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Differential gene expression in response to cold stress in *Lepidium apetalum* during seedling emergence

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Abstract

Germination of *Lepidium apetalum* Wild. seeds is invariably arrested by cold stress. cDNA-amplified fragment length polymorphism (AFLP) technique was used to isolate genes relevant to chilling stress (4 °C) during seedling emergence. 43 transcript-derived fragments (TDFs) were found to be up-regulated and 17 down-regulated during chilling stress. Eighteen TDF of up-regulated genes were cloned and sequenced. Some of these genes are involved in the stress response, some play important roles in energy and substrate metabolism, and some encode unknown proteins such as TDF119. Two sequences, designated TDF217 and TDF223, may correspond to novel genes. The expression profiles of 6 from 18 TDFs were analyzed by quantitative real-time PCR under chilling and abscisic acid (ABA) stress. It was demonstrated that all 6 genes were significantly induced by chilling and their expression was decreased when the temperature was shifted from 4 to 25 °C. The transcriptional levels of 5 TDFs were strongly enhanced also in response to exogenous ABA. Based on the characteristics of genes isolated from seedlings exposed to cold stress, we conclude that *Lepidium* adapts to cold stress by regulating many signal transduction pathways, including both ABA-dependent and ABA-independent signaling pathways.

Additional key words: AFLP, chilling, quantitative real-time PCR.

Introduction

Lepidium (*Lepidium apetalum* Wild.), a member of *Cruciferae*, is distributed throughout Asia and Europe (Zhou and Guo 1987). It is a traditional Chinese herb with the effects on relieving asthma, inducing diuresis and diminishing swelling (Xiao 2002). Lepidium is a of spring ephemeral plants that could germinate under low temperatures. Therefore it is regarded as a good material to study the cold tolerance mechanisms during plant germination (Zhao *et al.* 2010).

To survive stresses, efficient resistance is required for the plants (Hu *et al*. 2010). The tolerance of plants to low temperature during seedling emergence is mainly determined by genetic factors that were selected during plant evolution in existing environments (Ismail and Hall 2002). Acclimation to cold stress involves a number of biochemical and physiological changes, including increased contents of soluble sugars, proteins, amino acids and organic acids, as well as modifications in membrane lipid composition (Guy 1990, Walker *et al.* 2010). Many of these changes are regulated by the altered expression of genes encoding lipid transfer proteins, late-embryogenesis-abundant proteins, alcohol dehydrogenase, translation elongation factor, and other proteins with unknown functions (Thomashow 1999, Seki *et al.* 2001, 2002, Fowler and Thomashow 2002). For example, the overproduction of glycine betaine (GB) could enhance cold-stress tolerance of wheat by protecting plasma membranes (Zhang *et al.* 2010). Although it is recognized that the mechanisms by which plants cope with low-temperature stress are complex and multigenic (Hughes and Dunn 1996, Thomashow 1998), the complex molecular changes that occur after cold stress remain unclear.

Gene expression profiles can be obtained and

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Abbreviations: AFLP - amplified fragment length polymorphism; qRT-PCR - quantitative real-time polymerase chain reaction; TDFs - transcript-derived fragments
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compared using various methods (Donson *et al.* 2002). cDNA-amplified fragment length polymorphism (AFLP) is an efficient, sensitive, and reproducible technique for the isolation of differentially expressed genes (Bachem *et al.* 1996, Vuylsteke *et al.* 2006). In this study, this method was used to characterize and analyze gene expression during *Lepidium* seedling emergence under 4 °C and to identify genes and pathways that may play a role in chilling tolerance. The resulting survey of overall differential gene expression patterns in *Lepidium* to cold stress provides deeper insight into gene function and a more comprehensive knowledge of the complex processes that occur during the environmental responses of these and other ephemeral plants during seedling emergence.

Materials and methods

Lepidium apetalum Wild. seeds were collected from Liyu Mountain in Urumqi, Xinjiang Uygur Autonomous Region and kept at 4 °C after drying at room temperature. Sterilized seeds were sown in half-strength Murashige and Skoog (MS) medium and stored in the cold (4 °C) for 2 d for stratification. Then the seeds were allowed to germinate at 25 °C in a plant growth room for 60 h, at which time cotyledons emerged from the seed capsule.

For cold treatment, seedlings were exposed to temperature of 4 °C for 3 and 6 h, respectively. The control plants were kept at 25 °C. All treatments were done under the same irradiance of 14.5 W m^{-2}.

An additional experiment involving quantitative real-time polymerase chain reaction (qRT-PCR) was conducted. After the cold exposure for different time periods (1, 3, 6, 12, and 24 h), total RNA was isolated from half of the seedlings in each sample. The other half of the seedlings in each sample was treated at 25 °C for 6 h before total RNA isolation. Some additional plants were treated with ABA (100 μM) (Cheng *et al.* 2009a,b) for 0, 1 or 6 h, and then were used for total RNA isolation.

Total RNA was isolated from about 100 mg of fresh seedlings using the *Trizol*™ extraction method (*Invitrogen*, Carlsbad, CA, USA). Double-stranded cDNA was synthesized using the *M-MLV* RTase cDNA synthesis kit (*TaKaRa*, Kyoto, Japan) from 3 μg total RNA according to the manufacturer's instructions.

About 500 ng of double-stranded cDNA was subjected to standard AFLP template production using the method of Vos *et al.* (1995). The restriction enzymes used for digestion of cDNA were *Eco*RI and *Mse*I (*New England Biolabs*, Beverly, MA, USA). The digested products were ligated to adapters with sequences as follows: *Eco*RI adapter, 5′-CTCGTAGACTGCGTACC-3′, 3′-CTGACGCATGGTTAA-5′; *Mse*I adapter, 5′-GAC GATGAGTCCTGAG-3′, 3′-TACTCAGGACTCAT-5′. The ligated products were pre-amplified using the corresponding pre-amplification primers (*Eco*RI: 5′-GACTGCGTACCAATTC-3′, *Mse*I: 5′-GATGAG TCCTGAGTAA-3′). Preamplified products were amplified with 64 combination primers corresponding to selective nucleotides at the 3` end (*Eco*RI: AA, AC, AT, AG, CA, CC, CTCG; *Mse*I: CC, CG, CA, CT, TA, TC, TG, TT). The AFLP products were analyzed on 8 % polyacrylamide gels in TBE electrophoresis buffer. The gels were silver stained using silver nitrate according to Guo *et al*. (2006).

Polymorphic transcript-derived fragments (TDFs), identified based on their presence, absence or differential intensity, were cut from the gel and eluted. Eluted DNA (0.005 cm^3) was amplified in the pre-amplification PCR cycle described above, using the same primers that were used for the pre-amplification reaction of cDNA-AFLP analysis. The PCR products which ranged from 100 to 300 bp were isolated, eluted and ligated into the *pMD18-T* simple vector (*TaKaRa*). After the isolated cold-induced TDFs were cloned, each was sequenced and its distinct nucleotide sequence was subjected to a homology search of the *NCBI* database using *BLAST*.

qRT-PCR using *Power SYBR* green dye (*Applied Biosystems*, Shanghai, China) was performed to select TDFs representing unknown genes that may play a role in cold responses. The elongation factor 1-*alpha*, a constitutive protein, was used as an internal control. PCR was performed in triplicate for each sample. The amount of transcript of each gene of interest normalized to that of the internal control gene was analyzed using the 2-Delta DeltaC(T) method (Livak and Schmittgen 2001). The primers used for qRT-PCR were designed based on the selected sequences of the TDFs using *Primer Express v. 3.0* (Table 1).

Results and discussion

Identification of cold-induced transcripts: All 64 selected primer combinations were used for AFLP analysis of the cDNA from *Lepidium* seedlings at three different stages. A representative silver-stained cDNA-AFLP gel with clear and unambiguous bands (TDFs) is shown in Fig. 1. A total of 217 TDFs were isolated based on their presence/absence (qualitative variants) or on differences in their levels of expression (quantitative

Table 1. Primers used for real-time PCR amplification and the resulting amplicon size.

TDFS	Forward primers 5′-3′	Reverse primers 5′-3′	Products length [bp]
ef1	CAAGGCTAGGTACGAT	CAATCATGTTGTCTCCCT	119
107	CGGAACAGTAGATGACA	TAGTTCATGCCTGATGTC	125
119	GTTCAATGTCATCTTCTTC	GTGAATACCATAGCAGTC	109
217	TAACCACCAAATCACACC	ACTGTGAAAATGCTCATA	98
223	TGAGATGCATGATTGC	TAGTAGAAACAGAACT	127
205	CCAAAGAACTCTAGAGA	TGAAAGGTGTCTCAGCTGT	105
120	CACGACTATATATAGCAC	CCGTTGTGAGTAGACAAA	164

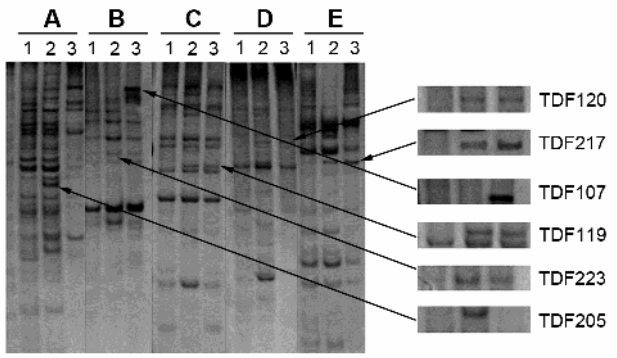

Fig. 1. Partial detail of a silver-stained cDNA-AFLP gel showing the differential expression of genes detected with 5 of 64 primer combinations. Six TDFs isolated from cDNA-AFLP were selected for qRT-PCR analysis. 1 - seedling chilled at 4 °C for 0 h; 2 - seedling chilled at 4 °C for 3 h; 3 - seedling chilled at 4 °C for 6 h. A, B, C, D, E - cDNA-AFLP with different primer combinations. *Arrows* designate TDFs that differ significantly in expression after chilling and that were selected for analysis expression profiling.

variants). Of 217 TDFs, we found 60 based on qualitative expression differences and 157 that showed quantitative differences in expression. Of these 60 TDFs, 43 were present in cold treated plants and 17 were present in control plants.

Sequencing and sequence analysis: In our study, 18 expected differentially expressed TDFs were successfully eluted from polyacrylamide gels, reamplified and sequenced. The results of databank searching (Table 2) revealed that these TDFs are related to other genes involved in stress responses. The biological role of some of the cloned genes can be inferred from their sequence similarities to genes that code for previously studied proteins of both plant and non-plant origin. We found that some of the cloned genes are involved in stress responses and some play important roles in energy and substrate metabolism. However, the majority of the cold-induced or repressed genes encode proteins whose roles cannot be predicted from their primary amino acid sequences; the biological activity of these proteins remains to be determined. Two of the sequences, TDF217 and TDF223, resulted in few hits, suggesting that they may correspond to novel genes.

TDF102 encodes a putative protein that shows high sequence similarity to the photosystem 2 (PS 2) polypeptide. It has been reported that exposure to low temperature results in the decomposition of PS 2 D1 protein, decrease in electron tranport activity and so depression of PS 2 activity (Allen and Ort 2001, Liu *et al.* 2002, Van Heerden *et al.* 2003, Strauss *et al.* 2006). TDF102 enhanced translation may help *Lepidium* seedlings adjust photosynthesis under cold stress.

TDF105 showed 91 % sequence similarity with eukaryotic translation initiation factor 3E (EIF3E). The eIF3 is the largest of 12 known translation initiation factors. It associates with the 40S ribosome and stimulates the binding of Met-tRNA$_i^{Met}$ in partial reactions reconstituted *in vitro* with purified mammalian eIFs (Trachsel and Staehelin 1979). Recent reports show that eIF3 is a five-lobed particle, an 750-kDa complex that controls the assembly of 40S ribosomal subunits on messenger RNAs (mRNAs) bearing either a 5'-cap or an internal ribosome entry site (IRES) and promotes initiation complex assembly (Siridechadilok *et al.* 2005, Baird *et al.* 2007, Zhou *et al.* 2008). TDF105 was up-regulated significantly when *Lepidium* seedlings were exposed to 4 °C; this implies that eIF3 plays an important potential role during low-temperature stress. However, the details of its role in translation initiation under cold conditions are still unknown.

Glutaredoxin was first identified by Holmgren (1976). Recent progress in our understanding of the glutaredoxin system has linked redox potential in cells to the regulation of multitude processes, including sulfhydryl homeostasis, signaling transduction, cell apoptosis and cell proliferation. In plant cells under chilling, the elimination of the excess generation of reactive oxygen species (ROS) is important in plant resistance mechanisms. One of the TDFs identified in this study, TDF114, which was up-regulated when the seedlings were stressed at 4 °C for 3 or 6 h, showed homology to a gene encoding a putative glutaredoxin family protein. This implies that increased TDF114 expression may improve the ability of *Lepidium* to tolerate low-temperature stress by reducing the toxicity of ROS brought about by cold stress.

Table 2. Nucleotide homologies of the transcript-derived fragments (TDFs) to known gene sequences in the database using the *BLASTN* algorithm along with the expression pattern.

TDFs	Homology to gene (*GeneBank* accession number)	Organism	E-value	Identities
102	putative photosystem 2 polypeptide (AY084512.1)	*Arabidopsis thaliana*	7e-048	172/196 (87 %)
105	EIF3E (eukaryotic translation initiation factor 3E (NM_115589.4)	*Arabidopsis thaliana*	5e-024	84/92 (91 %)
107	adenylate kinase family protein (AL161563.2)	*Arabidopsis thaliana*	5e-011	47/50 (94 %)
112	thioesterase family protein (NM_103729.3)	*Arabidopsis thaliana*	7e-034	100/108 (92 %)
114	glutaredoxin family protein (NM_115566.3)	*Arabidopsis thaliana*	4e-017	83/92 (90 %)
117	universal stress protein (NM_180232.2)	*Arabidopsis thaliana*	3e-010	67/77 (87 %)
119	unknown protein (NM_118635.4)	*Arabidopsis thaliana*	5e-032	103/113 (91 %)
120	peroxisomal targeting signal type 1 receptor protein (NM_125012.4)	*Arabidopsis thaliana*	9e-010	52/56 (92 %)
123	SCARECROW transcriptional regulator-like (NM_124630.4)	*Arabidopsis thaliana*	3e-038	189/224 (84 %)
125	glycine dehydrogenase (decarboxylating) (NM_119455.2)	*Arabidopsis thaliana*	3e-059	161/175 (92 %)
127	armadillo/beta-catenin repeat family protein (NM_111006.2)	*Arabidopsis thaliana*	6e-026	95/103 (92 %)
205	ETT (ETTIN); transcription factor (ETT) (NM_128946.3)	*Arabidopsis thaliana*	3e-030	91/98 (92 %)
206	ribulose bisphosphate carboxylase (RBCS1A) (NM_202369.2)	*Arabidopsis thaliana*	3e-050	125/132 (94 %)
207	chitinase, putative (NM_129921.5)	*Arabidopsis thaliana*	2e-044	136/150 (90 %)
217	a novel zinc finger protein (CU464125.6)	zebrafish	0.002	28/29 (96 %)
223	mitogen-activated protein kinase 1 (EU332852.1)	*Homo sapiens*	0.041	23/23 (100 %)
228	wound-induced basic protein (NM_111603.2)	*Arabidopsis thaliana*	3e-037	109/118 (92 %)
232	basic leucine zipper transcription factor (AY322555.1)	*Cucumis sativus*	9e-019	114/135 (84 %)

A putative partial protein encoded by TDF107 has a sequence similar to those of adenylate kinase family proteins. The adenylate kinase (AK, EC 2.7.4.3) prevents marked increases in the ATP/ADP ratio at the site of energy generation and marked decreases in the ratio at the site of ATPase, doubling the efficiency of the diffusion of ATP in the transfer of energy and maintaining efficient intracellular energy flow (Dzeja and Terzic 2003). Recently, a series of studies showed that AK regulates the membrane K^+-ATP channel (Stanojevic *et al.* 2008) and ATP-binding cassette (ABC) transporter activity (Randak and Welsh 2005) and is required for extracellular ATP synthesis (Choo *et al.* 2008).

The sequence of TDF205 is highly similar to that of transcription factors (ETT). Evidence shows that overexpression of transcription factors can markedly enhance the stress tolerance of plants (Kasuga *et al.* 1999, Winicov and Bastola 1999, Kong *et al.* 2004, Yu and Tang 2004). Increased levels of transcription factors CBF1 and CBF2 and 3 induced CBF-targeted gene expression and increased low-temperature resistance in plants (Jaglo-Ottosen *et al.* 1998, Maruyama *et al.* 2004, Vogel *et al.* 2005). TDF205 was responsive to low temperature in this experiment. Further study is required to determine whether TDF205 enhances *Lepidium* seedling endurance at low temperature.

The TDF232 sequence is similar to that of the gene for basic leucine zipper transcription factor. Basic leucine zipper (bZIP) transcription factors are present exclusively in eukaryotes; they constitute one of the largest and most diverse transcription factor families. In plants, bZIP transcription factors have been reported to regulate a variety of biological processes including pathogen defense (Thurow *et al.* 2005), photomorphogenesis and light signaling (Mallappa *et al.* 2006), and seed maturation and germination (Chern *et al.* 1996). The bZIP transcription factors are characterized by the presence of a conserved bZIP domain (Hurst 1995).

The sequence of TDF119 is fairly similar to an *Arabidopsis thaliana* sequence that codes for an unknown protein. The question of whether elevated TDF119 expression is specific to chilling-resistant *Lepidium* during seedling emergence should be further explored.

The sequences of TDF217 and TDF223 are not highly similar to any sequences in the *NCBI* database. However, TDF217 possesses a small degree of homology to a gene for a novel zinc finger protein. It is reported that zinc finger protein can increase plant survival in adaptation to stresses, particularly biotic stresses and play important roles in gene expression, cellular differentiation, embryogenesis, and disease (Bellefroid *et al.* 1998, Holmes *et al.* 1999). TDF223 has some homology with the gene for mitogen-activated protein kinase 1 (MAPK1). Several studies have shown that protein kinases with high sequence similarity to mammalian MAPKs are found in plants, and it has become apparent that MAPK cascades play very important roles in signal transduction pathways of plants, including those involved in abiotic and biotic stress signaling (Ichimura *et al.* 2000, Nuhse *et al.* 2000, Petersen *et al.* 2000).

Gene expression analysis: Cold acclimation involves the remodeling of cell and tissue structures and the reprogramming of metabolism and gene expression (Thomashow 1999, Viswanathan and Zhu 2002). QRT-PCR was performed on six of the TDFs (TDF107,

119, 217, 223, 205 and 120) to validate the changes in mRNA abundance in chilling seedlings and ABA-treated seedlings. It was found that the expression pattern of these cDNA fragments was similar in cDNA-AFLP and qRT-PCR analysis. The results demonstrated that these six TDFs showed various levels of differential expression after 0 - 24 h of cold stress and 6 h of ABA exposure.

It is universally believed that plants have two ways to adapt to abiotic stresses: ABA-dependent and ABA-independent signaling pathways (Yamaguchi-Shinozaki and Shinozaki 2005). Both the two ways involve different transcription factors from stress signal perception to gene expression (Agarwal and Jha 2010). With the exception of TDF217, the six TDFs identified in this study showed strongly enhanced transcriptional levels in response not only to chilling but also to exogenous application of ABA. Therefore, these five genes appear to be involved in ABA-dependent signaling pathways in response to chilling stress. Many cold-regulated genes in plants are induced by ABA as well as by cold. ABA-responsive genes invariably possess an ACGT motif, which is essential for ABA regulation (Nag *et al.* 2005). Additionally, low-temperature activation of the C-repeat element activation (CRT) may also occur through a novel ABA signaling pathway (Thieringer *et al.* 1998). Sequence analysis of the six cold-responsive genes identified in this study showed that TDF119 possesses an ACGT motif; whether the other five TDFS possess ACGT or CRT motifs is still unknown based on the partial sequences that we have identified.

TDF217 was responsive only to low temperature; treatment with ABA did not change its transcriptional level. This suggests that TDF217 is part of an ABA-independent signaling pathway that functions in cold stress.

The expression levels of the six identified cold-responsive genes declined when the seedlings were transferred to 25 °C for 6 h after 4 °C chilling; however, their expression levels did not decrease to that of the control.

Expression of TDF119 on exposure to low temperature at different growth stages: The protein encoded by TDF119 is unknown (Table 2). We determined that the expression of TDF119 increased between 1 and 24 h after 4 °C chilling and was up-regulated by ABA, implying that the protein encoded by TDF119 plays a role in an ABA-dependent signaling pathway that is affected by cold stress at seedling emergence. We further tested whether the increased expression of TDF119 in *Lepidium* in response to chilling occurs only during seedling emergence. The study showed that TDF119 was induced at high levels by exposure to low temperature during seedling emergence (radicle protrusion, radicle protraction, hypocotyl protrusion); TDF119 expression showed lower sensitivity to cold stress during plant growth than during seedling emergence, although its expression increased to a small extent at the stages of third leaf emergence and flowering. This result shows that TDF119 plays an important role in responses to low temperature during germination of *Lepidium*.

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