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ORIGINAL RESEARCH

Proteome expression patterns in the stress tolerant evergreen *Ammopiptanthus nanus* under conditions of extreme cold

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Abstract Low temperature is one of the important environmental changes that affect plant growth. The cold resistance capabilities of evergreen plants are the result of long-term adaptation to extreme environmental conditions. To investigate the responses of *Ammopiptanthus nanus*, a rare stress-tolerant evergreen plant, to extreme cold stress, we analyzed the proteome expression patterns of stressed plants; this is the first study to report these patterns for *A. nanus*. We collected adult *A. nanus* leaves under two conditions of cold stress: extreme cold (−29°C) and relatively less extreme cold (−5°C). Total crude proteins were extracted from leaf blades, separated by two-dimensional gel electrophoresis, and stained with Coomassie brilliant blue. Of the 500 protein spots detected in each of the samples, eight of the spots that exhibited clear changes under the different conditions were identified by MALDI-TOF analyses. Our results suggest that cold stress-related proteins may play diverse roles in the resistance to multiple environmental stresses.

Keywords Extreme cold conditions · Two-dimensional gel electrophoresis · *Ammopiptanthus nanus* (Popov) · Proteome analysis

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Introduction

Plants are often exposed to various environmental stresses during their life cycles, including high salinity, desiccation, and low temperatures (Hashimoto and Komatsu 2007). In order to defend themselves against such stresses, plants use several strategies, which entail the regulation of genes involved in the cellular response to stresses, such as components of signal networks (Amme et al. 2005), and determining the level of enzyme activities (Apel and Hirt 2004; Shinozaki et al. 2003).

Low temperature is one of the most serious threats to plant growth, and results in rolled and withered plant leaves (Cui et al. 2005). A number of studies have investigated stress-response systems in plants during cold stress (Gao et al. 2009; Xia et al. 2009; Hashimoto and Komatsu 2007; Nozu et al. 2006; Yan et al. 2006). Phytohormones and salicylic acid have been suggested to play important roles in sustaining the growth and development of plants at cold temperatures (Xia et al. 2009). The hypothesis that cold-responsive proteins are likely to be involved in cold tolerance has generated considerable interest in the study of gene-expression profiles during cold stress (Gao et al. 2009; Chen et al. 2002; Fowler and Thomashow 2002). Proteomics has emerged as a powerful tool for studying the responses of plants to environmental stress. A global protein expression profile can be investigated and compared using a two dimensional (2-D) gel-based protein separation method coupled with protein identification by mass spectrometry (MS) (Cui et al. 2005). However, these studies were mostly focused on rice (Hashimoto and Komatsu 2007; Nozu et al. 2006) and *Arabidopsis thaliana* (Ruelland et al. 2009; Gao et al. 2009). Very few investigations have been conducted in tree species such as peach (Bassett et al. 2006), poplar(Benedict et al. 2006; Welling et al. 2002), rubber tree,

white spruce and maritime pine (Rossignol et al. 2006; Xu et al. 2009). In general, proteins that exhibiting increased expression in response to low temperature, such as heat-shock proteins (HSPs), dehydrins, detoxifying enzymes, chaperones, uridine diphosphate (UDP)-glucose pyrophosphorylase and malate dehydrogenase), were related to defense and amelioration of abiotic stress or to energy metabolism (Renaut et al. 2008). There are no published reports on the proteome expression patterns of *Ammopiptanthus nanus* (Popov) Cheng f. during cold stress.

Ammopiptanthus nanus (Popov) Cheng f., a relic plant from the Tertiary subtropical evergreen forests, is a rare broadleaved evergreen that is endemic to the Xinjiang region of China (Pan and Huang 1993; Pan et al. 1992). It is one of the two *Ammopiptanthus* (Leguminosae) species found in China's northwestern desert, and is noteworthy in that it can survive throughout the winter, a period characterized by long-term extreme cold during which temperatures can fall well below −20°C. The tolerance of *A. nanus* to extreme environmental perturbations makes it a good candidate for the study of the molecular evolution and phylogenetics associated with stress acclimation (Chen et al. 2007; Yang et al. 2008).

In order to investigate the responses of *A. nanus* to extreme cold stress, changes in the protein expression of leaves exposed to extreme cold and relatively less extreme cold conditions were analyzed using a proteomic approach. Total crude proteins were extracted from the leaf blades of *A. nanus* and separated using 2-D gel electrophoresis (2-DE). Selected cold stress-responsive proteins were subsequently analyzed by MS.

Plant materials and growth conditions

Extreme cold condition: Samples were collected from a population of *A. nanus* growing in a natural habitat in Wuqia county, Xinjiang region, northwest China, which is approximately 100 km southeast of the Kyrgyzstan border. This is an area that has, to this day, remained relatively undisturbed. The climate of the area is characterized by very cold winter temperatures. When the samples used in this investigation were collected, the temperature had fallen to below −29°C.

Relatively less extreme cold condition: In addition to the above, samples were also collected at the same time from Turpan Eremophytes Botanic Garden (TEBG), where the prevailing temperature was −5°C. The TEBG site is also located in the Xinjiang region of China; however, the winter climate here is characterized by less extreme cold conditions, with a mean winter temperature of −5°C (Fig. 1).

The sampling was performed using gloves in order to avoid sample contamination. In addition, the samples were

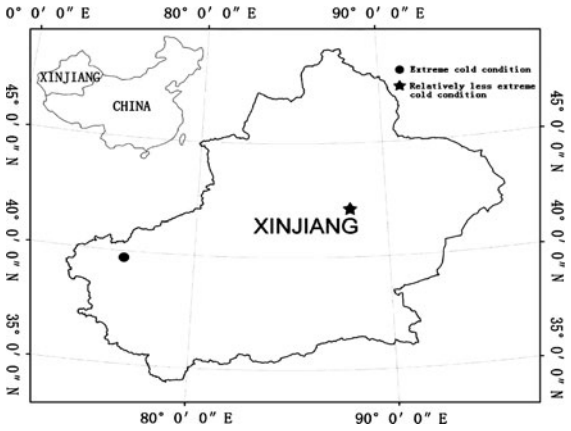

Fig. 1 Sites of *Ammopiptanthus nanus* Cheng f in the Xinjiang region of northwest China. Plant tissue samples were collected from extreme cold and relatively less extreme cold conditions

washed with double-distilled water to prevent contamination from foreign organisms and all the experiments were replicated in order to reduce errors. Following collection, the samples were packed in gauze and immediately frozen in liquid nitrogen and stored at −80°C.

Protein extraction

Plant material was washed two times with cold ether, and then ground in liquid nitrogen using a pug mill (IKA, Germany). For protein extraction, approximately 1 g (1 part) of the resulting fine powder was mixed with 10 ml (10 parts) of precipitation solution containing 10% w/v TCA and 0.07% w/v 2-mercaptoethanol in acetone according to Damerval et al. (1986).

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) of protein extracts in the first dimension and separation by SDS–PAGE in the second dimension were performed as previously described (Subramanian et al. 2005). Briefly, 300 μg of protein in 200 μl of rehydration/sample buffer [8 M urea, 4% (W/V) CHAPS, 40 mM DTT, and 0.2% Bio-Lyte] was used to passively hydrate 7 cm IPG strips (pI 3–10 linear; Bio-Rad). A PROTEAN IEF unit was programmed to provide an optimum maximum field strength with a 50 μA limit/IPG strip at 4,000 V for 20,000 Vh. Prior to the focusing step, the strips were held at 250 V for 30 min to remove charged contaminants and at 500 V for 1 h after focusing to eliminate artifacts due to over- and under-focusing. Prior to the second dimension SDS–PAGE, in order to solubilize the focused proteins and allow SDS binding, the focused IPG strips were equilibrated in buffers containing DTT for the reduction of sulfhydryl groups, followed by a second

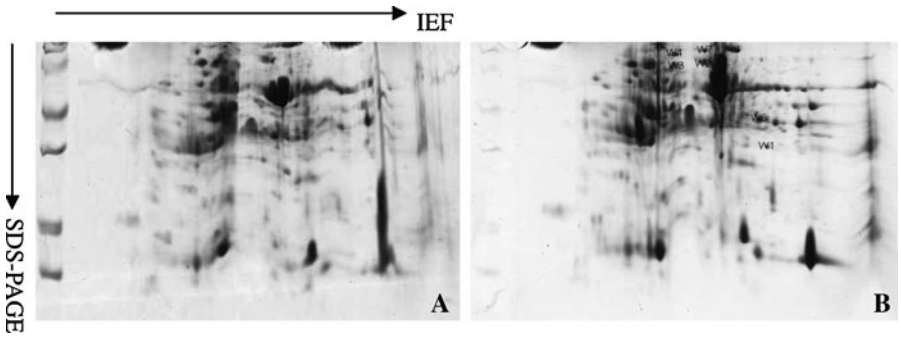

Fig. 2 Separation of *Ammopiptanthus nanus* leaf proteins by 2-DE. **a** is the sample collected under relatively less extreme cold conditions. **b** is the sample collected under extreme cold conditions

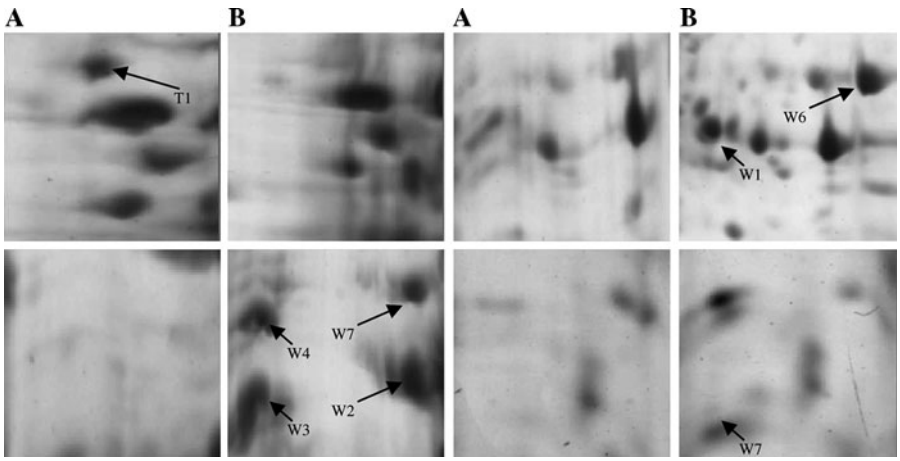

Fig. 3 A closer view of differentially expressed protein spots affected by extreme cold condition in Fig. 2. The spots from the sample under relatively less extreme cold condition (**a** and extreme cold condition **b**)

incubation in buffer containing iodoacetamide, which alkylates the reduced sulfhydryl groups. Second dimension electrophoresis was carried out on 12.5% polyacrylamide gels (7 cm × 7 cm, 1 mm thickness) using a PROTEAN mini system (Bio-Rad) at 45 V/gel until 2 h after the dye front had reached the bottom of the gel. The gels were stained with Coomassie brilliant blue (CBB) and images of the stained gels were acquired using a GS-800 calibrated densitometer (Bio-Rad). For each time-point, images from at least three gels obtained from three independent inoculation experiments were compared.

Image analysis

Two-dimensional gels of the protein extracts of leaf tissues collected in extreme cold and relatively less extreme cold conditions were analyzed using PDQuest software (Bio-Rad). Gels from three independent biological replicates were used to form match sets and individual spots were matched (added or deleted) using software tools. Each set of gels from the three replicates were analyzed simultaneously using the Student's *t*-test feature of this software in order to identify protein spots with statistically significant differences in levels as a result of pathogen challenge. Spot intensities of the control and inoculated gels at different time points were determined using the spot quantification tool and the fold changes from the controls were calculated. These spots were excised from the gels using a sterile scalpel and the proteins were identified by MS.

MS analysis and database search

In accordance with the method of Hashimoto (Hashimoto and Komatsu 2007), the spots on the gels stained with CBB were excised, and then digested with trypsin for 20 h. The digests were then collected, desalted, concentrated using a ZipTip, and finally subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. MALDI-TOF MS analysis was performed using a Bruker-Daltonics AutoFlex TOF-TOF LIFT Mass Spectrometer (Fig. 4). MASCOT software was used for protein identification by searching against NCBInr with other green plants from the taxonomic category.

Results and discussion

Two dimensional gel electrophoresis analysis of *Ammopiptanthus nanus* leaf proteins

Using PDQuest, we identified approximately 500 protein spots in the total crude proteins from the samples separated

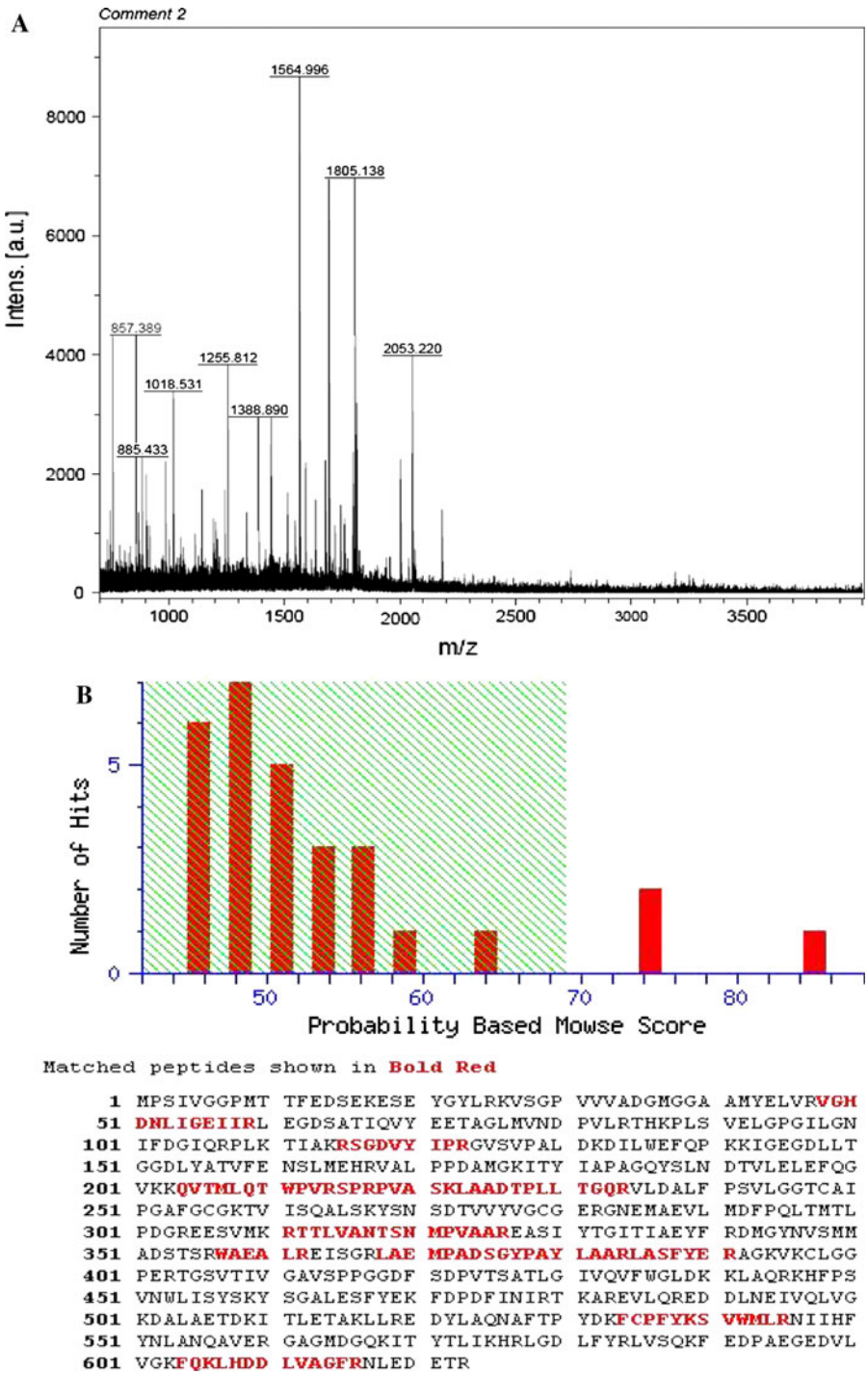

Fig. 4 MALDI-TOF–MS analysis pattern of protein spot W3 **a** Peptide mass fingerprinting of protein spot W3. **b** Mowse score and Database query result of spot W3. Protein score is −10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 69 are significant ($P < 0.05$)

by 2-DE (Fig. 2). Differences in expression patterns were found between the two samples: 39 proteins changed in abundance under the extreme cold conditions, with 19 proteins increasing and 20 proteins decreasing (Fig. 3). Common proteins related to photosynthesis and energy production exhibited high abundance patterns in both samples.

Identification of extreme cold stress-responsive proteins in *Ammopiptanthus nanus* leaves

We subsequently selected 10 good quality protein spots with high reproducibility, and with expression levels more than 2.5 times greater than control levels, for analysis by MALDI-TOF–MS. Eight of these spots (W1, 2, 3, 4, 5, 6, 7

Table 1 Differentially expressed proteins identified by PMF query

Spot no.	Protein name	Accession no	Experimental		S[a]	C[b]	Species
			Mass (kDa)	PI			
W1	AJ792158 *Antirrhinum majus*, mRNA sequence	gil51107486	26.639	9.28	103	27	*Antirrhinum majus*
W2	Unknown	gil118487947	81.094	5.97	87	19	*Populus trichocarpa*
W3	Vacuolar H+ -ATPase A1 subunit isoform;	gil27883932	68.812	5.20	85	19	*Lycopersicon esculen*
W4	Unknown	gil118488171	92.819	5.36	155	23	*Populus trichocarpa*
W6	Glycine cleavage system T protein	gil3915699	44.656	8.79	90	23	Pea
W7	Predicted protein	gil145353196	84.514	5.41	73	14	*Ostreococcus lucimarinus*

and 8) were expressed only under extreme cold conditions, whereas the remaining two spots (T1, 2) were expressed exclusively under the relatively less extreme cold conditions (Figs. 2, 3, 4).

The results of MS analyses are summarized in Table 1. Proteins that were up-regulated under the extreme cold conditions were an mRNA sequence regulated by DEF (W1), vacuolar H+-ATPase (V-ATPase) A1 subunit isoform (W3) (Fig. 4), proteins of unknown function (W2, W4), glycine cleavage system T protein (W6), and predicted protein (W7). The mRNA sequence regulated by DEF is associated with regulating petal morphogenesis. (Bey et al. 2004). It has been reported that in response to salinity, the levels of V-ATPase A1 subunit isoform in leaves were nearly doubled (Bageshwar et al. 2005). We can therefore conclude that this protein is expressed in response to both salt and cold stress. It has been observed that proteins W2 and W4 are associated with defense against insects; the physical clones will serve as useful reagents for functional genomics research in areas such as analysis of gene functions in defense against insects and perennial growth. (Ralph et al. 2008), in addition to being associated with insect defense, these proteins are also expressed under conditions of extreme cold. In plants, T protein plays a strategic role in the oxidative photosynthetic carbon cycle since it is also responsible for the release of NH4+ and catalyzes the first step of the photorespiratory nitrogen cycle (Givan et al. 1988; Keys et al. 1978, Bourguignon et al. 1993). The steady-state level of the mRNA corresponding to the T protein was high in green leaves compared to the level in etiolated leaves, and then the presence of a single gene encoding the T protein of the glycine decarboxylase complex in the haploid genome (Bourguignon et al. 1993).

W7 could be involved in siderophore biosynthesis (Palenik et al. 2007). In our experiment, it was up-regulated under extreme cold conditions.

Numerous studies have reported that phytohormone, regulatory proteins and genes are associated with environmental stress(Xia et al. 2009; Zhang et al. 2008; Pandey et al. 2008; Liu et al. 2007), and plants have developed diverse pathways that regulate stress resistance. Low temperature stimulates the activity of cytokinin-oxidase (Veselova et al. 2005); the increasing content of abscisic acid induces expression of cold acclimation related genes (Xiong et al. 2001); long-term exposure to cold stresses alters phytohormone levels, triggers cold acclimation signaling pathways, and results in selection of certain stress response genes that are most beneficial to plant survival. The present results demonstrate that *A. nanus* leaves contain proteins with energy metabolism, which play a role in energy production and resistance to extreme low temperature, and form a special defense strategy. This probably could explain why *A. nanus* can smoothly survive winter. At the same time, we propose that the specific proteins which are encoded by several stress-response genes, such as W2, W3, W4 W6 and W7, which regulate the tolerance of *A. nanus* under extreme cold environments.

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