Mol. Cells, Vol. 25, No. 2, pp. 258-264

Molecules and Cells

Expressed Sequence Tag Analysis of Antarctic Hairgrass *Deschampsia antarctica* from King George Island, Antarctica

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(Received September 19, 2007; Accepted October 30, 2007)

Deschampsia antarctica **is the only monocot that thrives in the tough conditions of the Antarctic region. It is an invaluable resource for the identification of genes associated with tolerance to various environmental pressures. In order to identify genes that are differentially regulated between greenhouse-grown and Antarctic field-grown plants, we initiated a detailed gene expression analysis. Antarctic plants were collected and greenhouse plants served as controls. Two different cDNA libraries were constructed with these plants. A total of 2,112 cDNA clones was sequenced and grouped into 1,199 unigene clusters consisting of 243 consensus and 956 singleton sequences. Using similarity searches against several public databases, we constructed a functional classification of the ESTs into categories such as genes related to responses to stimuli, as well as photosynthesis and metabolism. Real-time PCR analysis of various stress responsive genes revealed different patterns of regulation in the different environments, suggesting that these genes are involved in responses to specific environmental factors.**

Keywords: Abiotic Stress; Antarctic; *Deschampsia antarcitca*; Expressed Sequence Tags (ESTs); King George Island; Quantitative Real Time Reverse Transcription PCR (qRT-PCR).

Introduction

The Antarctic is the coldest region of the world, with 86% of the earth's ice (Alberdi et al., 2002), and it is a most extreme environment for terrestrial plants. Maximum usually range between 0 and 6°C in January (summer), while the minimum range is between 0°C and −6°C with a mean of 0°C (Alden and Hermann, 1971). Critical factors for plant growth in this habitat are day length and irradiance. Due to the high latitude, significant differences in levels of solar radiation and in day length occur between summer and winter. In the growing season (December to February), day length is about 20 h, and irradiance in the Maritime Antarctic can reach > 2,000 $\mu Mm^{-2}s^{-1}$ in summer and 50 $\mu Mm^{-2}s^{-1}$ in winter (Schroeter et al., 1995).

Environmental conditions have restricted the number of native flowering plants observed to only two species - *Colobanthus quitensis* (Caryophyllaceae) and *Deschampsia antarctica* Desv. (Poaceae). *D. antarctica*, Antarctic hairgrass, is the only natural grass species growing in the Maritime Antarctic (Edwards and Lewis-Smith, 1988). Abiotic factor effects on physiological and biochemical processes have been the focus of intensive studies using *D. antarctica*. Edwards and Lewis-Smith (1988) investigated the photosynthetic temperature response in this species and observed a system well adapted to low temperatures; the optimum temperature for net photosynthesis was 13°C in detached leaves, and leaves retained 30% of their maximum photosynthetic capacity at 0°C. Accumulation of the amino acid proline and non-structural carbohydrates (sucrose and fructans) was observed in leaves during the growth period (summer), and these macromolecules helped maintain a high degree of stress tolerance (Bravo et al., 2001). Antifreeze activity was observed in the non-acclimated state, and it increased after cold acclimation, indicating that the plant produces antifreeze proteins, which are secreted into the apoplast (Bravo and Griffith, 2005). Polar lipid content and degree of fatty acid unsaturation in lipid fractions did not significantly differ from other Gramineae species (Zúñiga, 1994). Convincing evidence of unique metabolic adaptation or survival strategies, differing from other cold- or frost-tolerant plants, is lacking for *D. artarctica*. Recently, Perez-Torres et al. (2007) suggested the presence of a

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photoprotective mechanism under excess light where oxygen is a sink for excitation energy.

Expressed sequence tags (ESTs), generated by large-scale single-pass sequencing of random cDNA clones, are essential tools for identifying novel genes and for evaluating environmental stimuli that affect gene expression. They can be screened with DNA and protein sequence databases in order to assign putative functions based on homology. EST analysis is widely used to gain insight into complex plant processes that are affected by, or resistant to, various stress-associated conditions including salt tolerance (Wang et al., 2007), cold acclimation (Brautigam et al., 2005), and abiotic stress (Ramalingam et al., 2006). Gidekel et al. (2003) identified three genes that were differentially regulation during cold-acclimation, using a combination of proteomics and mRNA differential display. In the present study, we used the EST approach to identify genes in *D. antarctica* that are involved in adaptation to the Antarctic environment. Our focus was on the identification of stress-responsive genes differentially expressed under Antarctic conditions.

Materials and Methods

Plant materials *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, during two Antarctic summers (January 2006 and 2007). The sampling site was about 30 m from the seashore near an area, frequented by Antarctic animals, that is enriched with nutrients and subjected to sea spray and flooding. Samples were immediately ground in TRI reagent (Molecular Research Center, USA), but some plants were placed in plastic containers and transported to Korea, where they were cultivated in clay sand with day/night cycles of natural sunlight to produce greenhouse samples. The temperature of the greenhouse was maintained at 20-25°C and plants were watered three times a day during the experimental period.

RNA extraction and cDNA library construction Total RNA was extracted from whole plants using TRI reagent according to the manufacturer's protocol supplemented by an RNase-free DNase (Promega, USA) treatment. RNA concentration was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, USA), and its integrity was assessed by formaldehyde gel electrophoresis. A cDNA library of the greenhouse samples was constructed using the lambda-ZAP system (Stratagene, USA). Because of the small amount of sample available, the cDNA library for the Antarctic samples was generated using a SMART cDNA library construction kit (Clontech, USA).

Single-pass sequencing Clones for 5′ end sequencing were randomly selected from the Antarctic and greenhouse libraries, plasmid DNA was purified from each clone and sequencing reactions were run on an ABI 3730 XL DNA sequencer (Perkin-Elmer Applied Biosystems, USA).

Sequence processing Processing was conducted using GeneMaster Ver. 3.0 (Ensoltech, Korea). Each EST was first processed individually using a multimodule custom pipeline that linked sequence backup, base calling, elimination of sequences shorter than 100 bp (and low-quality sequences), vector trimming and sequence assembly. The BLASTX algorithm (Altschul et al., 1990) was used to assign predicted functions to assembled consensus sequences based on homology, as described previously (Lee et al., 2004). Three categories of sequences were defined: 'Known', 'Unknown', and 'No hit'. 'Known' indicates a query sequence with a BLAST match score > 80 and an e-value of < 10^{-14}. 'Unknown' sequences had a score of 40−80, and an e-value of 10^{-2}–10^{-14}. 'No hit' sequences had a score < 40 with an e-value > 10^{-2}. Finally, functional categories were defined using the Gene Ontology (GO) Database (www.geneontology.org) and the BLASTX algorithm. In order to compare the distribution of genes in the various functional categories in the two libraries, we applied Fisher's exact test as described previously (Schmitt et al., 1999).

Quantitative, real-time RT-PCR Quantitative real-time PCR (qRT-PCR) was performed to analyze the expression of selected genes in the two samples using gene-specific PCR primers (Table 4). Total RNA (1 μg) was used as template for reverse transcription with the QuantiTect system (Qiagen, USA) and qRT-PCR was conducted using a QuantiTect SYBR Green PCR Kit (Qiagen), in a total volume of 10 μl on a Rotor-Gene 6000 PCR machine (Corbett, Australia). A melting curve was recorded at the end of every run to assess product specificity. PCR conditions (primer concentrations and cDNA quantity) were optimized for each target gene, and PCR efficiency was determined. Experimental samples were evaluated in triplicate and qRT-PCR reactions for each were run in duplicate. Target gene expression was normalized with α-tubulin gene and corrected according to the PCR efficiency value. The products of qRT-PCR were run on agarose gels and consistently had the predicted molecular weights.

Results and Discussion

Plant morphologies from different habitats *D. antarctica* has been the object of many studies due to its broad ecological range in the Antarctic and the unique climatic conditions of the Antarctic region. Gielwanowska et al. (2005) reported anatomical and ultrastructural differences between the leaves of *D. antarctica* plants from the wild, and those grown in a greenhouse; these included decreased length, irregular mesophyll cell structure and atypical ultrastructures. We also observed remarkable differences in morphology between greenhouse plants and

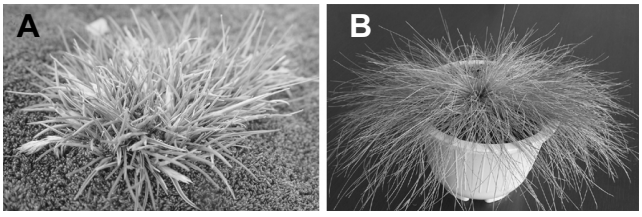

Fig. 1. The morphology of *D. antarctica* plants from different habitats. (A) a wild plant from the natural habitat of the Antarctic, and (B) a plant cultivated in the greenhouse.

those exposed to Antarctic conditions (Fig. 1).

Qualitative and cluster analyses of EST sequences A total of 2,112 cDNA clones were randomly selected from the libraries made from the two sets of plants, and 5′-ESTs were generated. Poor quality sequences, including sequences of < 100 bases, were removed, and the final EST number was 1,969. The Antarctic and greenhouse libraries produced 1,022 ESTs and 947 high-quality ESTs, with mean lengths of 551 and 668 bp, respectively (Table 1). Cluster analysis revealed 83 Antarctic library contigs and 697 singletons, with 780 unigenes and a redundancy level of 31.8%. Of all the Antarctic ESTs, 58% were longer than 500 bp. The greenhouse library ESTs contributed 163 contigs and 285 singletons, with 448 unigenes and a redundancy level of 69.9%. 77% of all the greenhouse ESTs, were longer than 500 bp. As for the combined EST database, a total of 1,969 original sequences were assembled and edited into 243 contigs and 956 singletons, representing 1,199 unigenes. Of these, 747 transcripts were present only in the Antarctic library and 420 others only in the greenhouse library, with 32 ESTs (2.67%) represented in both libraries. This points to a remarkable differences in the pattern of gene expression in Antarctic-grown plants compared to those grown in the greenhouse.

Dhanaraj et al. (2004) reported that a similar percentage (4.3%) of the total unigenes was shared between control and experimental libraries from blueberry floral buds, and this could be an underestimate. Another study using a larger (3X) EST dataset than we used (Bhalerao et al., 2003), found that 9.1% of total unigenes were shared between datasets consisting of ~5,000 ESTs in summer (young) and autumn (senescing) aspen leaf cDNA libraries. Collectively, these observations support the conclusion that the EST datasets obtained in the present study reflect the abundant genes expressed in the libraries.

Unigene identification The sequences of 1,199 unigenes, generated from the EST clustering, were translated in all possible reading frames and compared to the NCBI non-redundant protein database. The annotation results were exported into Microsoft Excel data sheet and manually

Table 1. EST cluster collection statistics and distribution according to the BLASTX search results.

cDNA library	Antarctic	Greenhouse	Combined
No. of cDNA sequenced	1,022	947	1,969
Cluster summary			
No. of singletons	697	285	956
No. of contigs	83	163	243
No. of unigenes	780	448	1,199
Annotation of unigenes			
Known	439 (56.3%)	280 (62.5%)	702 (58.5%)
Unknown	114 (14.6%)	43 (9.6%)	151 (12.6%)
No Hit	227 (29.1%)	125 (27.9%)	346 (28.9%)

sorted. Some 56.3–62.5% and 9.6–14.6% of the unigenes from the two libraries exhibited similarity to genes encoding ‘Known’ or ‘Unknown’ proteins, respectively. In addition, 27.9–29.1% of the ESTs had no similarity to any sequences in the database and were deemed ‘No hit’ (Table 1). It is worth noting that the sum of the ‘Unknowns’ and ‘No hits’ constitutes a higher percentage (43.7%) of the Antarctic library than of the greenhouse library (37.5%) (Table 1). The BLASTX search results for the 702 ‘Known’ transcripts showed a bias towards monocotyledon sequences (79.3%), especially those of rice (61.5%).

The most abundant ESTs Genes were defined as highly abundant when more than ten ESTs in the combined EST database were assigned to them. We did not perform a comparative analysis of the EST counts in the two libraries because of the large difference in redundancy beaten the libraries. The 13 most highly represented genes in the combined library are listed in Table 2. The most abundant were RuBisCO large chain and small subunit genes with 160 and 14 EST counts, respectively. In addition, chlorophyll a/b binding protein precursor and photosystem II 10K encoding genes were represented by 40 and 13 ESTs, respectively, suggesting that photosynthesis continues to provide the basic energy requirements for growth, since these genes are known to be highly expressed in plants. The third most abundant unigene encoded a RING zinc finger protein, represented by 20 ESTs. This protein family is involved in many developmental processes induced by stresses such as cold, dehydration, high salt and abscisic acid (Sakamoto et al., 2000). Metallothionein (MT) is a major cysteine-rich, metal-binding protein, and while there are some reports that it is induced by metal ions (e.g. copper), more evidence is needed to establish a relationship between environmental copper and cadmium levels and MT transcription (Hall, 2002). In *D. antarctica*, MT type 3 was represented by 19 ESTs, and was the fourth most abundant EST, consistent with an MT analysis in the

Table 2. Transcripts specific to Antarctic ESTs in the GO category 'Response to stimulus'.

Strongest BLASTX Hit	EST No.	Organism
pTACR7, cold-regulated protein	5	*Triticum aestivum*
Pathogenesis-related 1a	3	*Triticum monococcum*
Cold acclimation protein WCOR413-like protein	1	*Triticum aestivum*
Os12g0548700	1	*Oryza sativa* (japonica)
Unknown cold induced protein	1	*Deschampsia antarctica*
Conserved hypothetical protein	1	*Gibberella zeae* PH-1
Ozone-responsive stress-related protein-like	1	*Oryza sativa* (japonica)
Heat shock protein cognate 70	1	*Oryza sativa* (japonica)
MLA1-2, resistance protein	1	*Hordeum vulgare* subsp. Vulgare
HSP like protein	1	*Arabidopsis thaliana*
Leucine Rich Repeat family protein, expressed	1	*Oryza sativa* (japonica)
Cytosolic glutathione peroxidase	1	*Triticum monococcum*
ATP-dependent Clp protease ATP-binding subunit clpC	1	*Oryza sativa* (japonica)
Dehydrin	1	*Triticum turgidum* subsp. durum
Histone H2A	1	*Podospora anserina*
Os01g0170600	1	*Oryza sativa* (japonica)
Leucine Rich Repeat, putative	1	*Oryza sativa* (japonica)
Heat shock protein HSP81-1	1	*Arabidopsis thaliana*
Peroxidase 5 large chain	1	*Triticum monococcum*

alkali grass *Puccunellia tenuiflora* (Wang et al., 2007).

Functional classification using the Gene Ontology database The unigenes were matched to GO Consortium annotations, and GO terms were obtained for 592 unigenes. GO graphs using percentages of second level GO terms are presented under biological process categories. The three functional groups with the highest percentage of cDNAs in both libraries, in descending order, were: Cellular process > Cellular physiological process > Metabolism (Fig. 2). The most outstanding feature was a significant decrease in photosynthesis-associated genes (Fisher's exact test; $p = 0.043$) in the Antarctic library relative to the greenhouse library, suggesting defects in photosynthetic activity in the Antarctic, as might be expected. Expression of genes associated with cellular physiological processes, metabolism and stimulus response (88.4% of total) appeared to be increased in the Antarctic plants, as did stress response genes. Genes associated with localization, photosynthesis, regulation of biological process, other physiological process and development decreased in the Antarctic samples.

The relative abundance of "responses to stimuli" increased in the Antarctic samples as expected. Nineteen transcripts in the Antarctic EST library were in the category "Response to stimulus" (Table 2). They included genes thought to be related to cold (pTACR7 and WCOR413-like protein from wheat; unknown cold induced protein from *D. antarctica*), heat (Hsp70, Hsp81, and Hsp like protein), other abiotic stresses like ozone and drought (dehydrin), and biotic stresses (pathgenesis-related 1a, MLA1-2).

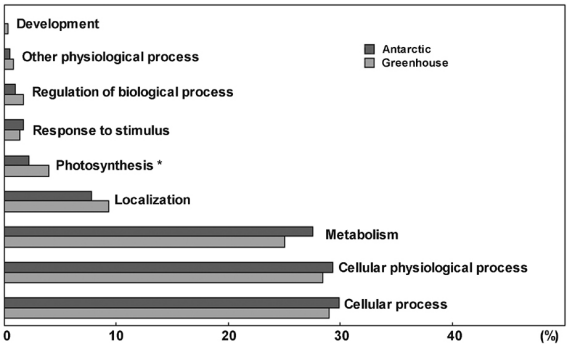

Fig. 2. Ten functional categories among the 592 identified unique transcripts. Annotated ESTs are distributed according to the functional categories developed by GO. The asterisk indicates significance ($p < 0.05$).

These results indicate that the Antarctic plants express these stress-related genes to survive in the severe Antarctic conditions.

qRT-PCR assessment of gene expression in the Antarctic and greenhouse samples In order to focus on stress response genes of plants from the Antarctica, candidate genes were manually selected from the EST database obtained in this study. Six genes designated DaURA1-6 (*D. antarctica* transcripts up-regulated in the Antarctic) were selected from the functional category 'Response to stimulus' (Fig. 2 and Table 2), based on published literature detailing plant stress responses, and used as targets for qRT-PCR differential expression analysis. The α-tubulin

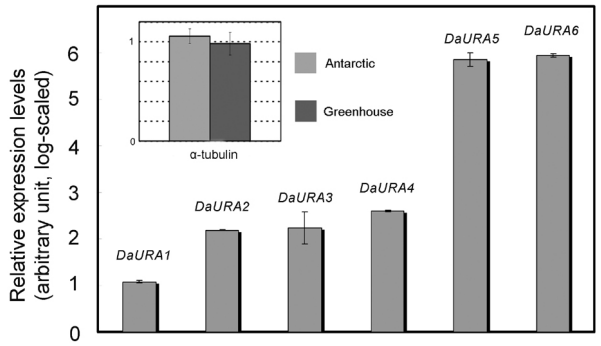

Fig. 3. qRT-PCR data for expression of selected genes in samples from the two different habitats. Relative levels were calculated by comparing gene expression in the Antarctic plants with the greenhouse plants (control) after normalization with the α-tubulin gene. Data are means of six experiments for Antarctic plants and presented as $\log_2$-scaled fold-differences of gene expression relative to greenhouse plants. Thus a value of 1 corresponds to two fold-overexpression in the Antarctic plants. The inset shows the expression of the α-tubulin gene in the Antarctic and greenhouse *D. antarctica* plants. *DaURA1*, ozone responsive protein; *DaURA2*, unknown cold resistant protein; *DaURA3*, heat shock protein 70; *DaURA4*, wheat cold resistant 413; *DaURA5*, pathogenesis related 1; *DaURA6*, wheat cold responsive protein.

gene was expressed at a similar level in both samples and was used as an internal control (Fig. 3). Of the six genes, three, *DaURA2, DaURA4* and *DaURA6,* were annotated as cold responsive genes. Expression of *DaURA4* increased by four-fold in the Antarctic plants relative to greenhouse plants. *DaURA4* showed 85% similarity to a cold acclimation protein gene *WCOR413* from wheat. *WCOR* genes were originally identified as low-temperature responsive (dehydrin-like), and were shown be involved in plasmalemma cryoprotection or dehydration protection (Danyluk et al., 1994). *WCOR413* was annotated as a cold-tolerance protein and also showed a cold induced expression pattern (Danyluk and Sarhan, 1996). Expression of *DaURA6* was increased 60-fold in the Antarctic plants, implying a role in cold-tolerance. *DaURA6* showed 63% similarity with *pTACR7* from wheat, which was originally cloned from hard red winter wheat where its expression increased in freeze-resistant plants relative to freeze-susceptible plants (Gana et al., 1997).

Several genes encoding response proteins to various stresses such as ozone (*DaURA1*, ozone responsive protein), heat (*DaURA3*, heat shock protein 70), and pathogen attack (*DaURA5*, pathogenesis related 1, PR-1) were identified. *DaURA1* exhibited 67% identity with an ozone-induced protein from Arabidopsis, and was two-fold induced in the Antarctic plants. Arabidopsis ozone-induced protein is known to be induced by ozone treatment and also by bacterial exposure (Sharma and Davis, 1995). It may be a broad range stress responsive gene involved in environmental stress protection. Heat shock proteins play a major role in mitigating the deleterious effects of heat-induced protein denaturation (Pellham, 1986; Yu et al., 2005), but many other functions have been assigned to this well conserved protein family. For example, *Hsp70* genes are induced by UV-B and by wounding in Arabidopsis (Swindell et al., 2007), and by low temperature in spinach and tomato (Li et al., 1999). *DaURA3*, showed 85% similarity with the heat shock protein 70 gene from rice, and its expression increased four-fold in Antarctica plants, indicating that *D. antarctica* plants face complex stress conditions. The PR-1 family is highly conserved and appears to be represented in every plant species investigated to date (van Loon and van Strien, 1999). *PR-1* mRNA is enriched by microbial infection (van Loon et al., 2006), by abiotic stresses such as UV (Green and Fluhr, 1995) and by cold stress (Hong et al., 2005) in a wide range of monocots and dicots. *DaURA5* was highly induced in the Antarctic plants (60-fold) suggesting that the activation mechanism of *PR-1* transcription may be well-conserved across plant species. *DaURA5* is therefore a strong marker for tolerance to Antarctic environmental conditions.

Table 3. The most abundant transcripts found in this study.

Strongest BLASTX Hit	EST No.	Percentage
RubisCO large chain	160	8.13
Chlorophyll a/b binding protein precursor	40	2.03
RING zinc finger protein	20	1.02
Metallothionein-like protein type 3	19	0.96
Chloroplast hypothetical protein	16	0.81
60S ribosomal protein L18a	15	0.76
RubisCO small subunit	14	0.71
Os04g0653000	14	0.71
Photosystem II 10K protein	13	0.66
Hypothetical protein PhapfoPp091	11	0.56
Hypothetical protein CaO19.10303	11	0.56
ATP synthase gamma chain, mitochondrial precursor	11	0.56
Phosphoribulokinase; ribulose-5-phosphate kinase	11	0.56

Conclusion

We generated 1,969 high quality *D. antarctica* EST sequences in this study. Sequence analysis indicated the presence of 1,199 unigenes in the combined data set. The majority of the unigenes exhibited similarity to known genes, making them useful for further analyses. Microarrays can now be designed using either cDNA microarrays or oligo-based platforms. The unigene dataset should en-

Table 4. Genes and primer pairs used in the qRT-PCR analysis.

Name	Description	Primers (5′ → 3′)	
DaTUB	α-tubulin	F ;	AGCTGAGAAGGCTTACCATGAGCA
		R ;	ACTGGATAGTGCGCTTGGTCTTGA
DaURA1	Ozone-responsive stress-related protein	F ;	TGGGCTAGTATGGCGATGTCTGTA
		R ;	CCAGATTGCCATGACAAGAGTACC
DaURA2	Unknown cold induced protein	F ;	TCGCCTCTCTGCTCTGTTCTTTGA
		R ;	TGACTCTGTTGACGGCAGAGAAAC
DaURA3	Heat shock protein 70	F ;	AAGAAGATCGAGGAGGCTGTTGAC
		R ;	GAGCTCCTTCATCTTGTCCTCGAA
DaURA4	Cold acclimation protein WCOR413-like	F ;	TGTTTCCCGCGGTTGTTTGGTATG
		R ;	GCACAAATTGAGGAAACAAGGCCC
DaURA5	Pathogenesis related 1 protein	F ;	TGCATGCGTGTGTCTGTGTAGGTA
		R ;	GCACCACATATGAGCCGTACATGA
DaURA6	pTACR7, wheat cold responsive protein	F ;	TTTAGACTTCGAGCTAGCACCGC
		R ;	ACAGACGCTCAATAAGGATCACGG

hance the effectiveness of molecular studies, including those focused on marker screening and gene expression profiling under stressful environmental conditions. Finally, the cluster analysis and redundancy information should be useful for subtracting abundant transcripts in the cDNA library, so facilitating tailoring of the data set for future functional genomics studies.

Acknowledgments This work was supported by the Korea Research Foundation Grant (KRF-2006-351-B00034) funded by the Korea Government (MOEHRD) and by a grant PE08050 from the Korea Polar Research Institute.

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