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Effects of cold acclimation on proteome expression patterns related to freezing tolerance in a Tibetan alpine plant *Saussurea laniceps*

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Abstract

Saussurea laniceps is a perennial herbal alpine plant possessing strong cold tolerance. To get a deeper insight into its cold tolerance mechanisms, freezing tolerance and the proteomic profiles of cold-acclimated *S. laniceps* plantlets were analyzed. The survival rate of plantlets with height of 8-9 cm were recorded after exposure to chilling stress (2 ºC) and compared to plant material kept at a control temperature (23 ºC). The survival percent at -10°C increased from 0% to 40% during the 12 d of acclimation. Proteomic analyses, by two-dimensional gel electrophoresis (2-DE), performed during this stage revealed that 9 proteins were up-regulated, while 5 proteins were down-regulated. Among the proposed accumulating or appearing proteins, chlorophyll a-b binding protein 2 (LHCP-2), ribulose-1, 5-bisphosphate carboxylase/oxygenase activase (RCA), 33kDa manganese stabilizing chloroplast protein (33kDa MSP), and oxygen-evolving complex protein 1(OEC-1) were all related to photosynthesis, Maturase K is involved in gene expression regulation and galactinol synthase (GS) related to raffinose synthesis. Cold exposure induced a decrease in the candidate proteins including microtubule plus-end binding protein, ribosomal protein S13, O-acetylserine (thiol) lyase and photosystem I assembly protein ycf4. These results suggest changes in proteins associated with energy production, microtubule dynamic, raffinose synthesis and gene expression regulation process allow *S. laniceps* to enhance its freezing tolerance in the chilling environment.

Keywords: Alpine plant, Cold acclimation, Freezing tolerance, Functional proteome, *Saussurea laniceps*.
Abbreviations: 2-DE_two-dimensional gel electrophoresis; LHCP-2_chlorophyll a-b binding protein 2; RCA_ribulose-1, 5-bisphosphate carboxylase/oxygenase activase; 33kDa MSP_33kDa manganese stabilizing chloroplast protein; OEC-1_oxygen-evolving complex protein 1; GS_galactinol synthase; MALDI-TOF MS_Matrix-assisted laser desorption/ionization time of flight mass spectrometry; RFO_raffinose family oligosaccharides.

Introduction

The adaptive process, by which plants develop low temperature tolerance, is known as cold acclimation (Thomashow, 1999). Cold acclimation is associated with marked changes in protein composition and can affect both the amount and the type of polypeptides produced by the plants (Guy, 1990). Plants have evolved a range of adaptive strategies that help to prevent or to endure them to the adverse conditions. Most are based on the regulation of gene expression. Products of stress-inducible genes are usually the proteins either directly connected to stress response or implicated in the regulation of gene expression and signal transduction.

Proteomics, which combines two-dimensional gel electrophoresis (2-DE) with mass spectrometry (MS) and database mining, is recognized as a powerful approach for comparing proteomes under various stress conditions. Proteome analyses of cold responses have been carried out in different plant organisms such as model plant *Arabidopsis thaliana* (Goulas et al., 2006), rice (Cui et al., 2005), wheat (Kamal et al., 2010), woody species (Abril et al., 2011), sunflower (Balbuena et al., 2011), strawberry (Fang et al., 2011), moss (Wang et al., 2009) and bean (Badowiec et al., 2014). Identified proteins in these plants are involved in diverse biological processes including signal transduction, protein biosynthesis, defense response, energy metabolism and protein degradation etc.

However, most of the proteomic studies have focused on these common plants and less attention has been paid to plants living in extreme cold environments. *Ammopiptanthus nanus*, which is endemic to the Xinjiang region of China, has been studied by 2-DE to identify its tolerance proteomes and 10 proteins associated with salt stress, insect defense and redox equilibrium were identified (Lu et al., 2010). The transcriptome profiles of chilling-treated *Chorispora bungeana*, a subnival alpine plant possessing strong cold tolerance, has been analyzed by Illumina deep-sequencing and compared with Arabidopsis (Zhao et al., 2012).

Tibetan snow lotus, *Saussurea laniceps Hand.-Mazz* (*S. laniceps*), is a perennial and monocarpic species. It is endemic to the eastern Himalayas and has limited distributions on rocky habitats > 4,000m. Snow lotus is used in traditional Chinese and Tibetan medicine for the treatment

of headaches and high blood pressure and to regulate menstrual cycles and treat its problems (Law et al., 2005). In the natural environments, where *S. laniceps* is growing, snow and hail often occur during favorable growing seasons, and air temperature fluctuates frequently from 5 to 20 ºC during the daytime and 2 to -8 ºC during the nights. *S. laniceps* can survive, grow and flower in local environment even in snow. Therefore, *S. laniceps* is a suitable candidate for the study of the molecular evolution and phylogenetics associated with stress acclimation of alpine plants.

The Tibetan Plateau, generally called "the roof of the world" because of its very high altitude. It is characterized by its extreme environment (Dai et al., 2012). Here, we described the changes in the protein expression of *S. laniceps* planlets exposed to cold conditions using a proteomic approach. We aimed to understand the freezing tolerant mechanism of this plant living in the Tibet alpine.

Results

Freezing tolerance of non-acclimated and cold-acclimated plantlets

The survival rate of plantlets with height of 8-9 cm were recorded after exposure to chilling stress (acclimated at 2 ºC) and compared to plantlets kept at a control temperature (grown at 23 ºC). The survival rate (percent at -10 °C) increased from 0% to 40% during the 12 d of acclimation. Then, it remained unchanged with further increase in the acclimation period (15 d) (Fig. 1A). So, we chose 15 d as the best duration time for plantlets cold acclimation.

2-DE analysis of cold responsive proteins

Because of lacking of a fully sequenced genome, protein identification of *S. laniceps* was an analytical challenge. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is the most successful technique to identify proteins from organisms, where DNA sequence is unknown (Jouber et al., 2001). After 2-DE gel separation and coomassie brilliant blue staining more than 900 protein spots were reproducibly detected by digital image analysis (Fig. 2). The pIs of the protein spots ranged from 4.5 to 6.5 and the molecular masses ranged from 10 to 120 KDa.

The proteins of *S. laniceps* changed after cold acclimation for 15d at 2 ºC. We subsequently selected 15 high quality protein spots with reproducibility and with the expression levels more than 1.5 times greater than control levels for analysis by MALDI-TOF MS. Nine proteins (U1, U2, U3, U4, U5, U6, U7, U8, U9) were up-regulated and 6 proteins (D1, D2, D3, D4, D5, D6) were down-regulated (Fig. 2, Table 1).

Identification and functional annotation of differentially accumulated proteins

The changed proteins after cold acclimation were found to be involved in diverse biological processes. The identified proteins chlorophyll a-b binding protein 2 (LHCP-2), chloroplast ribulose-1, 5-bisphosphate carboxylase/ oxygenase activase (RCA), 33kDa manganese stabilizing chloroplast protein (MSP), oxygen-evolving complex protein 1(OEC-1) and photosystem I assembly protein ycf4 (chloroplast) were all related to photosynthesis. Four hypothetical or unknown proteins of the 15 protein spots were also identified as cold responsive proteins in this study. They are named U3, U7, D4 and D6. The remaining six proteins were categorized functionally. They were Os12g0120400, galactinol synthase (GS), O-acetylserine(thiol) lyase, Maturase K, ribosomal protein S13 and microtubule plus-end binding protein. Among these proteins, microtubule plus-end binding protein was related to the dynamics and organization of microtubules. Maturase K and ribosomal protein S13 were involved in transcription and post-transcription processes. The O-acetylserine(thiol) lyase catalyses the final step of cysteine biosynthesis. The cysteine plays an important role in maintaining intracellular redox equilibrium. In addition, the GS participates in formation of raffinoses which can protect cell membrane.

Discussion

Alpine plants have a great ability to withstand different environmental stresses (Zhao et al., 2012). In this study, 8-cm tall plantlets of *S. laniceps* were submitted to a chilling temperature treatment during active growth. The plantlets were able to acclimate the cold condition, as shown by increased freezing tolerance, when plants were exposed to a treatment at 2 °C (Fig.1). Similarly, in three-month-old poplar plants, the freezing tolerance of the adult leaves increased from -5.7 °C for the control plants (grown at 23 °C) to -9.8 °C after 14 days of exposure to 4℃ (Renaut et al., 2004). This cold acclimation indicates that *S. laniceps* is able to develop efficient tolerance mechanisms to survive freezing temperature.

Increased freezing tolerance during cold acclimation is associated with many metabolic changes (Kaplan et al., 2004). In order to determine the main pathways involved in cold acclimation of *S. laniceps*, proteome analyses were performed on 15 days of cold exposure. Usually, low temperatures destroy the balance between the source of energy and the metabolic sink. Therefore, adjustments of photosynthesis to maintain the balance of energy flow are required. Photosynthesis is generally suppressed at low temperatures. This suppression could be due to reduction in activities of enzymes of carbon metabolism, thylakoid electron transport rate, photochemical efficiency of the photosystem (PS) II, and stomatal conductance (Huner et al., 1998). However, in the present study, some enzymes and proteins associated with photosynthesis were up-regulated (Fig1, Table1), indicating that energy production was activated in the chilling environment in *S. laniceps*. Proteins that were up-regulated by cold acclimation were LHCP-2 (U4), RCA (U5), MSP (U8) and OEC-1 (U9). In contrast, photosystem I assembly protein ycf4 (chloroplast) (D5) was down-regulated during the course of cold acclimation.

The main roles of the light-harvesting chlorophyll a/b binding proteins (LHCB) are to collect and transfer light energy to the photosynthetic reaction center. The regulation of the LHCB expression is considered as one of the important mechanisms for plants to modulate chloroplast functions

Table 1. Cold-regulated proteins in *S. lanicep* plantlets identified by MALDI-TOF analysis coupled with database searches.

Spot No.	Protein name	Species	GI number	Theo.M/PI	Coverage	Score
pH3-10						
U1	Maturase K, partial (chloroplast)	*Gaultheria nummularioides*	gi|351583938	29.19/9.77	22%	48
U2	Os12g0120400	*Oryza sativa Japonica Group*	gi|115487064	13.77/9.42	46%	48
U3	Unknown protein	*Oryza sativa Japonica Group*	gi|55168150	20.80/10.65	41%	71
pH4-7						
U4	Chlorophyll a-b binding protein 2	*Populus euphratica*	gi|158562858	3.80/8.2	100%	71
U5	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Solenostemon scutellarioides*	gi|225580059	47.71/8.16	22%	68
U6	Galactinol synthase	*Luffa aegyptiaca*	gi|34550074	2.95/4.68	58%	41
U7	Hypothetical protein VOLCADRAFT_76220	*Volvox carteri f. nagariensis*	gi|302845521	54.70/9.72	19%	58
U8	33 kDa manganese stabilizing chloroplast protein	*Allium cepa*	gi|336041494	25.61/5.13	26%	60
U9	Oxygen-evolving complex protein 1	*Oryza sativa*	gi|739292	26.60/5.13	25%	78
D1	Microtubule plus-end binding protein	*Brassica napus*	gi|175940502	30.33/5.31	20%	46
D2	Ribosomal protein S13, partial (mitochondrion)	*Kniphofia linearifolia*	gi|376341315	13.13/10.78	43%	58
D3	O-acetylserine (thiol) lyase	*Arabidopsis thaliana*	gi|6899947	41.52/6.96	16%	60
D4	Predicted protein	*Arabidopsis lyrata subsp. lyrata*	gi|297788638	25.58/5.65	22%	60
D5	Photosystem I assembly protein ycf4 (chloroplast)	*Xeronema callistemon*	gi|372480425	21.70/9.51	21%	47
D6	Hypothetical protein SORBIDRAFT_10g003850	*Sorghum bicolor*	gi|242094734	49.45/8.07	13%	46

(Xu et al., 2012). Some studies have shown that LHCB protein family will down-regulate under low-temperature, high salt or drought stress conditions (Seki et al., 2002; Hazen et al., 2005; Guo et al., 2009). Down-regulating or destruction of LHCB protein family will hinder the stomatal response to ABA, resulting in declining in the drought resistance of plants (Xu et al., 2012). Therefore, increase in abundance of the LHCP-2 (U4) might reinforce the light absorption and energy transfer of PS (PS I, PS II) in the cold-acclimated *S. laniceps* plantlets and further affect the photosynthetic process. RCA is a nuclear-encoded, cytosol synthesized chloroplast protein that activates and maintains activity of Rubisco by promoting the ATP-dependent removal of inhibitory sugar phosphates from Rubisco active sites (Ristic et al., 2009). Plants either lacking Rubisco activase or having a very low level of activase cannot survive at atmospheric CO_2 levels (von Caemmerer et al., 2005). Up-regulation of Rubisco activase may improve the photosynthetic ability for cold-treated *Saussurea laniceps*. The 33-kDa extrinsic polypeptide, also referred to as MSP, plays a critical role in the structure and function of the OEC (Wyman et al., 2005). Transgenic potato plants with enhanced MSP expression levels showed that the relative oxygen evolution was directly proportional to the MSP expression (Gururani et al., 2012). Decreased amounts of the MSPs led to a loss of photo-autotrophy, decreased variable fluorescence yield, and a loss of PS II reaction center components in *Arabidopsis thaliana* (Yi et al., 2005). So, the enhancement of expression of MSP implies that cold acclimation results in adaptive alteration of PS II reaction center in *S. laniceps.* In this study, oxygen-evolving complex protein 1 was up-regulated after cold-hardening. Similar to our results, a putative oxygen-evolving complex protein was up-regulated by cold stress (5 °C, 48h) in rice leaf blades (Hashimono et al., 2007). However, OEC-1 was down-regulated in H_2O_2-treated rice seedling leaves (Wan and Liu, 2008), while up-regulated by NO_3^- in rice leaves (Wang et al., 2006). The difference in response of oxygen-evolving complex

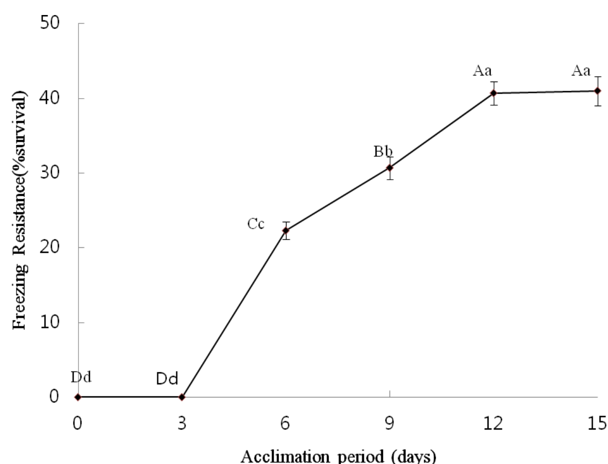

Fig 1. Increase in freezing resistance (percent survival at -10 °C) of *S. laniceps* plantlets during cold acclimation (2 °C). Each value is a mean of three replicates (10 plantlets were used for each replication) and vertical bars indicate the standard error of the mean. The capital letter indicates significant difference at P≤0.01. The lowercase indicates significant difference at P≤0. 05

protein1 to cold, H_2O_2 and NO_3^- is possibly due to distinctions in the plants' perception of the various stresses, especially for the alpine plant in our research. Photosystem I assembly protein ycf4 plays an essential role in PSI complex assembly in the green alga *Chlamydomonas reinhardtii* (Onishi et al., 2009). However, unlike in *Chlamydomonas*, Ycf4 is not essential for photosynthesis in tobacco and the *ycf4* knock-out mutants are capable of assembling sufficient amounts of PSI to allow for slow autotrophic growth (Krech et al., 2012). In the present research, unlike the up-regulated proteins related to photosynthesis, the expression level of Ycf4 was decreased in *S. laniceps* under cold treatment, implying that Ycf4 function is not essential or can be replaced by other factor(s) acting in PSI assembly and keeping the photosynthetic ability.

Therefore, up-regulation of LHCP-2, RCA, 33 kDa MSP and OEC-1 may be helping *S. laniceps* to retain a higher photosynthetic activity and adjust photosynthesis to maximize carbon gain at the risk of frost damage.

Regulation of transcription and translation plays an important role in stress alleviation (Miranda et al., 2003). As a development associated protein, Maturase K is not only involved in transcription and post-transcriptional modification but has also been implicated in regulation of gene expression through miRNA/siRNA formation in response to stress in plants (Sunkar et al., 2007). Enhanced levels of Maturase K in cold-acclimated *S. laniceps* correlate well with our conclusion that cold acclimation can increase the freezing tolerance of the alpine plant (Fig 1).

Raffinose family oligosaccharides (RFO) such as raffinose, stachyose, and verbascose accumulate in various plant species during seed desiccation and in leaves of plants experiencing environmental stress like cold, heat, drought or high salinity (Peterbauer et al., 2001). RFOs have been implicated in membrane protection and radical scavenging. Biosynthesis of RFO is initiated by the formation of galactinol from myo-inositol and UDP-galactose by GS (Krasensky et al., 2012). So, up-regulated GS after cold acclimation may lead to more raffinoses and to protect *S. laniceps* from freezing injury (Fig.1, Table 1).

Proteins that were down-regualted by cold acclimation were microtubule plus-end binding protein (D1), ribosomal protein S13 (D2), and O-acetylserine (thiol) lyase (D3).

Destabilization of microtubules or cytoskeletal rearrangements have increased Ca^{2+} influx in cold-shocked tobacco protoplasts (Mazars et al., 1997) and triggered low-temperature responses (Orvar et al., 2000). Microtubule plus-end binding proteins contribute to the dynamics and organization of microtubules during many cellular processes (Patel et al., 2012). Therefore, microtubule plus-end binding proteins may provide a possible point of integrating low-temperature calcium and calmodulin signaling with cold-regulated gene expression in *S. laniceps* and enhance its cold resistance.

Ribosomal protein S13 is a major component of the 40S rRNA preinitiation complex. Kim et al. (2009) demonstrated that RNA expression of the soybean S13 gene *STLI25* was induced by salt, ABA, or wounding stress, while reduced by dehydration stress. In the present research, the expression level of ribosomal protein S13 in *S. laniceps* was decreased by cold treatment, like the soybean S13 under dehydration stress (Kim et al., 2004), suggesting that this protein might function under low-temperature conditions in a new way.

Cysteine is a component in organic compounds including glutathione that have been implicated in the adaptation of plants to stresses (Romero et al., 2001). *O*-acetylserine (thiol) lyase (OAS-TL) catalyses the final step of cysteine biosynthesis (Shirzadian-Khorramabad et al., 2010; Heeg et al., 2008). In this study; however, the expression level of O-acetylserine(thiol) lyase was decreased after cold treatment, indicating that this protein might not aid in protection against cold stress in *S. laniceps* .

Materials and Methods

Plant material, growth conditions and treatments

Matured seeds of *S. laniceps* were collected from the snow mountain at an elevation of 4500m in east Tibet. Induction and culture of plantlets were performed as described by Chen et al. (2005). The seeds of *Saussurea laniceps* were cultured on hormone-free MS medium. Plantlets were formed from germinated seeds in 7–10 days under fluorescent light (100μmol m^{-2} s^{-}) at 23/20 ºC day/night temperatures, with a photoperiod of 14 h light and 10h dark. Then 0.5 cm × 0.5 cm leaf explants were transplanted to MS medium supplemented with 0.2 mg L^{-1} NAA and 1.5 mg L^{-1} 6-BA for 20 days. In the presence of 0.2 mg L^{-1} NAA in 1/2MS, 78% of the shoots formed roots.

Plantlets with height of 8-9 cm were selected for cold acclimation. The plantlets were transferred to the chambers for cold acclimation at 2 ºC and under 50μ mol m^{-2} s^{-1} photosynthetic photon flux density, with a photoperiod of 14 h light and 10h dark. After 0, 3, 6, 9, 12 and 15 days of cold acclimation, ten plantlets were frozen at a chamber (WD4005, ChongqingYin He) of desired freezing temperature: -5 ºC, -8 ºC, -10 ºC and -13 ºC, respectively. Then following a 30 min exposure to the target temperature, the plantlets were taken out for 1 night at 4 ºC. Then, they were transferred into chamber with a 10-h light/14-h dark cycle and a 23 ºC -day/20ºC-night temperature cycle. Plant survival was estimated after 10 days of treatment in both control and cold conditions by visual monitoring the fate of the frozen leaves and the progress of new root and leaf growth.

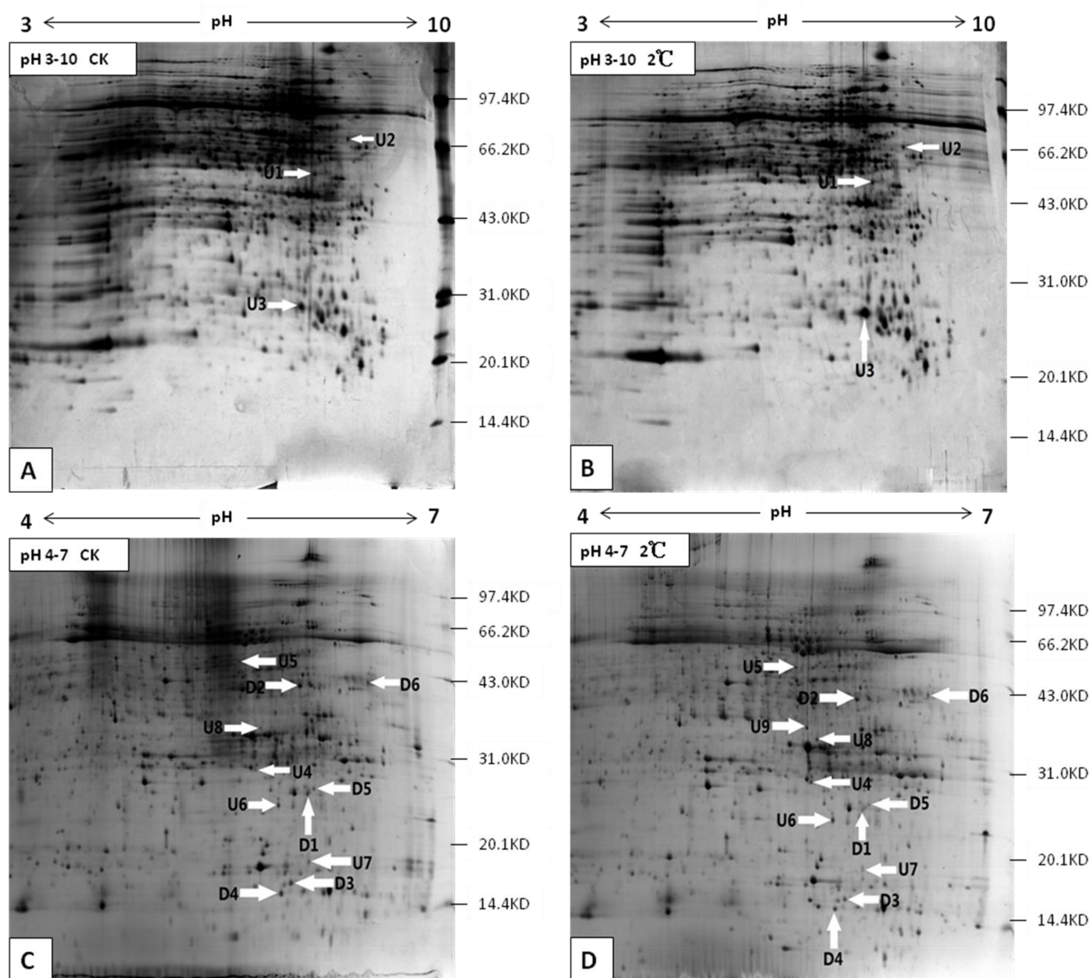

Fig 2. 2-DE of proteins extracted from *S. laniceps* leaves submitted to cold (2 ℃) after 15 days (C, D). The control plants were kept at 23 ℃ (A, C). In the first dimension (isoelectrofocusing), 720μg of protein were loaded on a 24 cm IPG strip with a linear gradient of pH 3–10 (A, B), or pH 4–7 (C, D). In the second dimension, 12.5% SDS-PAGE gels were used. Proteins were visualised by Coomassie brilliant blue staining. The arrows indicate 16 proteins that changed reproducibly and significantly in cold-exposed *S. laniceps* compared with controls.

The statistical analysis of the survival rate

The statistical results are presented as means ± SE. Statistical analysis was performed with 1% and 5% level of significant using the SPSS software (version 19.0).

Protein extraction

Collected plantlet leaves, both cultured at 23 ℃ and acclimated at 2 ℃ for 15 days, were frozen in liquid nitrogen. For protein extraction, a portion (2g) of each sample was pulverized with a pestle in a mortar that contained liquid nitrogen and homogenized in 3 mL extraction buffer (500mmol/L Tris-HCl, 50mmol/L EDTA, 700mmol/L sucrose, 100mmol/L KCl, 25mmol/L DTT, 1mmol/L PMSF) for 10 min. An equal volume of Tris-saturated phenol was then added followed by 10 min of vortexing and centrifuging at 5500g for 10 min at 4 ℃. The phenol phase was recovered and re-extracted with an equal volume of extraction buffer. After centrifuging, the proteins from the phenol phase were precipitated by addition of 4 vols of cold 0.1 mol/L ammonium acetate in methanol, and then incubated at -20 ℃ at least overnight. The sample was then centrifuged at 5500g at 4 ℃ for 15 min. The precipitate was washed three times with the cold ammonium acetate in methanol and once in cold acetone, and dried. Proteins were stored at -80 ℃.

2-DE process

Protein sample was finally dissolved in 500μL of the sample solution (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40mmol/L DTT). Protein concentrations were determined using Bio-Rad Protein Assay kit reagents (standard Bradford method) with bovine serum albumin as the calibration standard. Each sample contained 800μg protein in 500μL of

7mol/L urea, 2mol/L thiourea, 4% CHAPS, and 40mmol/L DTT was used for 2-DE. The protein samples were used to passively rehydrate the 24 cm pH 3-10, pH4–7 IPG strip. The sample of proteins was used to passively rehydrate for 20h.
IEF was done in a Protean IEF Cell (Bio-Rad) with the following protocol: 6h at 100V, 4h at 250V, 5h at 500V, 3h at 1000 V, increased from 1000 to 10,000 V over 9 h, and then 10,000V for 100,000 Vh. After IEF, the strips were equilibrated for 15 min in 8 ml of equilibration buffer I (6 M urea, 20 % glycerol, 2% SDS, 0.375 M TRIS–HCl, pH 8.8, 2% (W/V) DTT) followed buffer II (6 M urea, 20 % glycerol, 2% SDS, 0.375 M TRIS–HCl, pH 8.8, 2.5%(W/V) iodoacetamide) for 15 min, respectively. Second-dimensional SDS-PAGE was done in 12% polyacrylamide gels at 40 V for 1 h and then at 150 V for 7 h. At least three replicate gels were run for each sample. Following the electrophoresis, the gels were stained with Coomassie brilliant blue G-250, using the modified method of Neuhoff et al. (Neuhoff, 1998).

Image acquisition and analysis

The replicates of the 23 ℃ and 2 ℃ 2-DE gels were scanned using a calibrated densitometer (GS-800, Bio-Rad), and the spot patterns were characterized using PDQuest software (ver. 8.0.1, Bio-Rad). Image analysis steps included image filtration, spot detection and measurement, background subtraction, and spot matching. One 23 ℃ gel served as the reference, and the spots of the other five gels were referenced to it. Initially, spots were automatically matched, and the positions of unmatched spots were then manually determined.

MS analysis and database search

Protein spots of interest were excised and destained with 25mM ammonium bicarbonate, 50% ACN. Gels were then dried completely by centrifugal lyophilization. In-gel digestion was performed with 0.01 g/L trypsin (Promega) in 25 mM ammonium bicarbonate for 15h at 37 ℃. The supernatants were collected and the tryptic peptides were extracted from the gel sequentially with 5% TFA at 40 ℃ for 1h and with 2.5%TFA, 50%ACN at 30 ℃ for 1h. The extracts were pooled and dried completely by centrifugal lyophilization.

Protein Identification-Peptide mixtures were redissolved in 0.5%TFA, and 1 ul of peptide solution was mixed with 1 ul of matrix (4-hydroxy-cyanocinnamic acid in 30% ACN, 0.1% TFA) before spotting on the target plate. MALDI-TOF mass spectrometry was carried out on a 4700 Proteomics Analyzer (Applied Biosystems). Peptide mass maps were acquired in positive reflection mode, averaging 1500 laser shots per MALDI-TOF spectrum. Combined mass spectra were used to interrogate Viridiplantae (Green Plants) sequences in the NVBInr database using the MASCOT database search algorithms.

Conclusion

In conclusion, studies on the cold acclimation and freezing tolerance of *Saussurea laniceps* are important for understanding the adaptation to cold temperature of alpine plants. The results presented here indicate that *Saussurea laniceps* is a fast-responsive and chilling-tolerant species. Changes in proteins associated with photosynthesis, microtubule, raffinose synthesis and gene expression regulation indicate a unique insight into the process of cellular protection of freezing tolerance in *S. laniceps*. Combined with proteome analyses, *Saussurea laniceps* may be used to complete the understanding of the cold acclimation phenomenon, with the special features inherent in alpine plant species. In particular, it was shown that cold acclimation must be considered as a multigenic phenomenon that triggers a response network in relation to stress perception, and allows the plant to enhance its tolerance.

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