Author's personal copy

Planta (2013) 237:823–836
DOI 10.1007/s00425-012-1797-5

ORIGINAL ARTICLE

Transcriptome sequencing of the Antarctic vascular plant *Deschampsia antarctica* Desv. under abiotic stress

Jungeun Lee · Eun Kyeung Noh · Hyung-Seok Choi · Seung Chul Shin · Hyun Park · Hyoungseok Lee

Received: 4 July 2012 / Accepted: 23 October 2012 / Published online: 8 November 2012
© Springer-Verlag Berlin Heidelberg 2012

Abstract Antarctic hairgrass (*Deschampsia antarctica* Desv.) is the only natural grass species in the maritime Antarctic. It has been studied as an extremophile that has successfully adapted to marginal land with the harshest environment for terrestrial plants. However, limited genetic research has focused on this species due to the lack of genomic resources. Here, we present the first de novo assembly of its transcriptome by massive parallel sequencing and its expression profile using *D. antarctica* grown under various stress conditions. Total sequence reads generated by pyrosequencing were assembled into 60,765 unigenes (28,177 contigs and 32,588 singletons). A total of 29,173 unique protein-coding genes were identified based on sequence similarities to known proteins. The combined results from all three stress conditions indicated differential expression of 3,110 genes. Quantitative reverse transcription polymerase chain reaction showed that several well-known stress-responsive genes encoding late embryogenesis abundant protein, dehydrin 1, and ice recrystallization inhibition protein were induced dramatically and that genes encoding U-box-domain-containing protein, electron transfer flavoprotein-ubiquinone, and F-box-containing protein were induced by abiotic stressors in a manner conserved with other plant species. We identified more than 2,000 simple sequence repeats that can be developed as functional molecular markers. This dataset is the most comprehensive transcriptome resource currently available for *D. antarctica* and is therefore expected to be an important foundation for future genetic studies of grasses and extremophiles.

Keywords *Deschampsia antarctica* · Extremophile · Hairgrass · Pyrosequencing · Stress response

Abbreviations

AOX	Alternative oxidase
DEG	Differentially expressed gene
DHN	Dehydrin
EC	Enzyme commission
EST	Expressed sequence tags
GC	Guanine–cytosine
GI	GIGANTEA
GO	Gene ontology
IRIP	Ice recrystallization inhibition protein
IVD	Isovaleryl Co-A dehydrogenase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LEA	Late embryogenesis abundant protein
PEPC	Phosphoenolpyruvate carboxylase
ProDH	Proline dehydrogenase
qPCR	Quantitative reverse transcription polymerase chain reaction
SSR	Simple sequence repeat
TIM	Inner membrane translocase subunit

Introduction

The Antarctic is one of the most severe natural habitats, especially for terrestrial plants. Specific environmental

Electronic supplementary material The online version of this article (doi:10.1007/s00425-012-1797-5) contains supplementary material, which is available to authorized users.

J. Lee · E. K. Noh · S. C. Shin · H. Park · H. Lee (✉)
Division of Life Sciences, Korea Polar Research Institute, 12 Gaetbeol-ro, Yeonsu-gu, Incheon 406-840, Korea
e-mail: soulaid@kopri.re.kr; soulaid2@gmail.com

H.-S. Choi
Bio&Health Team, Future IT R&D Laboratory, LGE Advanced Research Institute, 38 Baumoe-ro, Seocho-gu, Seoul 137-724, Korea

Author's personal copy

conditions have restricted the number of native flowering plants to only two species: *Colobanthus quitensis* (Caryophyllaceae) and *Deschampsia antarctica* Desv. (Poaceae). Climate change, which is occurring more intensely and rapidly in the polar regions than elsewhere has already had a significant impact on terrestrial ecosystems. The *D. antarctica* population size has increased in response to global warming more rapidly than that of bryophytes (Lewis Smith 1994). Because of its successful adaptation to climate change and rapid spread, *D. antarctica* has been studied in a wide range of biological fields and has been suggested as an ecological marker of global warming and a valuable resource for the discovery of genes associated with stress tolerance (Xiong et al. 2000; Lee et al. 2008; John et al. 2009).

Stress acclimation and the acquisition of stress tolerance requires the orchestration of responses induced by multiple different abiotic stressors such as cold, drought, and high salinity, which can influence the development of plants and threaten crop yields worldwide. These abiotic stressors are often interconnected, and induce similar cellular damage (Shinozaki et al. 2003). Plants have evolved conserved mechanisms in terms of molecular, cellular, and physiological responses, such as the production of stress proteins, upregulation of antioxidants, and accumulation of compatible solutes, during their adaptation to unfavorable environmental conditions (Kreps et al. 2002; Iordachescu and Imai 2008; Gill and Tuteja 2010). Extensive studies have found that *D. antarctica* has a conserved mechanism of tolerance to various abiotic stressors. First, *D. antarctica* has a photosynthetic system that is adapted to low temperatures: maximal photosynthetic activity occurs at 13 °C in *D. antarctica*, and 30 % of its maximal photosynthetic capacity is retained at 0 °C (Edward and Lewis Smith 1988; Xiong et al. 2000). Second, both the amino acid proline and non-structural carbohydrates (sucrose and fructans), which confer a high degree of stress tolerance toward cold, accumulate in leaves during the growth period (summer) (Zuñiga et al. 1996; Bravo et al. 2001). Third, antifreeze compounds are produced in the non-acclimated state, and their levels increase after cold acclimation, indicating that the plant secretes antifreeze proteins into apoplasts (Bravo and Griffith 2005). In addition, *D. antarctica* has potent recrystallization inhibition activity, which inhibits the growth of small into potentially damaging large ice crystals at low temperatures (John et al. 2009).

A number of sequences and molecular markers from organisms across the three domains have improved our understanding of the genetic basis of traits and fine-scale genetic variation. Nevertheless, we lack genomic resources for most non-model organisms, and whole-genome sequencing is still largely impractical for most eukaryotes. Transcriptome analysis is an attractive alternative to whole-genome sequencing because the majority of eukaryotic genomes comprise non-coding DNA; furthermore, mRNA sequences lack introns and intragenic regions, which makes data analysis more difficult. In recent years, there have been significant advances in DNA sequencing technology. Among the currently used high-throughput techniques, pyrosequencing is a useful method for de novo transcriptome analysis of non-model species (Novaes et al. 2008; Vera et al. 2008; Parchman et al. 2010).

In our previous study, we presented the first snapshot of the *D. antarctica* transcriptome by comparing field-grown and greenhouse samples (Lee et al. 2008). Although a number of genes have been revealed to be inducible under Antarctic field conditions, the scale of that study was limited, and we could not specify the primary cause among the several abiotic factors that affect gene expression in the Antarctic. Thus, this study aimed at large-scale discovery of genes differentially expressed by independent abiotic stressors under a controlled environment. Here, we present the results of transcriptome analysis of *D. antarctica* grown under abiotic stress conditions. More than 370,000 sequence reads were generated by 454 pyrosequencing and assembled into 60,765 unigenes (28,177 contigs and 32,588 singletons). We identified numerous simple sequence repeats (SSRs) that will facilitate development of functional markers in the dataset, making it possible to link these genomic data with existing genetic information on *D. antarctica*. We also identified stress-responsive genes that were differentially expressed under various abiotic stress conditions and the enzymes involved in carbon fixation (which may be adapted to extreme environmental conditions). This transcriptome dataset is a valuable genetic resource for *D. antarctica*, which has adapted to an extreme natural environment, and may prove valuable for the discovery of novel genes associated with stress tolerance.

Materials and methods

Plant materials and abiotic stress treatment

Deschampsia antarctica Desv. (Poaceae) plants growing under natural conditions were collected in the vicinity of the Korean King Sejong Antarctic Station (62°14′29″S; 58°44′18″W), on the Barton Peninsula of King George Island in January 2007. The plants were transferred to the lab and grown hydroponically supplemented with 0.5× Murashige and Skoog (MS) medium containing 2 % sucrose under a 16:8 h light: dark cycle with a light intensity of 150 μmol m^{-2} s^{-1} at 15 °C (Control condition), a temperature that results in high Rubisco activity in *D. antarctica* (Pérez-Torres et al. 2006). Five individual

Author's personal copy

plants of 3–4 tillers, with the longest tiller 5 cm in length, were used in each experiment. For the low temperature stress treatment (cold), plants were transferred to a low temperature chamber and incubated at 4 °C. The temperature necessary to induce *D. antarctica* cold stress molecular responses was determined in two preliminary experiments. First, the average soil surface temperature of the *D. antarctica* habitat on the Barton Peninsula on King George Island was recorded as 4.4 °C (Fig. S1). Data were obtained over 71 days between December 9, 2008 and February 18, 2009. Additionally, two representative environmental stress-responsive genes (*DaURA5* and *DaURA6*; Lee et al. 2008) were induced 5- to 30-fold at 4 °C compared to at 15 °C (Fig. S2). Therefore, we selected 4 °C as the cold stressor to elucidate the genes responsible for cold resistance under laboratory conditions that mimic field temperatures. For dehydration (PEG) stress, plants were transferred to a dehydration test solution prepared at −0.6 MPa by dissolving 30 % PEG6000 in 0.5× MS medium using the formulae of Michael and Kaufmann (1973). Plants were transferred to 0.5× MS medium containing 300 mM NaCl for salt (NaCl) stress. Because *DaURA5* and *DaURA6* expressions were induced 5- to 30-fold at 4 °C and 24 h after cold treatment as compared at 15 °C (Fig. S2). The expression of *DaURA6* subsequently decreased, thus we determined that 24 h after the stress treatment was the most appropriate time point to induce *D. antarctica* stress-responsive genes. All stress treatments were performed over a 24-h period. Three biological replicates were performed for each stress treatment process; plant samples were then subjected to quantitative reverse transcription polymerase chain reaction (qPCR) analysis.

RNA extraction, cDNA preparation, and pyrosequencing

Total RNA for 454 sequencing was extracted from leaf tissues of plants grown under cold, PEG, NaCl, or control conditions (0.5× MS, 15 °C) and purified using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. mRNA was isolated from 50 μg of the total RNA sample using the FastTrack™ MAG Micro mRNA Isolation Kit (cat# K1580-01, Life Technologies, Gaithersburg, MD, USA), according to the manufacturer's protocol. Isolated mRNA was quantified using the Agilent RNA 6000 NANO CHIP (cat.# 5067-1511, Agilent Technologies, Palo Alto, CA, USA), and 200 ng of this material was used in the cDNA Rapid Library Preparation protocol. The first step of cDNA Rapid library preparation was mRNA fragmentation using $ZnCl_2$ and heat treatment. Then, we reverse transcribed the cleaved RNA fragments primed with random hexamers into first-strand cDNA using reverse transcriptase and random primers. Next, we removed the RNA template and synthesized a replacement strand to generate double-stranded (ds) cDNA. The ds cDNA ends were polished (blunted), and short adapters were ligated onto both ends. The adapters provided priming sequences for both amplifications. Sequencing of the sample library fragments and the "sequencing key", a short sequence of four nucleotides, was used by the system software for base calling, following purification and size selection using AMpure beads. Finally, the quality of the cDNA fragment library was assessed using a 2100 BioAnalyzer (Agilent Technologies), and the library was quantitated to determine the optimal amount to use as input for emulsion-based clonal amplification. Each of the four samples (control, cold, PEG, and NaCl) was run on a quarter-plate according to the manufacturer's instructions.

Sequence assembly and annotation

Sequence assembly was performed using the standard GS-FLX protocol (Macrogen, Seoul, Korea). Low-quality and low-complexity reads and adapter sequences were trimmed to a 100 bp parameter using SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) and Lucy software (Chou and Holmes 2001). All reads from the four libraries were pooled, clustered, and assembled into unigenes (contigs and singletons) using TGICL as produced by CAP3 with default parameters (Pertea et al. 2003). The assembled sequences were subjected to BLASTX or BLASTN searches against the non-redundant protein and nucleotide databases (nr) with an *E*-value threshold of 1×10^{-3} and an HSP length cutoff of 33 using the Blast2GO platform (Conesa et al. 2005). The transcript set of *D. antarctica* was analyzed for similarity/sequence conservation against *Brachypodium* expressed sequence tags (ESTs) at the Munich Information Center for Protein Sequences (http://mips.helmholtz-muenchen.de/plant/brachypodium) and EST databases of other plants at NCBI (http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/remote_blastdblist.html) by TBLASTX with an *E*-value threshold of 1×10^{-5}. Files containing the sequences and quality scores of reads were deposited in the NCBI Short Read Archive (Accession Number SRA051881). Gene ontology (GO) terms were assigned to each unique gene based on the GO annotations of the corresponding homologs in the UniProt database. GO mapping and annotation were performed with an annotation cutoff of $E < 1 \times 10^{-10}$. GO enrichment analysis was performed using AgriGO, a web-based GO analysis toolkit (Du et al. 2010) and Fisher's exact test ($P < 0.05$). The pathway assignments were mapped according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and

Author's personal copy

Goto 2000). Enzyme commission (EC) numbers were assigned to the sequences with a cutoff value of $E < 1 \times 10^{-10}$. The sequences were mapped to specific biochemical pathways according to the corresponding EC distribution in the KEGG database.

Identification of full-length cDNAs and SSRs

Putative full-length cDNAs were identified by comparison of BLASTX reports from the UniProt databases using FullLengther (Lara et al. 2007) and an ORF prediction algorithm based on BLAST alignment to locate protein-coding regions, with a cutoff value of $E < 1 \times 10^{-5}$. The Perl script program MISA (MIcroSAtellite; http://pgrc.ipk-gatersleben.de/misa/) was used to identify SSRs with the following minimum length criteria: ten repeat units for mononucleotides, six repeat units for dinucleotides, and five repeat units for all higher-order repeats (Thiel et al. 2003).

Differential gene expression profiling

We counted the number of raw reads that assembled into the consensus contigs, because the abundance of ESTs supporting a particular consensus sequence indicates the abundance of that transcript in the sampled tissue. To select differentially expressed genes (DEGs) under each experimental condition, the permutation *T* test was used after normalization of raw read counts with library sizes. The paired *T* test was used to evaluate the significance of differences in contig read counts between the control and treatment groups. The permutation test was used, since the distribution of the differences in read counts may not follow a normal distribution (Edgington 1995). Read counts for each contig in the permutation test were permuted 1,000 times by group-wise shuffling, and raw *P* values were converted to *Q* values for multiple test correction. Contigs with a *Q* value of false discovery rate <0.01 were identified as differentially expressed genes (Storey 2002).

qPCR analysis

Total RNA was extracted from leaves of plant samples and purified using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA extracted from samples using Superscript II (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were designed according to the sequences of the contigs and are listed in Table S1. Two reference genes, *DaCYP* and *DaEF1a1*, were used as internal controls: *DaCYP* forward primer 5′-GCGCATC ACCTTCCAAATTTCCCA-3′ and reverse primer 5′-ATA GAAGCTACCGTGCCTGGTTGT-3′, *DaEF1a1* forward primer 5′-TTTGTCCACTGCTACACTCGTGGT-3′ and reverse primer 5′-TCGAAGGCTGACGGACATAACCA A-3′ (Lee et al. 2010). qPCR was performed in triplicate with biologically replicated samples using SYBR® Premix Ex Taq™ DNA polymerase (Takara, Seoul, Korea) and the Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA).

Results

Pyrosequencing of the *D. antarctica* transcriptome

To better understand the stress response of *D. antarctica*, we performed 454 GS-FLX sequencing of cDNAs from controls, and cold-, PEG-, and NaCl-treated leaf tissues (as defined above). A total of 370,169 reads, averaging 212 bp, were generated from a pyrosequencing run. Trimming of the raw sequences (removal of primers, poly-A tails, etc.) gave 285,848 high-quality reads comprising 78.5 Mbp of sequence (Table 1). After pooling and clustering, these reads were assembled into 60,765 unigenes comprising 28,177 contigs and 32,588 singletons. The generated contigs measured 415 bp on average, with 6,553 (23 %) of them being longer than 500 bp, and 5,276 (18.7 %) of them being made up of more than 10 reads (Tables S2, S3). The average guanine–cytosine (GC) content of the assembled contigs was 48.94 %. The GC content distribution pattern of all *D. antarctica* unigenes was bimodal (range 30–75 %), and the proportions of assembled contigs with GC contents in the range 40–60 and 65–75 % were higher and lower, respectively, than those of singletons (Fig. S3).

Identification of SSRs

The discovery of molecular markers for *D. antarctica* is of great interest because it represents a potentially useful resource for determining the historical path of its colonization and functional genetic variation in the traits related to its adaptation. Among the various molecular markers, SSRs are highly polymorphic, and thus can be developed easily as molecular markers and provide rich resources of diversity. EST-SSRs are tandem repeats of 2- to 6-bp DNA core sequences (also known as microsatellites in transcribed sequences), which can be rapidly developed from an EST database at low cost (Morgante et al. 2002). To identify *D. antarctica* SSRs, 60,765 unigenes were examined for microsatellites containing motifs between one and six nucleotides in size. We identified a total of 2,064 SSRs in 1,916 unigenes (3.2 %) with a frequency of 1 SSR per 9.1 kb of sequence (Table 2). About 47 % of the SSRs were trinucleotides, 27 % were mononucleotides, 22.7 %

Author's personal copy

Table 1 Total number of reads for each tissue sample, as obtained by 454 sequencing

	Total reads	Total bp	Average length	High-quality reads
Control (Con)	103,142	21,967,920	213	72,977
Low temperature (cold)	76,554	15,460,786	202	45,084
Dehydration (PEG)	89,537	18,617,959	208	57,477
High salt (NaCl)	100,936	22,462,610	223	76,035
Total	370,169	78,509,275	212	285,848

Table 2 Statistics for SSRs identified in the *D. antarctica* transcriptome

Parameter	Value
SSR mining	
Total number of sequences examined	60,765
Total size of examined sequences (bp)	18,841,100
Total number of identified SSRs	2,064
Number of SSR containing sequences	1,916
Number of sequences containing more than 1 SSR	138
Number of SSRs present in compound formation	120
Frequency of SSR	One per 9.1 kb
Distribution of SSRs in different repeat type	
Mononucleotide	558 (27.0 %)
Dinucleotide	468 (22.7 %)
Trinucleotide	971 (47.0 %)
Tetranucleotide	46 (2.2 %)
Pentanucleotide	11 (0.5 %)
Hexanucleotide	10 (0.5 %)

were dinucleotides, and the remaining ~3 % were tetra-, penta-, and hexanucleotides.

Functional annotation of *D. antarctica* unigenes

All unigenes were blasted against the GenBank nr protein database using the BLASTX algorithm. Among them, 29,173 (48 %) had at least one significant alignment to an existing model with an *E*-value cutoff of 1×10^{-10} (Table 3). As expected, the most abundant sequences in the *D. antarctica* transcriptome corresponded to genes encoding photosynthesis-related proteins such as Rubisco small subunit, Rubisco activase, chlorophyll *a*/*b*-binding protein, PSII, and oxygen-evolving enhancer protein (Table S4). Sequences with significant similarities to metallothionein type 1, glyceraldehyde-3 phosphate dehydrogenase, *S*-adenosyl methionine decarboxylase, elongation factor 1-alpha, and non-specific lipid transfer protein 2 were also included in the ten most abundant sequences. As 27,396 (45 %) of all unigenes did not match any sequence in the nr protein database in BLASTX searches (*E*-value cut-off $<1 \times 10^{-3}$), we performed a BLASTN search against the EST nucleotide database (*E*-value cut-off $<1 \times 10^{-3}$). Among them, 19,709 sequences (32 %) matched the EST database, whereas 7,687 sequences (13 %) did not match any known sequences in the existing database (Table 3).

Putative full-length cDNAs were identified by alignment of the BLAST results using the UniProt database. Therefore, sequences were considered full-length when they included both 5′ and 3′ termini of the coding region of a protein in the UniProt database. Analyzing the fraction of full-length sequences in the transcriptome with the pool of 18,816 contigs with BLASTX matches, 4,337 contigs of the predicted transcripts were assigned as full-length or putative full-length sequences in the transcriptome (Table S5).

The *D. antarctica* unigene dataset was analyzed to identify sequences conserved in other plant species. The top-hit species distribution of BLAST matches to the nr protein database is shown in Fig. 1. The majority of the sequences (94 %) showed the best matches with grass species, predominantly with *Brachypodium distachyon* (37.4 %) and *Hordeum vulgare* (33.4 %) (Fig. 1a). The *D. antarctica* unigene dataset was also compared to EST datasets of other plant species in TBLASTX searches (*E*-value cut-off $<1 \times 10^{-5}$) (Fig. 1b). The largest number of *D. antarctica* transcripts (71.1 %) showed significant similarity with *B. distachyon*, followed by *Triticum aestivum* (69.7 %), *H. vulgare* (66.5 %), *Oryza sativa* (62.5 %), *Zea mays* (59.9 %), *Sorghum bicolor* (55.2 %), and *Glycine max* (51.2 %); the least similarity was with *Arabidopsis thaliana* (49.1 %). As expected, *D. antarctica* transcripts showed more significant similarity with monocot ESTs (55–71 %) than with dicot ESTs (49–51 %). In addition, they showed more significant similarity with ESTs from species of subfamily Pooideae in the BEP clade (*B. distachyon*, *T. aestivum*, and *H. vulgare*) than with those of subfamily Ehrhartoideae in the BEP clade (*O. sativa*), or with those from the PACMAD clade (*Z. mays* and *S. bicolor*).

GO distribution of the *D. antarctica* transcriptome

The unigene sequences with BLAST matches were assigned GO terms and EC numbers using the Blast2GO platform. A total of 26,467 unigenes (44 %) were assigned at least one GO term and were classified into different functional categories using the complete set of GO terms. Figure 2 shows the functional classification of *D. antarctica* unigenes into high-level GO terms within three categories: molecular function, biological process, and cellular

Author's personal copy

Table 3 Annotated summary of the *D. antarctica* transcriptome

	Contigs	Singletons	Unigenes
Significant BLASTX hits ($E < 1 \times 10^{-10}$)	17,322 (62 %)	12,356 (38 %)	29,173 (48 %)
Sequences with BLASTX hits ($E < 1 \times 10^{-3}$)	18,816 (67 %)	14,553 (45 %)	33,369 (55 %)
No hits by BLASTX Search ($E < 1 \times 10^{-3}$)	9,361 (33 %)	18,035 (55 %)	27,396 (45 %)
Sequences with BLASTN hits against est db ($E < 1 \times 10^{-3}$)	7,740 (27 %)	11,969 (37 %)	119,709 (32 %)
No hit with BLASTN Search	1,621 (6 %)	6,066 (19 %)	7,687 (13 %)
Sequences annotated with GO terms	15,451 (55 %)	11,026 (34 %)	26,477 (44 %)
Sequences assigned with EC numbers	5,854 (21 %)	3,988 (12 %)	9,842 (16 %)
Total	28,177	32,588	60,765

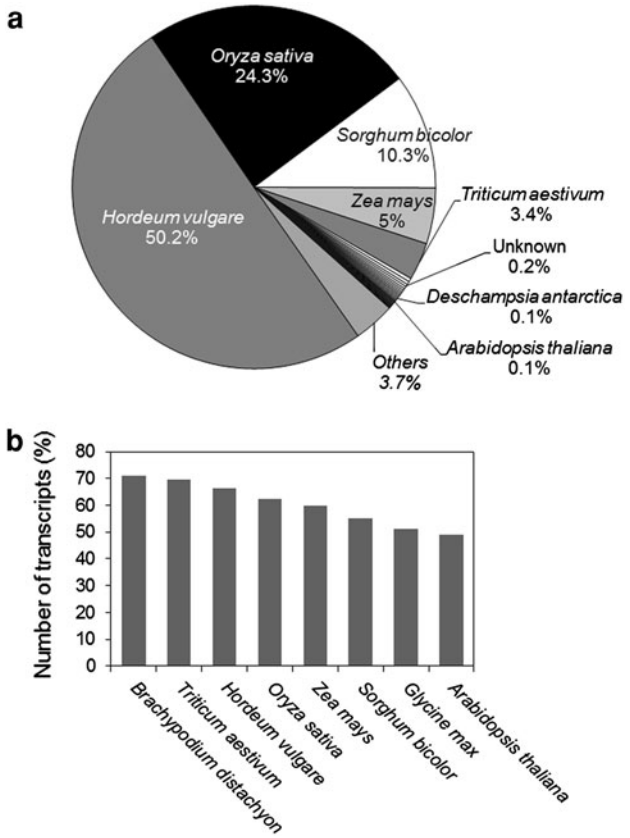

Fig. 1 Sequence conservation of *D. antarctica* transcripts in other plant species. **a** Species distributions of the top seven BLASTX matches to the nr protein database. **b** Sequence comparison of *D. antarctica* ESTs with reference proteins from other species. Percentages of transcripts showing a significant similarity ($E < 1 \times 10^{-5}$) to each transcriptome in TBLASTX searches are presented

component. Several branch child terms for antioxidant activity and the stress response were found, which included genes implicated in catalase (e.g., CL1Contig222) and superoxide dismutase (e.g., CL4750Contig1) as well as predicted transcription factors with putative roles in stress responses (e.g., CL1378Contig2) from the molecular function category, frequent sub-classification into the response to osmotic stress (e.g., CL9324Contig1: ABC transporter), and regulation of the response to stress (e.g., CL3108Contig1: SIZ1) from the biological process category. The abundance of stress-related annotations is not surprising, considering that a significant portion of the sequence reads were generated from tissues subjected to abiotic stressors.

Identification of DEGs by expression profiling

In principle, the higher the number of ESTs assembled in a particular contig, the higher the number of transcripts in a given tissue sample. To identify DEGs induced by abiotic stress in *D. antarctica*, the number of sequencing reads that supported the consensus contigs assembled from all libraries were counted and divided into four categories depending on the library of origin. The permutation *T* test with a multiple testing correction indicated that 2,097 transcripts were upregulated and 1,013 transcripts were downregulated under at least one abiotic stress condition compared to the control sample, with a Q value <0.01 (Table S6). Among the upregulated genes, 55 (2.6 %), 58 (2.8 %), and 81 (3.9 %) genes were in response to cold–NaCl, cold–PEG, and PEG–NaCl combinations, respectively. Only 21 transcripts (1.0 %) were upregulated in all three libraries. Among the downregulated genes, 273 (26.9 %), 208 (20.5 %), and 326 (32.2 %) genes were in response to cold–NaCl, cold–PEG, and PEG–NaCl combinations, respectively, and 154 were common to all three stress conditions (15.2 %) (Fig. 3; Table S6).

Then, GO analysis was conducted on the subsets of DEGs and whole unigenes with the complete list of GO terms. Among the 3,110 DEGs shown in Fig. 3, 2,099 transcripts (1,297 upregulated; 802 downregulated) were annotated with GO terms (Table 4). While the GO terms of response to an abiotic stimulus (GO:0009628), response to cold (GO:0009409), response to water (GO:0009415), and the starch metabolic stimulus (GO:0005982), and were enriched in the upregulated transcript group, the GO terms of ribonucleoprotein complex (GO:0030529), thylakoid (GO:0009579), photosynthesis (GO:0015979), chlorophyll binding (GO:0016168), and photosystem I (GO:0009522) were enriched in the downregulated transcript group.

Author's personal copy

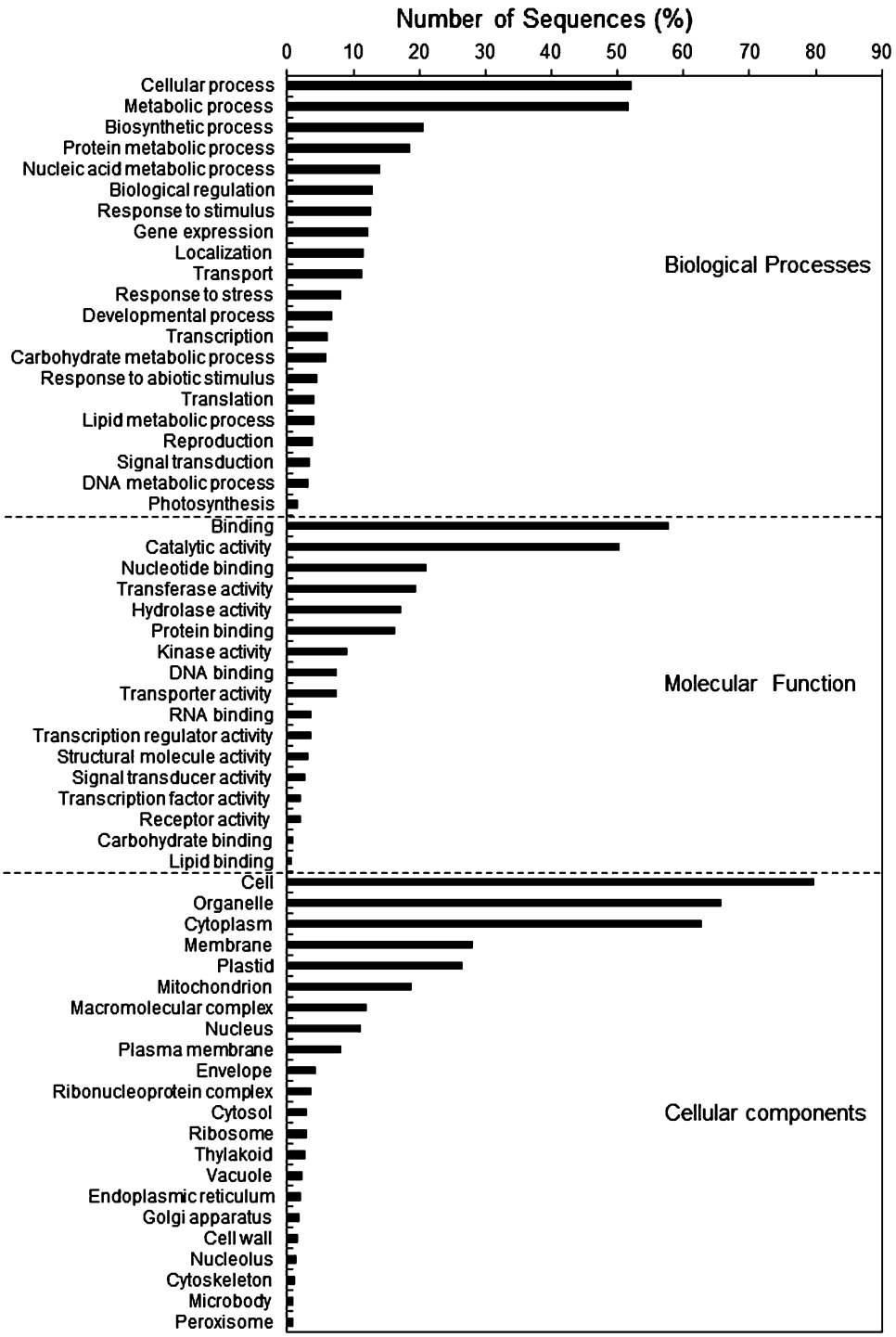

Fig. 2 Functional GO classification of the *D. antarctica* transcriptome. Sequences with BLASTX matches were assigned GO terms and classified into the following functional categories: biological process, molecular function, and cellular component

Carbon fixation enzymes

The optimum temperature for photosynthesis in *D. antarctica* is much lower than that in plants from warm regions, consistent with the hypothesis that carbon fixation in *D. antarctica* may have adapted to the extreme environment. To examine the sequences of cold-adapted carbon fixation enzymes in *D. antarctica*, we mapped the annotated sequences to KEGG using the Blast2GO platform. Transcripts encoding enzymes involved in the carbon fixation pathway in photosynthetic organisms were identified. We found 21 of the 24 known enzymes in the pathway, indicating that the gene function assignments were biologically meaningful and that the EC numbers had been correctly assigned to annotated sequences (Table 5). Among the enzyme-coding sequences identified, several

Author's personal copy

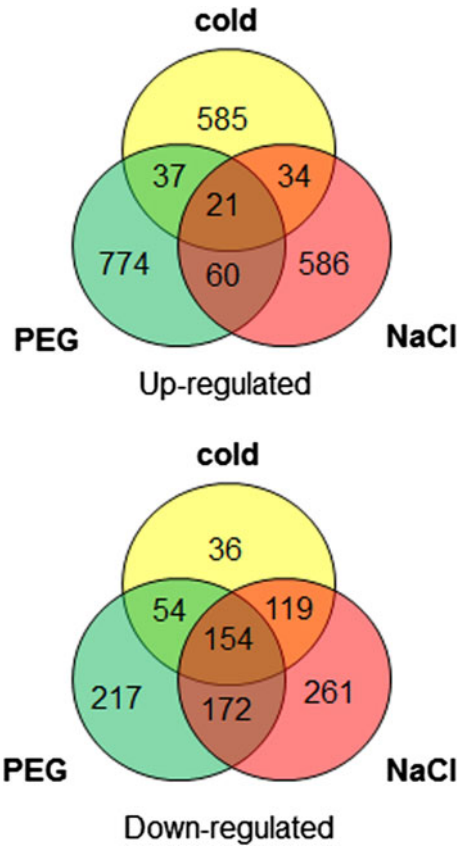

Fig. 3 Differential gene expression analysis of the *D. antarctica* transcriptome following exposure to three abiotic stressors. *Venn diagrams* showing differentially expressed transcripts selected using a permutation *T* test ($Q < 0.01$). Numbers of genes differentially expressed or shared by the cold, dehydration, or high salinity libraries are shown. The IDs of the transcripts in each group are listed in Table S6

sequences were included in the list of abiotic stress DEGs (Table S6). The expression of phosphoenolpyruvate carboxykinase (CL2236Contig1) was found to be increased by NaCl and that of PEPC (CL272Contig2) by cold. In contrast, the expression of fructose bisphosphatase (CL224Contig3) and that of phosphoglycerate kinase (CL1Contig342) was reduced by NaCl; and that of alanine transaminase (CL283Contig2) by cold. Comparative analysis of enzyme-coding sequences in *D. antarctica* and the model organisms *Arabidopsis*, rice, and barley by BLASTX showed that average overall sequence similarities were 81.2, 91.8, and 95.6 %, respectively (Table 5).

qPCR analysis of stress-induced transcripts

To validate the expression profiles obtained by DEG analysis, qPCR was performed on 43 genes selected due for their high probability of differential expression by abiotic stress ($Q < 10^{-5}$, Table S6). Among them, 42 (42/43) resulted in amplicons of the expected size, suggesting that our assembly process was successful. We also found that 35 genes (35/42, 83 %) were significantly induced by corresponding abiotic stressors (*T* test, $P < 0.05$, Fig. 4a). Among the induced genes, not only known stress-responsive genes (CL55Contig1: *COR410*, CL157Contig5: *DHN1*, CL342Contig3: *COR413*, CL76Contig2: *LEA*, CL315Contig2: *Lti*, CL183Contig1: *P5CS*) but also genes of unknown function were included. The digital expression analysis using the public gene expression database showed that some of these genes are also induced by abiotic stress in other plant species such as *Arabidopsis* and rice; for example, orthologs of CL3451Contig1 (U-box domain containing protein), CL1910Contig1 (electron transfer flavoprotein-ubiquinone), and CL1855Contig1 (F-box containing protein) (Fig. S4a). These results suggest conservation of the stress inducibility of these genes and that they might function in novel plant stress-tolerance mechanisms.

In contrast, to verify the gene expression profile under specific abiotic stress conditions, we amplified by qPCR well-known stress-responsive genes in diverse biological pathways and compared the results with the in silico data (Fig. 4b). For example, transcripts of ice recrystallization inhibition protein (*IRIP*; CL3383Contig1), actin depolymerizing factor-like protein (*ADF-like*; CL887Contig2), and GIGANTEA-like protein (*GI-like*, CL2959Contig2) exhibited strong (>30-fold) induction by cold. The levels of transcripts of inner membrane translocase subunit (*TIM*; CL2053Contig2) and isovaleryl Co-A dehydrogenase (*IVD*; CL1871Contig1) were increased by both NaCl and PEG. Additionally, several genes showed PEG-specific induction. The transcripts of dehydrin 1 (*DHN1*; CL157Contig4), late embryogenesis abundant protein (*LEA*; CL2607Contig1) and mitochondrial alternative oxidase (*AOX*; CL771Contig2) exhibited specific induction by PEG. These results were in agreement with the expected expression profiles obtained by DEG profiling, and with the fact that the stress response is mediated by various components of a number of gene regulatory pathways.

Discussion

Many wild plant species have evolved tolerance to cold, drought, and salinity. Such species represent an important genetic reservoir that can be exploited to improve crop yields in the face of unpredictable climate changes. *D. antarctica*, an Antarctic vascular plant, is an important extremophile that survives on marginal land and that has been studied extensively for several decades. An increase in the *D. antarctica* population in the maritime Antarctic is an important parameter for detection of global warming (Lewis Smith 1994; Alberdi et al. 2002). It is believed that

Author's personal copy

Table 4 GO enrichment analysis of differentially expressed transcripts in the stress-induced transcriptome

Term	ID	Category	DEG[b] (%)	Whole[a] (%)	*P* value
Up-regulated					
Response to water	GO:0009415	P	2	0.8	0.0003
Response to cold	GO:0009409	P	2.2	1	0.0011
Starch metabolic process	GO:0005982	P	1.1	0.3	0.0017
Response to abiotic stimulus	GO:0009628	P	6.6	4.4	0.0025
Response to water deprivation	GO:0009414	P	1.7	0.7	0.0027
Starch biosynthetic process	GO:0019252	P	0.7	0.2	0.0041
Multidrug transport	GO:0006855	P	0.9	0.3	0.0046
Response to temperature stimulus	GO:0009266	P	2.9	1.6	0.0051
Oxidoreductase activity	GO:0016705	F	1.5	0.6	0.0058
Amylase activity	GO:0016160	F	0.6	0.1	0.006
Response to drug	GO:0042493	P	0.9	0.3	0.0076
Lipid binding	GO:0008289	F	1.5	0.6	0.008
Response to chemical stimulus	GO:0042221	P	8.6	6.5	0.0084
Cellular amino acid catabolic process	GO:0009063	P	0.9	0.3	0.012
Glutamine family amino acid metabolic process	GO:0009064	P	1.2	0.5	0.013
Response to abscisic acid stimulus	GO:0009737	P	1.8	0.9	0.014
Down-regulated					
Ribonucleoprotein complex	GO:0030529	C	14.8	3.7	1.5×10^{-24}
Translation	GO:0006412	P	15.8	4.2	2.0×10^{-24}
Thylakoid	GO:0009579	C	9.8	2.8	3.0×10^{-14}
Photosynthetic membrane	GO:0034357	C	7.9	2.1	9.3×10^{-13}
Chloroplast part	GO:0044434	C	14.1	5.6	9.9×10^{-13}
Chloroplast	GO:0009507	C	22.9	12.7	8.4×10^{-11}
Photosynthesis	GO:0015979	P	6.4	1.7	2.5×10^{-10}
Gene expression	GO:0010467	P	22.2	12.3	2.5×10^{-10}
Photosystem I	GO:0009522	C	3	0.4	6.9×10^{-10}
Photosynthesis, light reaction	GO:0019684	P	4.3	0.9	2.8×10^{-9}
Photosynthesis, light harvesting	GO:0009765	P	3.2	0.5	4.5×10^{-9}
Chlorophyll binding	GO:0016168	F	2.3	0.3	1.6×10^{-7}
Protein-chromophore linkage	GO:0018298	P	2.3	0.3	2.0×10^{-7}
Photosystem II	GO:0009523	C	2.8	0.5	4.3×10^{-7}
Nucleosome	GO:0000786	C	2.1	0.5	8.9×10^{-5}
Chromatin assembly	GO:0031497	P	2.1	0.5	0.00015

Total number of down-regulated DEGs with GO terms: 802

P biological processes, *F* molecular function, *C* cellular components

[a] Total number of ESTs annotated with GO terms in this study: 26,477

[b] Total number of up-regulated DEGs (differentially expressed genes) with GO terms: 1,297

the Antarctic terrestrial environment inhibits successful colonization by new plant species because it consists of isolated islands interspersed with glaciers, with an extremely harsh climate. However, several uncontrolled experimental and accidental vascular plant introductions have occurred in the Antarctic; indeed, *Poa annua* successfully colonized King George Island (Chwedorzewska 2009). But its distribution is restricted to the colonization area, and *D. antarctica* remains the representative vascular plant in the maritime Antarctic. Despite the ecological importance of *D. antarctica*, like most other non-model plants, the genetic and genomic resources necessary for molecular studies are limited. In this study, we analyzed the *D. antarctica* transcriptome to generate molecular resources for further study of this Antarctic vascular plant. Gene annotation revealed that 13 % of sequences (7,687) did not

Author's personal copy

Table 5 Identification of carbon fixation enzymes in the *D. antarctica* transcriptome and three model organisms

Sequence ID	Sequence annotation	EC number	bp	% Amino acid identity corresponding enzymes in model organisms		
				Arabidopsis	Rice	Barley
CL3909Contig1	Ribulose-bisphosphate carboxylase chain	EC:4.1.1.39	896	91	98	98
CL20315Contig1	Ketose-bisphosphate aldolase	EC:4.1.2.13	347	75	93	95
CL1221Contig1	Malate dehydrogenase (decarboxylating)	EC:1.1.1.39	1212	74	92	97
CL18Contig1	Phosphoribulokinase precursor	EC:2.7.1.19	1627	81	92	97
CL73Contig2	Sedoheptulose- bisphosphatase	EC:3.1.3.37	1270	87	94	98
CL193Contig2	Malate dehydrogenase (nadp +)	EC:1.1.1.82	1318	86	93	96
CL261Contig1	Aspartate aminotransferase	EC:2.6.1.1	1304	83	94	–
CL1716Contig1	Malate dehydrogenase (oxaloacetate-decarboxylating)	EC:1.1.1.40	946	73	92	95
CL86Contig1	Ribulose-phosphate 3-epimerase	EC:5.1.3.1	1305	82	86	–
CL1Contig342	Phosphoglycerate kinase	EC:2.7.2.3	1502	88	94	96
CL224Contig3	Fructose-bisphosphatase	EC:3.1.3.11	1209	83	91	94
CL31Contig3	Triosephosphate isomerase	EC:5.3.1.1	1244	80	89	93
CL515Contig1	NAD-malate dehydrogenase	EC:1.1.1.37	1506	74	90	92
CL208Contig1	Pyruvate kinase	EC:2.7.1.40	1973	87	94	98
CL272Contig2	Phosphoenolpyruvate carboxylase	EC:4.1.1.31	737	85	94	96
CL11Contig1	Transketolase	EC:2.2.1.1	2587	83	91	97
CL2087Contig1	Pyruvatephosphate dikinase	EC:2.7.9.1	1498	80	91	96
CL302Contig1	Ribose-5-phosphate isomerase	EC:5.3.1.6	813	75	92	94
CL2236Contig1	Phosphoenolpyruvate carboxykinase	EC:4.1.1.49	633	80	88	96
CL1Contig409	Glyceraldehyde-3-phosphate dehydrogenase	EC:1.2.1.13	1648	82	91	95
CL283Contig2	Alanine aminotransferase	EC:2.6.1.2	1841	76	89	93

match any known sequences in the existing database, even in BLASTN searches. It is possible that these poorly conserved transcripts were derived from novel gene clusters, non-coding RNA genes, or novel spliced exons, which may be specific to *D. antarctica*. The overall GC content distribution pattern was more similar to that of rice, a monocot species, than those of dicots such as *Arabidopsis* and soybean, which have unimodal distributions (Wang and Hickey 2007).

Molecular markers in the *D. antarctica* transcriptome could potentially be applied to several different research fields, such as evolutionary genetics, comparative genomics, and functional genomics of adaptive traits. The order of *D. antarctica* SSRs based on base pairs is tri-, mono-, and dinucleotides (Table 2). The abundance of the tri-nucleotide repeat motif in cereal species is very high. It is considered a specific feature of monocot genomes, and may be due to codon usage bias (Thiel et al. 2003; Peng and Lapitan 2005). Although 454 sequencing is less accurate in homopolymer regions (Margulies et al. 2005), it is capable of generating a pilot pool of molecular markers for comparative mapping and evolutionary studies of the historical distribution of *D. antarctica*.

DEG screens of transcriptomes using next-generation sequencing technology have reported good correlations with qPCR and microarray-based analyses (Rothberg and Leamon 2008; Wang et al. 2009; Wilhelm and Landry 2009). Since several studies have shown that *D. antarctica* has high phenotypic plasticity in different habitats and its phenotype is strongly influenced by abiotic factors (Gielwanowska et al. 2005; Lee et al. 2008), screening DEGs specific to stress conditions is important for elucidation of the adaptive mechanisms. In this study, the expression of a number of genes (5.1 %, 3,110/60,765) was altered by cold, PEG, or NaCl (Table S6). The majority of transcriptome changes were stressor-specific and not part of a general response to abiotic stresses. Among the total of 3,110 DEGs, 5.6 % were shared by all three stressors. The shared responses were reduced to <1.0 % for upregulated DEGs. This picture of predominately stress-specific responses is analogous to that observed in a comparison of drought- and NaCl-stressed barley (Ozturk et al. 2002) and *Arabidopsis* transcriptome changes in response to cold, mannitol, and NaCl stresses (Kreps et al. 2002).

An important feature of this study was comparison of multiple stressors to identify potential stimulus-specific responses. In the case of cold, 585 stimulus-specific DEGs were identified from 677 total DEGs. Expression of the *IRIP* gene (CL3383Contig1), a known contributor to *D. antarctica* cryotolerance, was increased by cold in both

Author's personal copy

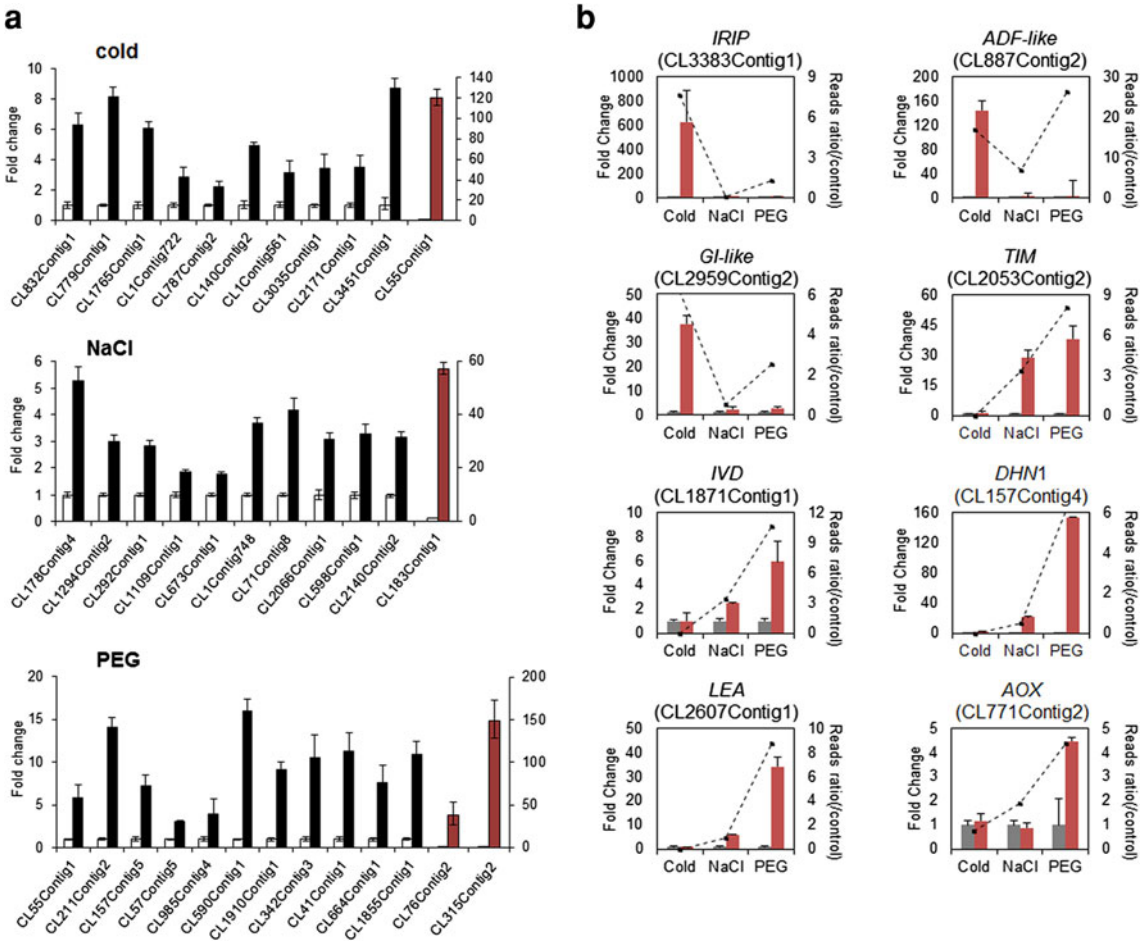

Fig. 4 qPCR analysis of selected *D. antarctica* genes following exposure to different abiotic stressors. **a** qPCR analysis results of 35 stress-induced genes randomly selected from among those with high expression levels ($Q < 10^{-5}$). The genes shown were significantly induced by abiotic stress (T test, $P < 0.05$). *White and black bars* indicate control and treatment samples, respectively. The *left vertical axis* indicates the relative ratio of the transcript abundance of the selected gene compared to the abundance of the transcripts of the internal *DaEF1A* and *DaCYP* controls. The *right vertical axis* is for genes represented by *red, filled bars*. **b** qPCR results of known stress-responsive genes under cold-, NaCl-, or PEG-stress conditions and comparison with the in silico data. *Grey* and *red bars* indicate the expression levels of the genes in the control and treatment samples, respectively. The *left vertical axis* indicates the relative ratio of the transcript abundance of the selected gene compared to the abundance of the transcripts of the internal *DaEF1A* and *DaCYP* controls. The *right vertical axis* represents the relative ratios of read counts between the control and treatment samples. Data and *error bars* represent means and standard deviations, respectively of three technical replicates. Total RNA was extracted from samples of plants grown under control conditions (15 °C, no treatment) or exposed to a low temperature (cold 4 °C), dehydration (PEG 30 %), or high salinity (NaCl 300 mM). Three biological replicates were performed for each experiment; similar results were obtained. The unigene IDs and primer sequences of the genes tested by qPCR are listed in Table S1

the in silico expression and qPCR analyses, which is in agreement with previous reports (John et al. 2009). *GI* (CL2959Contig2) encodes a novel nuclear protein that regulates several developmental processes, including circadian clock function, light responses, and carbohydrate metabolism (Dalchau et al. 2011). The possible involvement of *GI* in the regulation of the cold-stress response has been reported; *GI* mRNA expression is specifically induced by low temperatures in both *Brachypodium* and *Arabidopsis* (Cao et al. 2005; Hong et al. 2010). In addition, it has been suggested that *GI* may function in input pathways to the clock that transmits both light and temperature signals, because C-repeat binding factor gene expression is gated by a circadian clock (Cao et al. 2005). The strong induction of *GI* during cold acclimation in *D. antarctica* is consistent with the hypothesis of the transcriptional regulation of *GI* by the circadian clock and a cold signaling pathway. Therefore, investigation of the mechanisms by which Antarctic plants integrate seasonal environmental information, including photoperiod, which differs greatly between seasons (ranges from constant light to constant darkness), and freezing temperatures, is warranted.

Previous reports proposed that osmotic and salt stress share signaling components and target genes for activation,

Author's personal copy

and that each stimulus provides quite different information resulting in the need for multiple sensors and complex signaling mechanisms (Kreps et al. 2002; Xiong et al. 2002; Zhu 2002). In this study, 81 genes responded to the PEG–NaCl combination. Of these, *IVD* (CL1871Contig1) encodes an isovaleryl CoA dehydrogenase that is located in mitochondria and oxidizes intermediates of leucine and valine catabolism (Däschner et al. 2001). Its regulation by aluminum stress at the proteome level has been reported previously (Zhou et al. 2009). Another gene was similar to the mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family, which is important for stress tolerance (Lister et al. 2004). Nevertheless, the majority of NaCl or PEG changes appeared to be stimulus specific. PEG induced 892 genes, and NaCl induced 701 genes. Among the PEG-specific genes, *LEA* and *DHN1*, general molecular markers for the water stress response (Welin et al. 1994), showed a high level of induction by PEG. Additionally, expression of the gene encoding the mitochondrial protein AOX (CL771Contig2) was strongly induced by PEG. AOX is thought to regulate cellular stress responses by preventing excess mitochondrial reactive oxygen species production, and is transcriptionally regulated by multiple stressors (Van Aken et al. 2009). Thus, our findings suggest that the mitochondrial stress response is involved in stress tolerance in *D. antarctica* and that the stress-response system is conserved among plant taxa.

Carbon fixation is the process by which CO_2 is incorporated into organic compounds during photosynthesis. Higher plants usually have a broad optimal temperature range for photosynthesis, which generally corresponds to the average daytime temperatures in their natural climates (Salvucci and Crafts-Brandner 2004). The responses of photosynthesis and growth to changes in temperature differ between species. It has been proposed that improving photosynthesis would increase crop yields. There have been attempts to increase photosynthetic yields through bioengineered modification of carbon fixation enzymes (Kajala et al. 2011; Raines 2011). Previous studies reported that *D. antarctica* has a much lower optimum temperature for photosynthesis than other plants from temperate regions, supporting adaptation of enzyme function and evolution of the corresponding gene sequences (Edward and Lewis Smith 1988; Xiong et al. 2000). To identify differences among carbon fixation pathway components at the molecular level, we screened genetic sequences from the transcriptome data and identified 21 enzymes related to photosynthetic activity in *D. antarctica*. Among them, those encoding the enzymes NAD-malate dehydrogenase and alanine aminotransferase showed relatively low identity with the coding sequences in other species (Table 5), suggesting that they contribute to the differences in photosynthetic abilities among species. It would be interesting to compare the conservation of these sequences in species from different habitats and to determine the relationship between sequence differences and the temperature dependence of photosynthetic activity.

Expression of *PEPC* found to be increased by both cold and PEG in *D. antarctica*. PEPC (EC 4.1.1.31) is an important enzyme that acts at a crucial branch point in plant carbohydrate metabolism (O'Leary et al. 2011). Several reports have described a correlation between the induction of genes involved in carbon metabolism and external stimuli, including *PEPC*. While the regulation of enzyme activity according to developmental stage or by external stimuli is known to be mainly mediated at the post-translational level (Vidal and Chollet 1997), mRNA expression of the *Arabidopsis PEPC* family member *Atppc4* is induced by salt and drought stress (Sánchez et al. 2006), and expression of the wheat *PEPC* gene is induced by drought and cold stress (González et al. 2003). In maize, under short-term, moderate water stress induced by sorbitol, the activity of PEPC in maize leaf discs increases by 50 %; proline levels also increase (Rodriguez-Penagos and Munoz-Clares 1999). Based on these reports, upregulation of *PEPC* in *D. antarctica* appears to be an integral component of responses to Antarctic environmental stressors. Its molecular mechanism of action should be explored.

Transcripts of several *D. antarctica* genes involved in photosynthesis, such as chlorophyll *a*- and *b*-binding protein, Rubisco small subunit, and photosystem I reaction center subunit, were repressed by abiotic stressors. This agrees with previous transcriptome studies which demonstrated that plants under drought-, high salt-, and cold-stress downregulate the expression of genes involved in photosynthesis (Seki et al. 2002; Wong et al. 2006). The expression of these genes is significantly decreased in plants growing in the Antarctic, suggesting that defects in photosynthetic activity in the Antarctic are associated with abiotic stressors (Lee et al. 2008). Among other genes downregulated by abiotic stressors, proline dehydrogenase gene (*ProDH*, CL3059Contig1) encodes the first enzyme of the proline catabolism pathway, which catalyzes oxidation of proline to pyrroline-5-carboxylate in mitochondria. *ProDH* expression is downregulated by low water potential, dehydration, and salinity, which is accepted as a control point that promotes proline accumulation under stress conditions (Verslues and Sharma 2010). Antisense suppression of *ProDH* resulted in increased proline and stress tolerance in one study (Nanjo et al. 1999). Further research into the *D. antarctica ProDH* expression pattern in the Antarctic might supply further information regarding the proline biosynthesis feedback environmental adaptation strategy.

The generation of a transcriptome dataset provides an important tool for investigating how a non-model organism responds to environmental changes, and we hope that it

Author's personal copy

will stimulate genomic research. In this study, we conducted a global analysis of the stress-responsive transcriptome of *D. antarctica*, an Antarctic flowering plant, through massive parallel pyrosequencing. The *D. antarctica* stress-response profiles will aid understanding of how plants can achieve a high level of tolerance against environmental extremes and will enable us to address fundamental genetic questions underlying adaptation to environmental challenges during climate change.

Acknowledgments This work was supported by Functional Genomics on Polar Organisms grant (PE12020) funded by Korea Polar Research Institute (KOPRI).

References

Alberdi M, Bravo LA, Gutierrez A, Gidekel M, Corcuera LJ (2002) Ecophysiology of Antarctic vascular plants. Physiol Plant 115:479–486

Bravo LA, Griffith M (2005) Characterization of antifreeze activity in Antarctic plants. J Exp Bot 56:1189–1196

Bravo LA, Ulloa N, Zuñiga GE, Casanova A, Corcuera LJ, Alberdi M (2001) Cold resistance in Antarctic angiosperms. Physiol Plant 111:55–65

Cao S, Ye M, Jiang S (2005) Involvement of *GIGANTEA* gene in the regulation of the cold stress response in *Arabidopsis*. Plant Cell Rep 24:683–690

Chou HH, Holmes MH (2001) DNA sequence quality trimming and vector removal. Bioinformatics 17:1093–1104

Chwedorzewska KJ (2009) Terrestrial Antarctic ecosystems in the changing world: an overview. Pol Polar Res 30:263–276

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676

Dalchau N, Baek SJ, Briggs HM, Robertson FC, Dodd AN, Gardner MJ, Stancombe MA, Haydon MJ, Stan GB, Goncalves JM, Webb AA (2011) The circadian oscillator gene *GIGANTEA* mediates a long-term response of the *Arabidopsis thaliana* circadian clock to sucrose. Proc Natl Acad Sci USA 108:5104–5109

Däschner K, Couée I, Binder S (2001) The mitochondrial isovaleryl-coenzyme A dehydrogenase of Arabidopsis oxidizes intermediates of leucine and valine catabolism. Plant Physiol 126:601–612

Du Z, Zhou X, Ling Y, Zhang Z, Su Z (2010) agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res 38:W64–W70

Edgington ES (1995) Randomization tests, 3rd edn. Marcel-Dekker, New York

Edward JA, Lewis Smith RI (1988) Photosynthesis and respiration of *Colobanthus quitensis* and *Deschampsia antarctica* from the maritime Antarctic. BAS Bull 81:43–63

Gielwanowska I, Szczuka E, Bednara J, Gorecki R (2005) Anatomical features and ultrastructure of *Deschampsia antarctica* (Poaceae) leaves from different growing habitats. Ann Bot 96:1109–1119

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

González MC, Sánchez R, Cejudo FJ (2003) Abiotic stresses affecting water balance induce phosphoenolpyruvate carboxylase expression in roots of wheat seedlings. Planta 216:985–992

Hong S-Y, Lee S, Seo P, Yang M-S, Park C-M (2010) Identification and molecular characterization of a *Brachypodium distachyon GIGANTEA* gene: functional conservation in monocot and dicot plants. Plant Mol Biol 72:485–497

Iordachescu M, Imai R (2008) Trehalose biosynthesis in response to abiotic stresses. J Integr Plant Biol 50:1223–1229

John UP, Polotnianka RM, Sivakumaran KA, Chew O, Mackin L, Kuiper MJ, Talbot JP, Nugent GD, Mautord J, Schrauf GE, Spangenberg GC (2009) Ice recrystallization inhibition proteins (IRIPs) and freeze tolerance in the cryophilic Antarctic hair grass *Deschampsia antarctica* E. Desv. Plant Cell Environ 32:336–348

Kajala K, Covshoff S, Karki S, Woodfield H, Tolley BJ, Dionora MJA, Mogul RT, Mabilangan AE, Danila FR, Hibberd JM, Quick WP (2011) Strategies for engineering a two-celled C4 photosynthetic pathway into rice. J Exp Bot 62:3001–3010

Kanehisa M, Goto S (2000) KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27–30

Kreps J, Wu Y, Chang H, Zhu T, Wang X, Harper J (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol 130:2129–2141

Lara A, Pérez-Trabado G, Villalobos D, Díaz-Moreno S, Cantón F, Claros M (2007) A web tool to discover full-length sequences: full-lengther. In: Corchado E, Corchado J, Abraham A (eds) Innovations hybrid intelligent systems. Springer, Berlin, pp 361–368

Lee H, Cho HH, Kim I-C, Yim JH, Lee HK, Lee YK (2008) Expressed sequence tag analysis of Antarctic hairgrass *Deschampsia antarctica* from King George Island, Antarctica. Mol Cells 25:258–264

Lee H, Kim JH, Park M, Kim I-C, Yim JH, Lee HK (2010) Reference genes validation for qPCR normalization in *Deschampsia antarctica* during abiotic stresses. Antarct Sci 22:477–484

Lewis Smith RI (1994) Vascular plants as bioindicators of regional warming in Antarctica. Oecologia 99:322–328

Lister R, Chew O, Lee MN, Heazlewood JL, Clifton R, Parker KL, Millar AH, Whelan J (2004) A transcriptomic and proteomic characterization of the Arabidopsis mitochondrial protein import apparatus and its response to mitochondrial dysfunction. Plant Physiol 134:777–789

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376–380

Michael BE, Kaufmann MR (1973) The osmotic potential of polyethylene glycol 6000. Plant Physiol 51:914–916

Morgante M, Hanafey M, Powell W (2002) Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. Nat Genet 30:194–200

Nanjo T, Kobayashi M, Yoshiba Y, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett 461:205–210

Novaes E, Drost DR, Farmerie WG, Pappas GJ Jr, Grattapaglia D, Sederoff RR, Kirst M (2008) High-throughput gene and SNP discovery in *Eucalyptus grandis*, an uncharacterized genome. BMC Genomics 9:312

O'Leary B, Park J, Plaxton WC (2011) The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological functions and post-translational controls of non-photosynthetic PEPCs. Biochem J 436:15–34

Author's personal copy

Ozturk ZN, Talamé V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol 48:551–573

Parchman TL, Geist KS, Grahnen JA, Benkman CW, Buerkle CA (2010) Transcriptome sequencing in an ecologically important tree species: assembly, annotation, and marker discovery. BMC Genomics 11:180

Peng JH, Lapitan NL (2005) Characterization of EST-derived microsatellites in the wheat genome and development of eSSR markers. Funct Integr Genomics 5:80–96

Pérez-Torres E, Bascuñán L, Sierra A, Bravo L, Corcuera L (2006) Robustness of activity of Calvin cycle enzymes after high light and low temperature conditions in Antarctic vascular plants. Polar Biol 29:909–916

Pertea G, Huang X, Liang F, Antonescu V, Sultana R, Karamycheva S, Lee Y, White J, Cheung F, Parvizi B, Tsai J, Quackenbush J (2003) TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. Bioinformatics 19:651–652

Raines CA (2011) Increasing photosynthetic carbon assimilation in C3 plants to improve crop yield: current and future strategies. Plant Physiol 155:36–42

Rodriguez-Penagos M, Munoz-Clares R (1999) Response of phosphoenolpyruvate carboxylase from maize leaves to moderate water deficit. J Plant Physiol 155:631–638

Rothberg JM, Leamon JH (2008) The development and impact of 454 sequencing. Nat Biotechnol 26:1117–1124

Salvucci ME, Crafts-Brandner SJ (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of rubisco activase in plants from contrasting thermal environments. Plant Physiol 134:1460–1470

Sánchez R, Flores A, Cejudo FJ (2006) *Arabidopsis* phosphoenolpyruvate carboxylase genes encode immunologically unrelated polypeptides and are differentially expressed in response to drought and salt stress. Planta 223:901–909

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31:279–292

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417

Storey JD (2002) A direct approach to false discovery rates. J R Stat Soc B 64:479–498

Thiel T, Michalek W, Varshney RK, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor Appl Genet 106:411–422

Van Aken O, Giraud E, Clifton R, Whelan J (2009) Alternative oxidase: a target and regulator of stress responses. Physiol Plant 137:354–361

Vera JC, Wheat CW, Fescemyer HW, Frilander MJ, Crawford DL, Hanski I, Marden JH (2008) Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Mol Ecol 17:1636–1647

Verslues PE, Sharma S (2010) Proline metabolism and its implications for plant-environment interaction. Arabidopsis Book 8:e0140

Vidal J, Chollet R (1997) Regulatory phosphorylation of C4 PEP carboxylase. Trends Plant Sci 2:230–237

Wang H-C, Hickey D (2007) Rapid divergence of codon usage patterns within the rice genome. BMC Evol Biol 7:S6

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10:57–63

Welin BV, Olson A, Nylander M, Palva ET (1994) Characterization and differential expression of *dhn/lea/rab*-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. Plant Mol Biol 26:131–144

Wilhelm BT, Landry J-R (2009) RNA-Seq—quantitative measurement of expression through massively parallel RNA-sequencing. Methods 48:249–257

Wong CE, Li Y, Labbe A, Guevara D, Nuin P, Whitty B, Diaz C, Golding GB, Gray GR, Weretilnyk EA, Griffith M, Moffatt BA (2006) Transcriptional profiling implicates novel interactions between abiotic stress and hormonal responses in Thellungiella, a close relative of Arabidopsis. Plant Physiol 140:1437–1450

Xiong FS, Mueller EC, Day TA (2000) Photosynthetic and respiratory acclimation and growth response of Antarctic vascular plants to contrasting temperature regimes. Am J Bot 87:700–710

Xiong L, Schumaker KS, Zhu J-K (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14:S165–S183

Zhou S, Sauvé R, Thannhauser TW (2009) Proteome changes induced by aluminium stress in tomato roots. J Exp Bot 60:1849–1857

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273

Zuñiga GE, Alberdi M, Corcuera LJ (1996) Non-structural carbohydrates in *Deschampsia antarctica* Desv. from South Shetland Islands, maritime Antarctic. Environ Exp Bot 36:393–399