., 2007). Transcript-derived fragments (TDFs) of interest were excised from the gel, re-amplified with the respective primer combinations and sub-cloned after elution from an agarose gel for sequencing.

RESULTS AND DISCUSSION

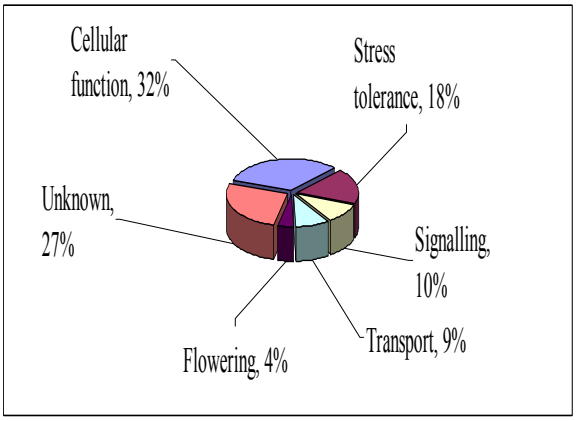
A number of LT-induced genes in wheat have been sequenced and characterized. However, our understanding of LT tolerance is still yet to be fully realized. Detailed quantitative expression patterns of several LT-induced genes in the wheat genotypes used in the present study have been reported under long duration acclimation conditions (Ganeshan et al., 2008). The study revealed that differences in expression of the LT-induced genes were variable and the roles of these genes were subject to varying interpretations depending on tissues or times during acclimation. In order to gain more insight into the expression profiles of these wheat genotypes, the cDNA-AFLP approach was used. A summary of the results is presented in Table 1 and Figure 1. While the expression patterns were highly variable among the four genotypes and times during acclimation, TDFs unique to any of the four genotypes were not clearly discernible. This could be due to expression actually occurring in all the genotypes irrespective of their LT tolerance. Since the amplification products were resolved at the end of 35 cycles, the PCR would have reached saturation and therefore differential expression among the genotypes would not have been detected. This could explain the non-detection of dehydrins and other cold-regulated (*COR*) transcripts from the sequenced TDFs, as these would have been assumed to be constitutive products on the gels due to their occurrence in all the genotypes. Resolution of amplified products prior to saturation phase would enable detection of differential expression.

Table 1. Summary of cDNA-AFLP results using 16 standard AFLP primers in all combinations. The primers used were: E32, E33, E35, E36, E37, E38, E40, E41, M47, M48, M49, M50, M59, M60, M61 and M62.

Primer combinations used	64
Number of TDFs (number of fragments excised from gel)	548
Putative genes (1 band of the same size in different genotypes = 1 gene)	172
Blastx hits	355
Unique blastx hits	74

Among the TDFs that were selected based on apparent occurrence in genotypes at certain times, BLASTX hits indicated 27% unknown and 18% stress-related protein

(Fig. 1). Again the non-representation of COR proteins attests to the PCR saturation precluding differential detection. Further studies will be conducted to specifically address this differential expression by



resolving the amplified products after different number of cycles during the PCR. Nonetheless the present study has given an indication that the cDNA-AFLP may be of value in expression profiling of wheat genotypes for LT-induced transcripts. The high percentage of unknown proteins indicates that in terms of gene discovery the cDNA-AFLP may be a valuable technique. Further studies are in progress to characterize these unknown TDFs and also to resolve amplification products after different number of cycles, preferably coinciding with the exponential phase of amplification.

ACKNOWLEDGEMENTS

Financial support from Genome Canada/Genome Prairie (DBF and RNC), Ducks Unlimited (DBF), Canada Research Chairs (RNC) and Canada Foundation for Innovation (RNC) is gratefully acknowledged. The excellent technical assistance of Carli Holderness, Garcia Schellhorn and Twyla Chastain is greatly appreciated.

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