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ORIGINAL PAPER

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Identification and characterization of three novel cold acclimation-responsive genes from the extremophile hair grass *Deschampsia antarctica* Desv.

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Abstract *Deschampsia antarctica* Desv. is the only monocot that thrives in the harsh conditions of the Antarctic Peninsula and represents an invaluable resource for the identification of genes associated with freezing tolerance. In order to identify genes regulated by low temperature, we have initiated a detailed analysis of its gene expression. Preliminary 2-D gels of in vivo-labeled leaf proteins showed qualitative and quantitative differences between cold-acclimated and non-acclimated plants, suggesting differential gene expression. Similarly, cold-acclimation-related transcripts were screened by a differential display method. Of the 38 cDNAs initially identified, three cDNA clones were characterized for their protein encoding, expression pattern, response to several stresses, and for their tissue-specific expression. Northern blot analysis of *DaGrx*, *DaRub1*, and *DaPyk1* encoding a glutaredoxin, a related-to-ubiquitin protein, and a pyruvate kinase-like protein, respectively, showed a distinct regulation pattern during the cold-acclimation process, and in some cases, their cold response seemed to be tissue specific. All three transcripts seem to be responsive to water stress as their levels were up-regulated with polyethyleneglycol treatment. *DaRUB1* and *DaPyk1* expression was up-regulated in leaf and crown, but down-regulated in roots from cold-acclimated plants. The significance of these results during the cold-acclimation process will be discussed.

Keywords Analysis · Cold acclimation · *Deschampsia antarctica* · Gene expression analysis

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Introduction

Low temperature is one of the major limiting factors of growth, development, and geographical distribution of plants (Levitt 1980; Sakai and Larcher 1987; Thomashow 1999). Some plants are able to acclimate by increasing their freezing tolerance upon exposure to low non-freezing temperature (Levitt 1980; Thomashow 1998, 1999). This process involves physiological and biochemical changes, such as increases in some soluble carbohydrates, proteins, organic acids, and modification of membrane lipid composition (Guy 1990). Many of these changes are regulated through qualitative and quantitative modification of gene expression, leading to the accumulation of newly synthesized proteins and mRNAs (Guy 1990; Cativelli and Bartels 1992; Lea 1993; Thomashow 1990, 1998, 1999). A number of genes, whose expressions are induced by low temperature, have been isolated and characterized from a wide range of plant species (Thomashow 1994; Hughes and Dunn 1996). However, the precise contribution that these changes play in gene expression in the cold-acclimation process and the biological function for most low-temperature-responsive genes remain to be determined.

It has been suggested that some of them mediate biochemical and physiological changes required for growth and development at low temperature (Thomashow 1998). Although the expression of some cold-inducible genes seems to be specifically regulated by low temperature, sometimes it can be also regulated by other types of stress and by abscisic acid (ABA) (Hughes and Dunn 1996; Thomashow 1998, 1999). For example, drought and exogenous ABA treatments can mimic the effects of low temperature on the freezing tolerance of several species (Chen and Gusta 1983).

Most studies of gene expression during cold acclimation have been performed in plants from temperate and semi-temperate climates. *Deschampsia antarctica* Desv. (Poaceae), a highly freezing-tolerant plant (Bravo 2001; Alberdi 2002), is one of the two vascular plants that have naturally colonized the Maritime Antarctic (Edwards and Lewis-Smith 1988; Alberdi 2002). The optimal photosynthetic activity of *D. antarctica* occurs at 13°C, and it can maintain 30% of this rate at 0°C (Xiong 1999). A high accumulation of soluble carbohydrates, especially sucrose and fructans, was found in leaves of this species during the growth period in the Antarctic summer (Zúñiga 1996). Total protein extracts from leaves of *D. antarctica* growing in the Antarctica have a high cryoprotective activity on barley chloroplasts (Triviño 1998). Membrane lipid contents and the degree of unsaturation of fatty acids in the leaves do not differ significantly from plants in temperate zones (Zúñiga 1994). Cold-acclimation experiments showed that *D. antarctica* is able to acclimate from −14.8 (LT_{50} at 13°C) to −26.8°C when growing at 2 ± 1.5°C for 21 days in a solid substrate in the laboratory (Casanova 1997; Bravo 2001). Under these conditions, some leaf anatomical features of this plant species changed when compared to plants growing in the Antarctic (Romero 1999). Since *D. antarctica* has a high cold-acclimation capacity, it is likely that this plant species has a differential expression of cold-responsive genes during cold acclimation with respect to non-acclimated plants. Gene expression during cold acclimation in *D. antarctica* was studied, as well as gene expression under salt- and osmotic stress treatments and the expression of three genes in different tissues from plants collected during the Antarctic summer. The possible functions of these genes are discussed.

Materials and methods

Plant material and growth conditions

Deschampsia antarctica Desv. (Poaceae) plants were collected in the Coppermine Peninsula on Robert Island, Maritime Antarctic (62°22′S; 59°43′W) and transported in plastic bags to the laboratory. Plants were propagated vegetatively in plastic pots using a soil: peat mixture (3:1), fertilized with 0.12 g/l Phostrogen (Solaris, Buckinghamshire, UK) once every 2 weeks, and maintained at 13 °C in a growth chamber (Forma Scientific, Inc., Marietta, OH, USA) with a photon flux density of 180 μmol m^{-2} s^{-1} at the top of the canopy and 21/3 h light/dark. The light source consisted of cool-white fluorescent tubes F40CW (General Electric, Charlotte, NC, USA). Relative humidity was around 60–70%. Plants growing under these conditions were considered as controls.

For differential display experiments, plants were cold acclimated at 4°C at the same light and photoperiod conditions of control plants for different times (0–21 days). Deacclimation was performed by returning cold-acclimated plants to normal growth conditions for 1 day. For stress response studies, control plants were transferred to hydroponic cultures and preconditioned for a week in nutrient solution (Phostrogen). Salt-stressed plants were obtained by growing the plants in nutrient solutions containing 50 mM NaCl or 250 mM NaCl. Osmotic stress was induced with polyethylene glycol, PEG (Sigma P-2139), added to the nutrient solution to reach osmotic potentials of -0.5 MPa or −2.0 MPa, according to the calculation of Michael and Kaufmann (1973). Salt and osmotic stress treatments were performed over a period of 7 days.

In vivo labeling and extraction of soluble protein

Intact plants were labeled to avoid wound-induced changes in protein synthesis that could occur if excised roots segments were used (Theillet 1982). Control, 7-day and 21-day cold-acclimated plants were transferred to 250 μl of fresh nutrient solution (Phostrogen) containing 10 μl of a mixture of [^{35}S]-methionine and [^{35}S]-cysteine (EXPRE^{35}S^{35}S Protein Labelling Mix, NEN Life Science, 1175 Ci/mmol at 2.0 mCi/ml). Plants were then incubated for 6 h either at 4°C (cold acclimation) or 13°C (control) at the same light intensity and relative humidity of soil-grown plants. Leaf proteins were prepared according to Pérez-Molphe-Balch (1996). Protein extracts were run immediately on gels or stored at 70°C.

Two-dimensional gel electrophoresis and fluorography

Two-dimensional electrophoresis was performed according to Celis (1994) with the Protean II 2D Multi-Cell gel apparatus (BioRad, Hercules, Calif., USA). Sample volumes of approximately equal amounts of radioactivity were applied to isoelectric focusing (IEF) gels and the gels were run for 3.5 h at 750 V. Second dimension electrophoresis was carried out in 15% polyacrylamide sodium dodecylsulphate (SDS) gels at 30 mA for 2–3 h until the dye front reached the edge of the gel. Following electrophoresis, gels were prepared for fluorography according to Bonner and Laskey (1974). Gels were then dried over Whatman 3MM paper and exposed to Kodak X-ray film at 70°C for 72 h.

Purification of total RNA and Northern blot analysis

Total RNA was extracted using the Trizol (Invitrogen, Carlsbad, Calif., USA) reagent (following the manufacturer's instructions) from leaves, crown, and roots of control and cold-acclimated plants at different times and from hydroponically-grown plants treated with different kind of stresses. For Northern blot analysis, total RNA (10°μg) from various tissues or organs was electrophoresed on denaturing formaldehyde-1% agarose gels. After electrophoresis, RNA was transferred onto Hybond-N membranes (Amersham Pharmacia Biotech, Little Chalfont, UK), UV-cross-linked, and hybridized with the different ^{32}P-labeled cDNA inserts at 65°C for 16 h in hybridization solution (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, 1% BSA). Filters were washed at high stringency and autoradiographed on Kodak Biomax MS films with intensifying screens at -80°C. All RNA blots were hybridized with a 28S rRNA probe to confirm uniform RNA loading.

mRNA differential display

Differential display of reverse-transcribed mRNA was performed as described by Liang and Pardee (1992) and modified by Gutiérrez (1997). Four two-base anchor primers, $T_{11}GC$, $T_{11}CG$, $T_{11}CA$, and

$T_{11}CC$, were used to subdivide the mRNA population, and a total of 20 different 5′ arbitrary 13-mer primers was employed for the subsequent polymerase chain reaction (PCR) amplification. Ordinarily, 0.5°μg of DNA-free total RNA was used for the first-strand cDNA synthesis (Gibco/BRL, Life Technologies, Breda, The Netherlands). In the subsequent PCR amplification, the reaction mixture contained 1.3 μM of a [^{32}P]-phosphorylated anchor primer, one of the arbitrary primers, and 1 U of *Taq* Polymerase (Gibco/BRL). The amplified cDNAs were then separated on a 6% (w/v) DNA sequencing gel. Differentially-expressed cDNAs were excised and the DNA was recovered and reamplified using the same PCR conditions. The resulting product was subcloned into the pGEM-T Easy vector (Promega, Madison, Wis., USA). Multiple plasmid preparations were performed for each transformed clone using the standard alkaline lysis method (Birnboim and Doly 1979), and analyzed by digestion with *Eco*RI for presence and size of the cDNA insert. cDNA band inserts were purified from agarose slices, random-radiolabeled ,and used in RNA blot analysis. Those detecting differentially expressed mRNA were sequenced.

Construction of cold-acclimation cDNA library

Total RNA isolated from 24- and 48-h cold-acclimated *D. antarctica* leaves were pooled and used for cDNA library construction by means of the SMART cDNA Library Construction Kit (Clontech, Palo Alto, Calif., USA) and Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif., USA), following the manufacturers' instructions. The primary library contained an estimated 5×10^6 independent clones with an average insert size of 1,200 bp. The library was amplified and stored in 7% DMSO at −80°C. The cDNA library was screened by in situ plaque-hybridization with a random-primed ^{32}P-labeled probe of differential display clone D0-B1.

Rapid amplification of cDNA ends

The 5′-end of DACOR-0.7 was cloned using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions and using gene-specific primers derived from the differential clone D0-611. A similar approach was followed to gain more sequence information of DACOR-2.0 from the differential clone D0-531.

Sequencing analysis

cDNA fragments differentially induced by cold acclimation were sequenced in an automatic sequencer using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Promega, Southampton, UK). The obtained sequences were analyzed for homology at the Web site of the NCBI data banks running the blast programs: http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html. Multiple sequences alignments and dendrograms were obtained with the CLUSTAL W algorithm (Thompson 1994) incorporated in the Mac Vector sequence analysis software (Oxford Molecular Group, Oxford, UK), using the BLOSUM series matrix, an open gap penalty of 10, an extended gap penalty of 0.05, and a divergent delay of 40%.

Results

Protein synthesis in *D. antarctica* shows qualitative and quantitative changes with cold-acclimation regime

To determine if specific protein synthesis changes occur during cold acclimation, the pattern of newly synthesized leaf proteins of *D. antarctica*, both control and cold-acclimated, was analyzed. A comparison of the protein electrophoretic patterns showed that cold acclimation leads to quantitative and qualitative changes in at least nine proteins (Fig. 1). Eight of these polypeptides with molecular masses between 35 and 75 kDa (pI ranging from 4.2 to 7) were found to increase either at 1 week or after 3 weeks of cold acclimation. Six of them seemed to be de novo-synthesized, while two were already present at much lower levels in control plants. One polypeptide was found to decrease transiently at 1 week and to regain control levels by the 3rd week of treatment (ca. 68 kDa, pI 7).

Identification of cold-acclimation-responsive cDNAs by mRNA differential display

D. antarctica cold-acclimation gene expression was studied by mRNA differential display using leaf total

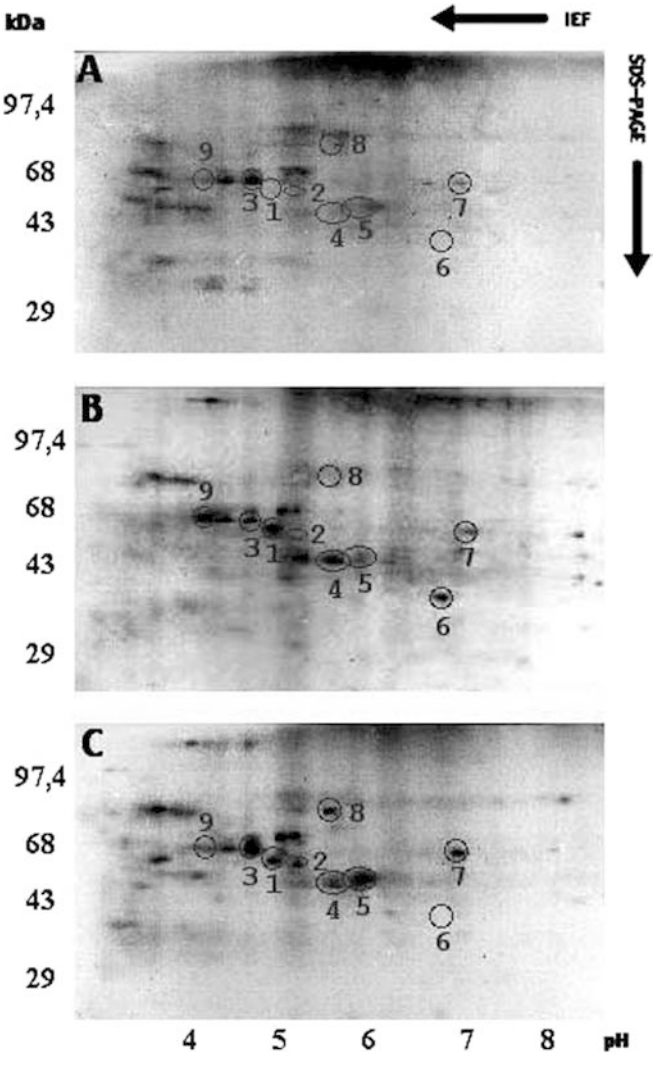

Fig. 1A–C Two-dimensional gel analysis of newly synthesized total leaf proteins in *Deschampsia antarctica*. Total leaf proteins were labeled and extracted from control (**A**), 1-week (**B**) and 3-week (**C**) cold-acclimated plants. Protein spots that show changes upon cold treatment at 4°C are numbered from 1 to 9 and *circled*

Table 1 List of selected cold-induced cDNAs

Clone identity	Insert size (bp)	Sequence homology	Accession number
DACOR-1.3	316	*Brassica napus* Galactinol synthase	CA748463
DACOR-0.7	614	*Oryza sativa* Glutaredoxin	AF374461
DACOR-1.0	831	*Arabidopsis thaliana* UBQ15/UBQ7	AY090541
DACOR-2.0	1730	*A. thaliana* Piruvate kinase-like protein	AY090539
DACOR-0.9	184	Unknown	CA748460
DACOR-0.8	191	Unknown	CA748461
DACOR-0.65	346	Unknown	CA748462

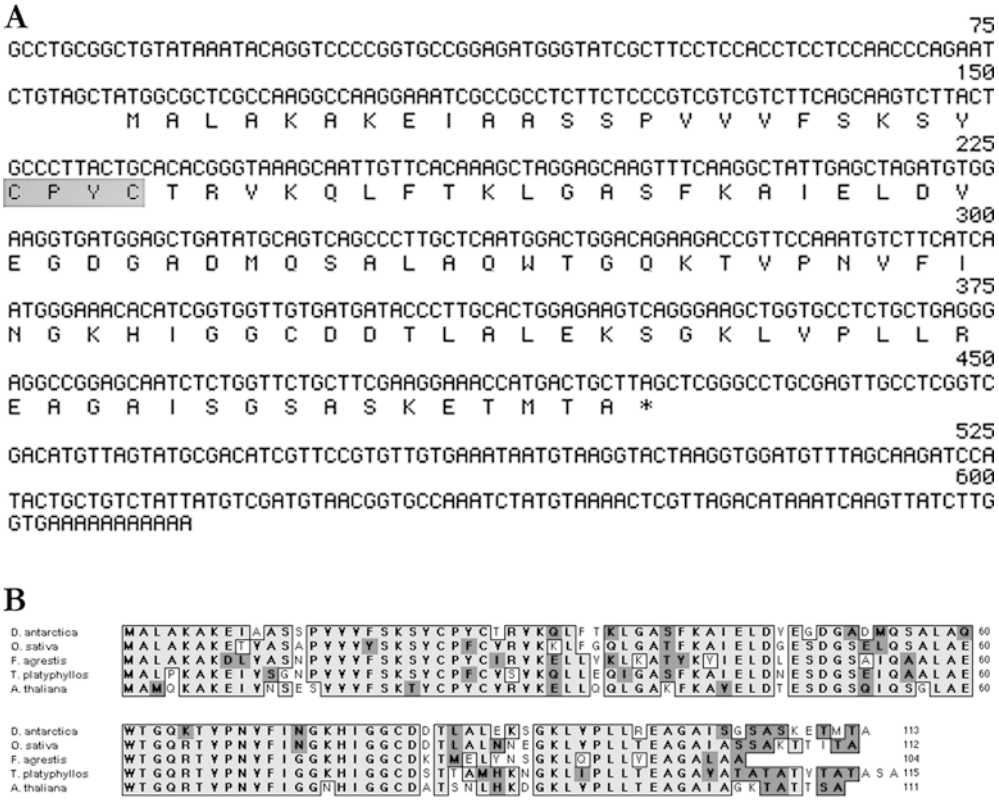

Fig. 2 **A** Nucleotide sequence of the cDNA encoding *DaGrx* and deduced amino acid sequence. The characteristic active site of glutaredoxins is *shaded*. **B** Multiple alignment of the protein sequence derived from the cDNA sequence DACOR-0.7 with other plant glutaredoxins from the GenBank databases: *Oryza sativa* (JC5445), *Fritillaria agrestis* (AAB92419), *Tilia platyphyllos* (AAL04507), *A. thaliana* (BAB11592)

RNA extracted from non-acclimated and cold-acclimated plants taken at different intervals of treatment. A total of 38 cDNA bands were found to exhibit differential expression. Fragments were cut out of the gels, reamplified by PCR, and subcloned into the pGEM-T Easy vector. Twenty-three partial cDNAs were shown by Northern blot analysis to be up- or down-regulated upon cold acclimation (data not shown). DNA inserts of seven clones, ranging in size from 200 to 500 bp, were sequenced and the resulting sequences were compared with the NCBI non-redundant sequence database. The database search assigned putative functions to four of them, but left three clones with unknown functions (Table 1). It was decided to proceed with a more thorough characterization of three partial cDNAs of known function, including their full-length sequence and their gene expression pattern.

Sequence analysis and characterization of three genes regulated during cold acclimation

DaGrx, a 210-bp differential clone, D0-611, was isolated from total RNA from cold-acclimated plants and revealed a ca. 0.7 kb transcript in Northern blots. Primers were designed and used in 5′-RACE experiments to finally obtain a 614 bp full-length cDNA clone, DACOR-0.7 (*D. antarctica co*ld *r*egulated transcript *0.7*). The cDNA clone was sequenced and found to consist of a 342 bp putative open reading frame, which begins 83 bp from the 5′ end and terminates at 425 (Fig. 2A). The open reading frame predicts a 113-aa-long polypeptide with a calculated molecular mass of 11 865 Da and a pI of 8.82. The deduced amino acid sequence possesses the conserved active site [CPCY], characteristic of the glutaredoxins (Grxs) (Rouhier 2002). There are two ATG codons, which could be translational start

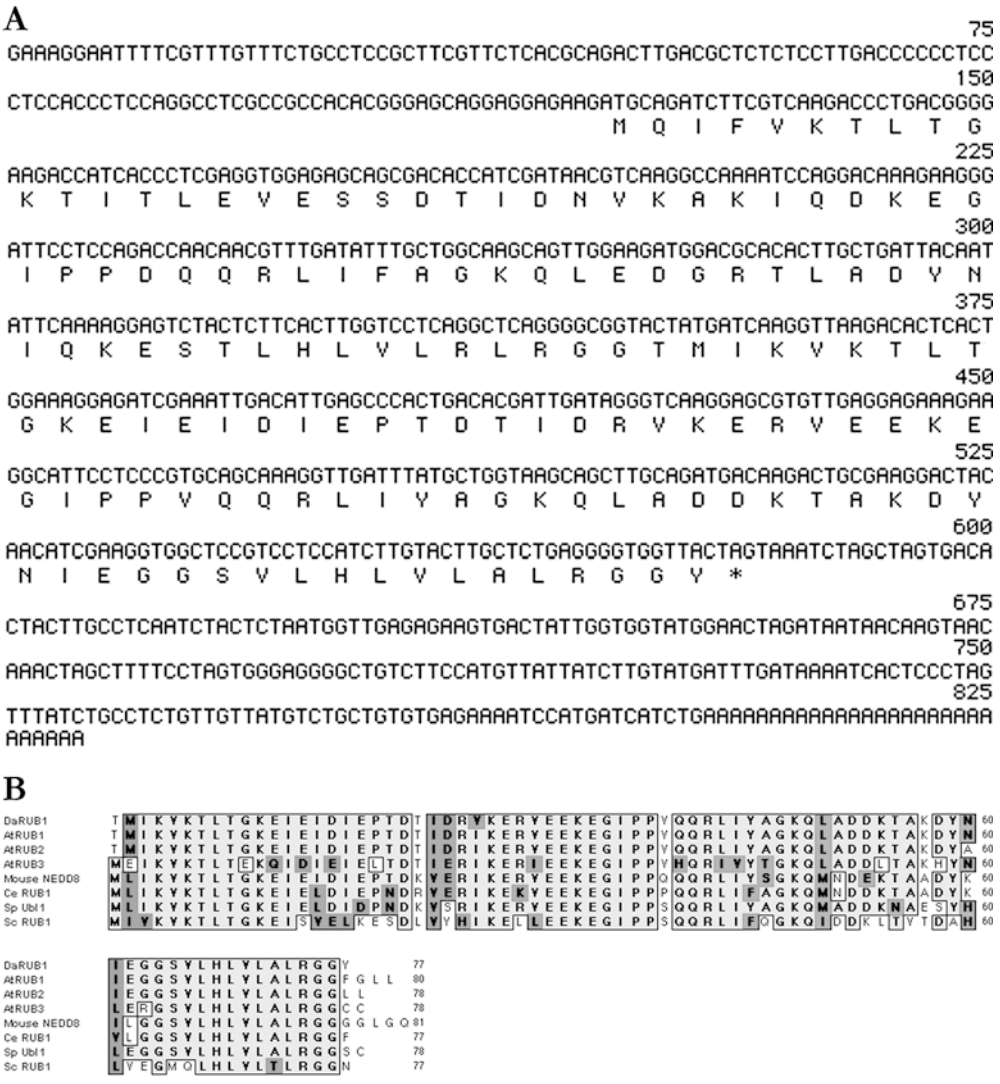

Fig. 3 **A** Nucleotide sequence of the cDNA encoding *DaRub1* and deduced amino acid sequence. **B** Multiple alignment of the RUB protein sequence derived from the cDNA sequence DACOR-1.0 with other RUB proteins from the GenBank databases: *Arabidopsis thaliana* (*AtRUB1, AtRUB2, AtRUB3*), *Mouse RUB1* (*NEDD8*), *C. elegans* (*CeRUB1*), *S. pombe* (*SpUbl1*), *S. cerevisiae* (*ScRUB1*)

codons; however, the latter ATG codon (AGCT *ATG*GCG) is in the monocotyledon consensus initiation context for the flanking regions of ATG (Joshi 1997; Kochetov 1998). Indeed, scanning the protein sequence against the databases showed a strong similarity to several plant glutaredoxins (Fig. 2B). The highest homology was found with the glutaredoxin from the monocotyledon *Oryza sativa* (GenBank accession number JC5445) with a 76% identity and 87% similarity. This *D. antarctica* cold-regulated gene was named *DaGrx*.

DaRub1, a 260-bp differential clone, D0-B1, was isolated from total RNA from cold-acclimated plants and unveiled a ca. 1.0 kb transcript in Northern blots. In order to isolate a full-length cDNA, a cDNA library (5×10^4 pfu), prepared from total RNA isolated from cold-acclimated *D. antarctica* leaves, was screened with this 260 bp insert as the probe. An 831 bp cDNA, DACOR-1.0, was isolated. This cDNA has a putative open reading frame (ORF) of 462 bp, flanked by a 120 bp 5′ untranslated region (UTR) and a 3′ UTR of 249 bp that includes a 28 bp poly-A tail (Fig. 3A). Database searches revealed that the putative ORF encodes a polypeptide of 153 amino acids that consists of a ubiquitin monomer fused in frame to a ubiquitin-like protein RUB (*r*elated to *ub*iquitin) (Rao-Naik 1998). BLAST analysis showed that this RUB moiety is almost identical to other plant members of the RUB family of proteins. Two of the three Arabidopsis *RUB* genes, AtRUB1 and AtRUB2, are also fusions of the RUB-coding region with that of ubiquitin. Like ubiquitin, RUB proteins are 76 amino acids in length, but only share 52–63% sequence identity with ubiquitin (Fig. 3B). In general, RUB proteins have one to five non-conserved COOH-terminal amino acids. These additional amino acids are presumably removed from the initial translation products in a manner similar to that seen for ubiquitin and other systems (Kamitani 1997). This cold-regulated gene has been named *DaRub1*.

DaPyk1, a 490-bp differential clone, D0-531, was isolated from total RNA from cold-acclimated plants by differential display. In Northern blots, it revealed a ca.

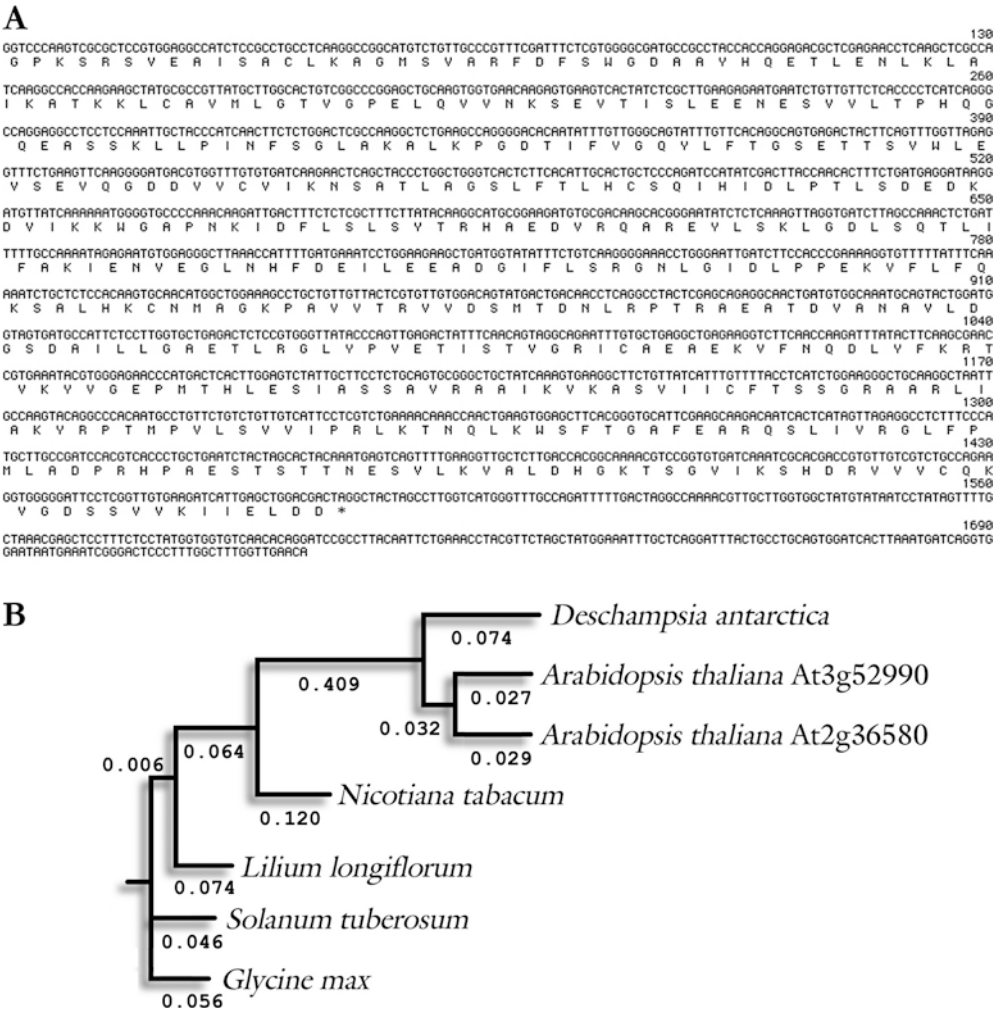

Fig. 4 **A** Nucleotide sequence of the partial cDNA encoding *DaPyk1* and deduced amino acid sequence. **B** Dendrogram based on the amino acid sequence alignment of the following pyruvate kinase-like proteins and cytosolic pyruvate kinases: *DaPyk1* (AAM22747), At3g52990 (AAK56244), At2g36580 (AAL47446), *N. tabacum* (Q42954), *S. tuberosum* (JC1481), G. max (AAM94349), *L. longiflorum* (AAF44707)

2.0 kb transcript. Based on its sequence, primers were designed and used in two rounds of 5′-RACE experiments to obtain a 1,730 bp partial cDNA clone, DACOR-2.0 (Fig. 4A). Unfortunately, repeated screening of our cold-acclimated cDNA library did not result in a full-length cDNA. A search in the databases showed a high homology, 85–87% identity and 91–94% similarity, with two still undescribed *Arabidopsis thaliana* 527-amino acid proteins (GenBank accessions AAK56244 and AAL47446) annotated as pyruvate kinase-like protein and putative pyruvate kinase, respectively. Based on the size of their mRNAs and number of amino acids, we predict that DACOR-2.0 is approximately 40 amino acids short of a full-length *DaPyk1* sequence. Next to these two *Arabidopsis* highly homologous proteins, BLAST searches found a much lower homology, 40–42% identity and 58–62% similarity, with a family of cytosolic pyruvate kinases. A dendrogram constructed with available sequences (Fig. 4B), shows *DaPyk1* as member of a well-resolved branch of pyruvate kinase-like proteins separate from the cytosolic ones, suggesting that they could be functionally distinct. This *D. antarctica* cold-regulated gene has been named *DaPyk1*.

Regulation of transcript levels by cold acclimation

To investigate the regulation of *DaGrx, DaRub1* and *DaPyk1* during cold acclimation, total RNA was extracted from *D. antarctica* leaves at various time points of treatment. There was no detectable signal for any of the three genes in total RNA samples from young leaves, although a rather low signal was found in older leaves (data not shown). Due to the presence of the ubiquitin encoding moiety in the gene *DaRub1*, which in other systems seems to be encoded by a family of genes, the Northern blots were probed with the 3′-UTR of the cDNA clone DACOR-1.0. Cold-acclimated leaves accumulated mRNAs for all three genes as early as 30 min (Fig. 5). The *DaGrx* message was particularly different in that it seemed to disappear after 1 h, before coming back up by 6 h of treatment in a steady manner up to 12 h. In contrast, the messages for the other two genes, *DaRub1* and *DaPyk1*, appeared to have a more uniform expression pattern during the first 12 h (Fig. 5).

Northern blots at other time points of the 21-day cold-acclimation treatment indicated that although these three genes are up-regulated during cold acclimation,

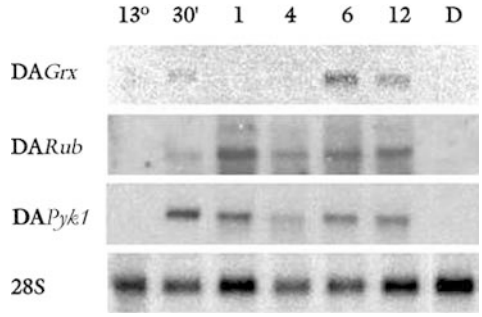

Fig. 5 Northern blot analysis of *DaGrx*, *DaRub1* and *DaPyk1* transcript levels in leaves of *Deschampsia antarctica* cold-acclimated plants at different times: 30 min, 1–12 h. Control plants were kept at 13°C. Deacclimated plants are 24 h at 13°C. Total RNA (10 μg) were separated on formaldehyde/1% agarose gels and probed with the corresponding cDNA clones except for *DaRub1*, which was probed with the 3′-UTR region. A 28S ribosomal RNA probe was used as loading control on the same membranes

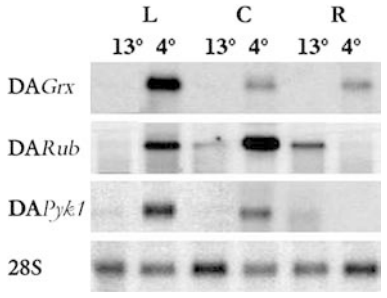

Fig. 6 Northern blot analysis of *DaGrx, DaRub1* and *DaPyk1* transcript levels in leaves of *Deschampsia antarctica* plants treated with two PEG concentrations for different times: 7–24 h and 7 days. Control plants are at 13°C. Total RNA (10 μg) were separated on formaldehyde/1% agarose gels and probed with the corresponding cDNA clones except for *DaRub1*, which was probed with the 3′-UTR region. A 28S ribosomal RNA probe was used as loading control on the same membranes

their products may not be needed to the same extent during the cold-acclimation process. Thus, *DaGrx* messages were back to control levels by 4 days, whereas *DaPyk1* signals were detected up to 5 days. Conversely, *DaRub1* messages were found throughout the whole 21-day cold treatment (data not shown). In all three cases, the transcripts declined rapidly to control levels 24 h after the plants were returned to 13°C.

Osmotic stress affects transcription of *DaGrx, DaPyk1* and *DaRub1* in *Deschampsia* leaves

In order to test if water or salinity stress affects the transcription of any of these three genes, *D. antarctica* plants were grown hydroponically in the presence of NaCl (50 mM or 250 mM) and PEG (−0.5 or −2.0 MPa) for a week. Total RNA of these plants was extracted at different times and analyzed in Northern blots. Saline stress did not affect the transcription of these three genes (unpublished results) at the two NaCl concentrations tested. However, their transcripts began to accumulate after 7 h in the presence of 0.5 Mpa PEG (Fig. 6). The accumulation level reached a peak after 24 h and declined at 7 days of treatment. When plants were exposed to −2.0 MPa PEG, transcripts began to accumulate after 7 h and progressively increased up to the end of the treatment.

Cold-acclimation response is tissue-specific

Tissue-specific expression of *DaGrx, DaRub1* and *DaPyk1* was examined at two different temperatures by Northern blot analysis in leaves, crown, and roots from *D. antarctica* plants. Transcripts for *DaGrx* accumulated at 4°C mainly in leaves and slightly in the crown and roots. Few *DaGrx* transcripts, if any, were observed at 13°C in all investigated tissues. *DaRub1* and *DaPyk1*

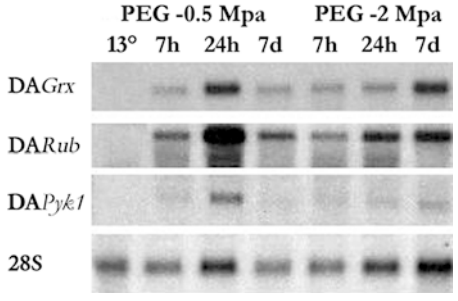

Fig. 7 Northern blot analysis of *DaGrx*, *DaRub1* and *DaPyk1* transcript levels in different tissues of *Deschampsia antarctica* plants at 4° and 13°C. *L* Leaves, *C* crown, *R* roots. Total RNA (10 μg) were separated on formaldehyde/1% agarose gels and probed with the corresponding cDNA clones except for *DaRub1*, which was probed with the 3′-UTR region. A 28S ribosomal RNA probe was used as loading control on the same membranes

transcripts accumulated in leaves and in the crown at 4°C. The response in roots, however, was the opposite. The level of transcripts of both genes was lower at 4°C than at 13°C (Fig. 7).

Discussion

Most of the reports in cold-acclimation research have dealt with plants from temperate and semi-temperate climates and not with plants from cold and extreme environments, such as the hair grass *D. antarctica*. The native Antarctic vegetation certainly must have one or various mechanisms that allow the maintenance of metabolism at low temperature during the Antarctic summer (growing season) and survival during the winter (Bravo 2001). This paper is the first report of changes affecting both protein synthesis and gene expression during cold acclimation of *D. antarctica*, including induction and characterization of three cold-responsive genes.

Exploratory 2-D analyses of control and cold-acclimated plants showed changes in at least nine proteins. The apparent low number of proteins showing changes upon a cold-acclimation regime could be explained by the fact that we sampled from long-term treatments —1 and 3 weeks— rather than the first 24 h. On the whole, however, these results suggest that cold acclimation in *D. antarctica* is related to a differential regulation of protein synthesis. In order to determine if these changes parallel differential expression of genes, it was decided to go one step further and analyze gene expression by differential display. Using this technology, we have been able to isolate 23 partial cDNAs, up- or down-regulated, during the cold-acclimation process. The number of truly differential clones is similar to those isolated in other systems using more primer combinations (Heindenreich 2001; Huang 2001; Suzuki 2001; Kaijalainen 2002).

DaGrx cold-regulated expression suggests a possible redox regulation during cold acclimation

Glutaredoxins, also known as thioltransferases, are heat-stable proteins of approximately 12 kDa that use glutathione as a cofactor for reduction of disulfides in prokaryotes and eukaryotes. They seem to play an important role in redox regulation in eukaryotic and prokaryotic cells (Holmgren and Åslund 1995). In prokaryotic and animal cells, glutaredoxin acts as a reductant for many enzymes, including ribonucleotide reductase (Holmgren 1979), pyruvate kinase (Axelsson and Mannervik 1983), and PAPS reductase (Lillig 1999). Likewise, some glutaredoxins have been shown to have an additional dehydroascorbate (DHA) reductase activity (Wells 1990; Ahn and Moss 1992). Recently, it has been reported that glutaredoxin may participate in cell transduction cascades through the redox regulation of some transcription factors. Thus, in *Escherichia coli*, a glutaredoxin has been involved in the enzymatic reduction of the active form of the OxyR transcription factor. This transcription factor activates the expression of antioxidant genes in response to hydrogen peroxide only after being activated through the formation of an intrachain (Cys^{199} – Cys^{208}) disulphide bond (Zheng 1998).

The presence of glutaredoxins was demonstrated for the first time in plants in the aleurone layer from *Oryza sativa* (Minakuchi 1994). In *Spinacia oleracea* leaves, a cytosolic glutaredoxin was found to cross-react with antibodies raised against *E. coli* glutaredoxin (Morell 1995). It was also established in castor bean that glutaredoxin is one of the major polypeptides of the phloem sap, which suggests that it could play a role in the signal transduction mechanism over long distances. However, unlike rice and spinach glutaredoxins, the *Ricinus communis* glutaredoxin did show DHA reductase activity (Szederkenyi 1997). More recently, Chevalier (1999) isolated a glutaredoxin cDNA from young tomato fruits preferentially expressed at the division phase versus the expansion phase.

The cold regulation of the *DaGrx* gene in *D. antarctica* opens several questions regarding the possible function of this protein during the first stages of cold acclimation in plants. Although it is very tempting to speculate as to the function of this protein in the redox regulation of some transcription factors, or its role as a reductant for some enzymes during the cold-acclimation process, these functions could be limited to *Deschampsia* or, at the most, to monocots. A recent transcriptome profiling of cold acclimation in *Arabidopsis* failed to detect either long-term or transient up-regulation for any *Grx*-like gene (Fowler and Thomashow 2002). However, it should be noted that differences in plant culture conditions, environmental treatments, or the expression-profiling methods used could affect these set of experiments since four of the genes designated as being cold regulated by Seki (2001) were not cold regulated in the most recent study.

The putative thioltransferase and possible DHA reductase activities of purified recombinant *DaGrx* is currently being investigated. Also, the ability to produce specific antibodies will facilitate a comprehensive protein expression analysis for *DaGrx* during the first stages of the cold-acclimation process. The use of a recently cloned 1.5 kb genomic fragment (upstream of the *DaGrx* coding sequence) in GUS fusion constructs will make it possible to gain an understanding of its tissue-specific response and the regulation of its gene expression by cold acclimation as well as dehydration.

DaRub1 cold-regulated expression suggests a role for RUB modification during cold acclimation

Covalent addition of ubiquitin and other ubiquitin-like (UBL) proteins to substrate proteins is apparently widely used in plants, to such an extent that is difficult to name any biological process without direct or indirect connection to a ubiquitylation step (Bachmair 2001). In general, ubiquitylation of substrate proteins involves the activation of ubiquitin's C-terminal Gly by linkage to a Cys residue of a ubiquitin-activating enzyme (E1), the transfer of ubiquityl moieties to a Cys residue in a ubiquitin-conjugating enzyme (E2), and the final formation of an isopeptide bond to a Lys residue of a substrate with the help of a ubiquitin-protein ligase (E3) (Hershko and Ciechanover 1998; Vierstra and Callis 1999; Glickman and Ciechanover 2002).

In the past few years, the list of UBL proteins has expanded rapidly to include RUB, SUMO (*s*mall *u*biquitin-like *mo*difier), and APG12 (Vierstra and Callis 1999). The RUB family of proteins and their mammalian counterparts NEDD8 are ~50–60% identical to ubiquitin. In *Arabidopsis*, three RUB genes have been identified, two of which are fusions of the RUB-coding region with that of ubiquitin (Rao-Naik 1998).

Several studies in a number of models have shown that these RUB proteins are conjugated to target proteins through the sequential action of RUB-activating and RUB-conjugating enzymes in a manner similar to ubiquitin conjugation (del Pozo 1998;; Hochstrasser 1998, 2000; Lammer 1998; Liakopoulos 1998, 1999; Linghu 2002). In *Arabidopsis*, RUB is activated by a heterodimeric E1 enzyme composed of the AXR1 and ECR1 proteins and transferred to a RUB E2 enzyme called RCE1 (del Pozo and Estelle 1999). At present, the only known targets for RUB modification are members of the cullin protein family (Lammer 1998; Hori 1999; Liakopoulos 1999; Wada 1999). Cullins are subunits of the E3 ubiquitin-protein ligase complexes called SCFs (for *S*KP1, *C*DC53 or *c*ullin, and *F*-box proteins) (Patton 1998). Unlike ubiquitin modification, conjugation of RUB to cullin does not appear to modify its metabolic stability. Also, instead of chains being added, only a single RUB appears to be attached to the AtCUL1 subunit of the SCF^{TIR1} complex in Arabidopsis (Gray 1999). The precise role of the RUB modification remains unclear. The question is whether RUB modification is also important for other SCFs. This seems likely since RUB modification appears to be a general characteristic of all cullin proteins (Lammer 1998; Hori 1999).

In this paper, we describe a unique *D. antarctica* RUB1 gene which, unlike AtRUB1 and AtRUB2, is not constitutively expressed but rather is cold induced. *DaRUB1* expression in roots is down-regulated by cold, as opposed to that in leaves and crown, where it is up-regulated. Preliminary genomic blots (data not shown) seem to indicate that there are at least two RUB genes in the *Deschampsia* genome. It is too early to speculate about the nature of RUB targets, if any, during cold acclimation. The possibility of using RUB-specific antibodies to determine any RUB protein tagging change during cold acclimation is hampered by its poor antigenicity (J. Callis, personal communication). However, the recent cloning of a 1-kb *DaRUB1* promoter fragment in our lab will certainly help in the study of its gene expression.

DaPyk1 cold-regulated expression defines a novel pyruvate-kinase-like protein

Pyruvate kinase (PK) is a key regulatory enzyme of glycolysis that catalyzes the irreversible synthesis of pyruvate and ATP from phosphoenolpyruvate and ADP. Considerable evidence indicates that PK is a primary control site of plant glycolytic flux to pyruvate (Plaxton 1996). The enzyme has been demonstrated to be significantly displaced from the equilibrium in vivo and has pronounced regulatory properties in vitro. Plant pyruvate kinases exist as cytosolic and plastidic isozymes (PK_c and PK_p, respectively), which differ substantially in their molecular and kinetic/regulatory properties (Plaxton 1989; McHugh 1995).

We have reported the identification and characterization of a PK-like gene induced during the cold-acclimation process. At this point, it would be too premature to speculate if *DaPky1* has any enzymatic activity at all. The recombinant expression of *DaPky1* cDNA will allow the characterization of its biochemical properties and the nature of its putative substrates, if any. Similarly, further studies will be needed to verify if it indeed represents the ortholog of the two *Arabidopsis* PK-like protein-encoded genes. Relatively little is known about the existence, functions, or genetic basis for tissue- or developmental-specific isozymes of plant glycolytic enzymes. Several lines of evidence suggest that these isozymes could be important for cell-specific metabolism in higher plants (Plaxton 1996). Endosperm-, cotyledon-, and leaf-specific isozymes of *R. communis* PK_c that present noticeable differences in their respective physical and kinetic/regulatory characteristics have been purified and characterized (Hu 1995). These results are consistent with Southern blot studies, indicating the existence of at least six PK_c genes in potato (Cole 1992). The apparent differential *DaPky1* cold-induced response found in leaves and roots of *D. antarctica* could be due to the existence of two very close isozymes or to a very complex gene expression pattern driven by a single promoter. Preliminary Southern blots seem to indicate that *DaPyk1* is a single-copy gene (data not shown). The cloning of the *DaPky1* regulatory region and its use in promoter studies will surely clarify the nature of this differential response.

Some plant glycolytic enzymes may have nonglycolytic functions in vivo. Thus, enolase from *Echinochloa phyllopogons* is induced by anoxia as well as by cold and heat shock, and may act as a general stress protein that protects cellular components at the structural level (Fox 1995). Certainly more work will be needed to determine if this is the case with *DaPky1*.

In summary, we have shown that cold acclimation in *D. antarctica* brings about wide changes in the protein synthesis as well as in gene expression. In addition, we have characterized a set of three novel cold-induced genes previously unreported in the literature. Future work will clarify their function during the cold-acclimation process.

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