Phosphoproteomics of cold stress-responsive mechanisms in *Rhododendron chrysanthum*

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**Abstract**

**Background** As an alpine plant, *Rhododendron chrysanthum* (*R. chrysanthum*) has evolved cold resistance mechanisms and become a valuable plant resource with the responsive mechanism of cold stress.

**Methods and results** We adopt the phosphoproteomic and proteomic analysis combining with physiological measurement to illustrate the responsive mechanism of *R. chrysanthum* seedling under cold (4 °C) stress. After chilling for 12 h, 350 significantly changed proteins and 274 significantly changed phosphoproteins were detected. Clusters of Orthologous Groups (COG) analysis showed that significantly changed phosphoproteins and proteins indicated cold changed energy production and conversion and signal transduction.

**Conclusions** The results indicated photosynthesis was inhibited under cold stress, but cold induced calcium-mediated signaling, reactive oxygen species (ROS) homeostasis and other transcription regulation factors could protect plants from the destruction caused by cold stress. These data provide the insight to the cold stress response and defense mechanisms of *R. chrysanthum* leaves at the phosphoproteome level.

**Keywords** *Rhododendron chrysanthum* Pall. · Cold stress · Phosphoproteomics · Proteomics

**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| ALDH         | NAD(P)⁺⁺− dependent aldehyde dehydrogenases |
| CaM-BP       | CaM-binding protein |
| CaM          | Calcium |
| CAT          | Catalase |
| COG          | Clusters of Orthologous Groups of proteins |
| MAP3K        | Epsilon protein kinase 1 |
| NDKP2        | Nucleoside diphosphate kinase 2 protein kinase |
| OEC          | Photosystem II oxygen evolving complex |
| POD          | Peroxidase |
| SOD          | Superoxide dismutase |
| TMT Labeling | Tandem mass tags labeling |
| STP          | Serine/threonine-protein kinase |

**Background**

As one of the major environmental factors, cold affects plant growth and development broadly [1]. Cold stress directly leads to membrane rigidification and protein denaturation, but also indirectly reduces the activities of enzymes [2], inhibition of photosynthetic protein synthesis and degradation, as well as considerable membrane damage [3]. In order to reduce limit cold-induced damage, plants especially alpine plants have evolved multiple stress-tolerant strategies, which contribute to protecting plants from destruction caused by cold stress. Photosynthetic process of plants is inevitably changed in response to cold stress, especially Photosystem II (PSII). The Photosystem II photoinhibition appeared in order to protect Photosystem I at cold stress [4]. Cold stress induced the changes of a variety of protein kinases and transcription factors in plants [5]. According to reports, calcium plays a vital role in cold stress signaling. Ca²⁺ signals triggered are relayed by Ca²⁺ sensors, such as calmodulins (CaMs), Calmodulin-binding protein (CaMBP), CaM-like proteins (CMLs) [6, 7]. As one of the abiotic stresses, cold result in excessive accumulation of reactive oxygen species (ROS) in plants [8], and the effective antioxidant enzymes...
such as catalase (CAT), Superoxide dismutase (SOD) and peroxidase (POD) could eliminate excessive ROS [9].

As the major post-translational modification, protein phosphorylation plays a vital role in providing stress signal transduction and the regulation of diverse biological functions in plants [10]. Therefore, protein phosphorylation events mean a lot in designing strategies preventing plants from cold and other abiotic stresses, and it should be included when studying stress-induced related signal pathways [11]. Phosphoproteomic responses to cold stress have been investigated in different parts from different plants, including the leaves of Arabidopsis [10], tomato [12], Paper Mulberry [13], Tobacco [14].

*Rhododendron chrysanthum* Pall. (*R. chrysanthum*), an alpine plant, which is a valuable germplasm resource in the world with important ornamental and medicinal value *R. chrysanthum* only grows at altitudes around 1700 m at the Changbai Mountain in Jilin Province in China. In the long-term adaptive evolution, *R. chrysanthum* has evolved sophisticated mechanisms to respond to cold stress and protect itself from freezing injury [15].

Previous studies on the cold stress mechanism of cash crops and tropical plants are more thorough. Nevertheless, studies targeted on the molecular mechanism of cold response are lacking in alpine plants. In this study, quantitative proteomics and phosphoproteomics combining with traditional physiological analyses were employed to explore the cold response and defense mechanism in *Rhododendron chrysanthum* leaves at the phosphorylation level. These results have improved our understanding of how *Rhododendron chrysanthum* responds to cold stress and further unveils the specific phosphorylated proteins are related to potential pathways in *Rhododendron chrysanthum* under cold stress.

**Results**

**Changes of photosynthetic characteristics in response to cold stress**

The photosynthesis of *Rhododendron chrysanthum* is very sensitive to cold stress. The Fo and Fm significantly decreased under cold stress (Fig. 1A, B). Fv/Fo also decreased in response to the cold stress (Fig. 1E). In addition, qP and NPQ were considered as two indexes of utility rate of luminous energy. The decrease of qP and NPQ reflects the decrease of light energy utilization under cold stress (Fig. 1G, H). The parameters Fv/Fm and Fv'/Fm' represent the maximal and effective quantum yield of Photosystem II photochemistry, respectively. Fv'/Fm' was significantly reduced under cold stress (Fig. 1C, D). However, there was no significant change in ETR under cold stress (Fig. 1F).

**Effects of cold stress on antioxidant enzyme systems**

The H₂O₂ content significantly increased under cold stress conditions (Fig. 2A). The activities of the antioxidant enzymes were also affected by cold stress. Catalase (CAT) activity significantly increased with the cold stress.

**Cold stress-responsive proteome in Rhododendron chrysanthum leaves**

In total, 5192 protein species in leaves were identified in *Rhododendron chrysanthum*. The abundance of 350 proteins were significant changed (p < 0.05) under cold stress, of which 173 were up-regulated and 177 were down-regulated (Fig. 3A). The cold stress-responsive proteins were classified into 20 functional categories (Fig. 3B). The cold stress-responsive mainly functioned in Energy production and conversion, Signal transduction mechanisms. The majority of the cold stress-increased proteins in Signal transduction indicated that active signaling and metabolic networks have been initiated in *Rhododendron chrysanthum* leaves to response to the cold stress.

**Cold stress-responsive phosphoproteome in Rhododendron chrysanthum leaves**

In *Rhododendron chrysanthum* leaves, 2872 phosphopeptides corresponding 2508 phosphoproteins were identified (Table S1). The phosphorylation level of 274 phosphopeptides were significant changed (p < 0.05) under cold stress, of which 22 were up-regulated and 252 were down-regulated (Fig. 3A). A total of 274 cold stress-responsive phosphopeptides were classified into 8 functional categories. There were 252 phosphopeptides’ phosphorylation levels decreased under cold stress. The corresponding proteins were mainly involved in Energy production and conversion, Signal transduction mechanisms (Fig. 3B).

**Three-dimensional structure modeling of cold stress-responsive phosphoproteins**

In order to better understand the functions of the protein phosphorylation under the cold stress response, we predicted the molecular structure of cold stress-responsive phosphoproteins. In total, 6 statistically acceptable homology models were built through the SWISS-MODEL and their phosphorylation sites were located within the three-dimensional structure models (Fig. 4). The phosphorylation levels of Photosystem II protein D1 and light-harvesting complex II chlorophyll a/b binding protein 1(LHCB1) increased under cold stress and the
phosphorylation levels of phosphoglycerate kinase, fructose-bisphosphate aldolase, transketolase and mitogen-activated protein kinases decreased under cold stress. The cold stress-increased phosphorylation site of D1 protein occurred on Ser232, which was located in the PEST-like region [16] (Fig. 4A). However, the phosphorylation levels of phosphoglycerate kinase, fructose-bisphosphate aldolase, transketolase and mitogen-activated protein kinases decreased under cold stress. The cold stress-increased phosphorylation site of D1 protein occurred on Ser232, which was located in the PEST-like region [16] (Fig. 4A). However, the phosphorylation
sites of other proteins were not located in their functional domain.

**Discussion**

**Photoinhibition and phosphorylation of photosynthetic proteins in response to cold stress**

In this study, photosynthetic parameters [i.e., $F_{m}$, $F_{o}$, $F_{v}/F_{o}$ and $F_{v'}/F_{m'}$] of *Rhododendron chrysanthum* were obviously reduced with the cold treatment (Fig. 1), which indicated serious damage of the photosystem in *Rhododendron chrysanthum* leaves. However, there was no significant change in electron transport rate (ETR), which might mean that there are some strategies in *Rhododendron chrysanthum* to alleviate Photosystem II damage. Phosphorylation of Photosystem II centers increases the stability of Photosystem II complexes and concomitantly improves plant tolerance to cold stress [17]. In this study, as the center of Photosystem II, D1 protein was significantly phosphorylated under cold stress, which might result from the accumulation of ROS. The degradation of the D1 reduced following phosphorylation of protein [18], but if the phosphorylation of D1 protein can not be efficiently scavenged, D1 synthesis was inhibited and the rate of Photosystem II repair was reduced [19]. In addition, it has been reported that the phosphorylation of the lhcb4(CP 29) and lhcb1(LHC B 2) led to sustained thermal dissipation under cold stress [20]. We observed that the phosphorylation level of lhcb4(CP 29) and lhcb1(LHC B 2) dramatically increased (Fig. 5, Supplementary Table S1 and Supplementary Table S2). The nonphotochemical quenching (NPQ) was also increased (Fig. 1H) under cold stress in *Rhododendron chrysanthum*. Similar results had been illuminated at *Picea abies* in cold stress [21]. The decrease of phosphorylation level of PsbR indicated that Photosystem II oxygen evolving complex(OEC) might be damaged under cold stress [22]. However, NAD(P) + -dependent aldehyde dehydrogenases (ALDH) involved in oxidation of reactive aldehydes decreased in *Rhododendron chrysanthum* under cold stress [23]. The cold stress also decreased the phosphorylation levels of Calvin cycle enzymes e.g., fructose-bisphosphate aldolase and transketolase (Fig. 5), which might lead to the decrease of Calvin cycle activity under cold stress [24].

**ROS-scavenging pathways of Rhododendron chrysanthum are employed for cold Stress responses**

In *Rhododendron chrysanthum* leaves, cold stress caused the accumulation of $H_{2}O_{2}$ (Fig. 2A). In response to oxidative damage caused by the cold stress, *Rhododendron chrysanthum* has developed enzymatic detoxification systems to counteract reactive oxygen species (ROS) toxicity. Reactive oxygen species could also induce some secondary messengers like MAPK, and impact cold signaling [5]. As we know, in response to oxidative stress, plant tissues would increase the activity of SOD to reduce the reactive oxygen species level and generate $H_{2}O_{2}$ [9]. The SOD was significantly enhanced under cold stress (Fig. 6, Supplementary Table S1 and Supplementary Table S2), which is in agreement with the cold stress-increased SOD activity in rice [25] and rapeseed [26]. In addition, cold stress significantly enhanced the catalase (CAT) activity (Fig. 2B), which is supposed to break down $H_{2}O_{2}$ into water and oxygen [9]. Catalase (CAT) is also distinguished from many other peroxide-metabolizing enzymes due to its high specificity for $H_{2}O_{2}$ [27]. Enzymes capable of eliminating $H_{2}O_{2}$ include Prx. Phosphorylation of Prx reduced the peroxidase activity of this protein [28]. In this study, cold stress decreased the phosphorylation level of Prx, which might suggest that cold stress enhanced the activity of Prx. However, not all the antioxidant enzyme pathways were activated under cold stress.
Fig. 3 Functional categorization and abundance patterns of cold stress-responsive proteins and phosphopeptides in *R. chrysanthum* leaves. A A total of 350 cold stress-responsive proteins were classified into 20 functional categories, and a total of 274 cold stress-responsive phosphopeptides were classified into 20 functional categories. The percentage of proteins in each functional category is shown in the pie; B Abundance patterns of cold stress-responsive proteins and phosphopeptides in each functional category.
stress in *Rhododendron chrysanthum*. The POD pathway was inhibited in *Rhododendron chrysanthum* leaves as evidenced by the cold stress-decreased abundances of all the isoenzymes of POD. Similar results had been illuminated at rice under heat stress [29]. The NDPK is associated with H2O2-mediated mitogen-activated protein kinase signaling in plants [30]. In this study, the phosphorylation levels of NDPH and MAP3K are similar.

**Calcium-mediated signaling pathways are induced in the cold-tolerant *Rhododendron chrysanthum***

It is becoming clear that Ca2+ signaling plays a crucial role in conferring cold tolerance in plants [7]. In addition, the calcium would bind to the calmodulin [31] to phosphorylate CaM-binding protein (CaM-BP) to regulate gene expression [32]. In this study, the cold stress-phosphorylated CaM-binding protein implied they were possibly involved in calcium signaling events (Fig. 6, Supplementary Table S1 and Supplementary Table S2). It was reported that calreticulin and calnexin played a crucial role in calcium homeostasis and signaling [33]. The abundances of calreticulin and calnexin were increased under cold (Fig. 6.), which means calcium-mediated signaling...
pathways were activated in *Rhododendron chrysanthum* leaves under the cold stress. Serine/threonine-protein kinase BLUS1 functions as primary regulator of stomatal control to enhance photosynthetic CO₂ assimilation [34]. In this study, cold stress decreased the phosphorylation levels of the corresponding proteins, respectively. 3PGA 3-phosphoglycerate, F6P fructose 6-phosphate, FBA Fructose-bisphosphate aldolase, FBP fructose-1, 6-bisphosphate, G3P glyceraldehyde 3-phosphate, NDH NAD(P)H dehydrogenase, PS II photosystem II, Ru5P ribulose-5-phosphate, RuBisCO ribulose bisphosphate carboxylase/oxygenase, RuBP ribulose-1, 5-bisphosphate, TK transketolase, Xu5P ketose xylulose-5-phosphate.

**Fig. 5** Schematic representation of cold stress-responsive photosynthesis in *R. chrysanthum* leaves. Protein names in red and green represent increased and decreased protein abundances under cold stress, respectively. Arrows with solid and dashed lines represent direct stimulation/single-step reaction and indirect stimulation/multi-step reaction, respectively. A “P” in red and green circles indicates increased and decreased phosphorylation levels of the corresponding proteins, respectively. 3PGA 3-phosphoglycerate, F6P fructose 6-phosphate, FBA Fructose-bisphosphate aldolase, FBP fructose-1, 6-bisphosphate, G3P glyceraldehyde 3-phosphate, NDH NAD(P)H dehydrogenase, PS II photosystem II, Ru5P ribulose-5-phosphate, RuBisCO ribulose bisphosphate carboxylase/oxygenase, RuBP ribulose-1, 5-bisphosphate, TK transketolase, Xu5P ketose xylulose-5-phosphate.

**Fig. 6** Cold stress-responsive signal transduction in *R. chrysanthum*. Protein names in red and green represent increased and decreased protein abundances under cold stress, respectively. Arrows with solid and dashed lines represent direct stimulation/single-step reaction and indirect stimulation/multi-step reaction, respectively. A “P” in red and green circles indicates increased and decreased phosphorylation levels of the corresponding proteins, respectively; CaM calmodulin, CaMBP calmodulin-binding protein, CAT catalase, MAP3K epsilon protein kinase 1, NDPK2 nucleoside diphosphate kinase 2 protein kinase, POD peroxidase, Prx peroxiredoxin, ROS reactive oxygen species, SOD superoxide dismutase, STK serine/threonine-protein kinase, STP serine/threonine-protein phosphorylase.

In this study, cold stress decreased the phosphorylation levels of serine/threonine-protein kinase (Fig. 6), which might lead to stomatal closure in *Rhododendron chrysanthum* leaves. Similar results had been illuminated at *Jatropha Curcas* Seedling under cold stress [35]. All these results indicated that calcium-mediated signaling...
pathways were induced and led to cold-responsive gene expression.

**Conclusion**

As an alpine plant, *R. chrysanthum* is an excellent material for plants’ cold response research. Although our physiological results indicated that the photosynthesis of *R. chrysanthum* was inhibited, the proteomics and phosphoproteomics analysis implied that diverse ROS scavenging pathways and calcium-mediated signaling were triggered to alleviate damage under cold stress. All these results provide valuable information about the molecular mechanism of the cold tolerance of *Rhododendron chrysanthum* Pall.

**Materials and methods**

**Plant materials and experimental design**

Wild *Rhododendron chrysanthum* tissue seedlings were exposed to 4 °C for 12 h were used as the experimental group (EG). Wild *Rhododendron chrysanthum* tissue seedlings were exposed to normal atmospheric temperature for 12 h were used as the control group (CG). Both experimental group (EG) and control group (CG) leaves were excised from six-month-old plants of the experimental group (EG) and the control group (CG) were immediately used for protein extraction. To ensure adequate coverage, three biological replicates of each group (i.e., six plants) were collected.

**Chlorophyll fluorescence measurement**

Chlorophyll fluorescence induction parameters of experimental group (EG) and control group (CG) leaves were carried out with the Maxi-version of the Imaging-PAM (Walz, Germany).

We chose the fourth leaf from the top of each plant to detach and clamp onto the holder after keeping them in the dark for 30 min. The minimal fluorescence (Fo) of dark-adapted leaves was recorded during the weak measuring pulses of 0.5 μmol m⁻² s⁻¹ and the maximal quantum yield of PSII photochemistry (Fm) was obtained upon application of a 0.5 s saturation light pulse of 2800 μmol m⁻² s⁻¹. The intensity of actinic light setting used in all trials was 230 μmol m⁻² s⁻¹. The maximal quantum yield of Photosystem II photochemistry (Fv/Fm), effective quantum yield of PSII photochemistry (Fv’/Fm’), nonphotochemical quenching (NPQ), photochemical quenching (qP), and electron transport rate (ETR) were calculated using ImagingWin version 2.39 software (Walz). Statistical analysis was performed by using SAS 9.4. All data are represented as the means ± SD with three biological independent replications.

**H2O2 Content and antioxidant enzyme activity assays**

The activity of catalase (CAT) and the content of H₂O₂ within leaves were determined by Plant CAT ELISA kit and Plant H₂O₂ ELISA kit (Shanghai Enzyme Biotechnology Co., Ltd., China) according to the manufacturer’s instructions.

**Protein extraction**

Total leaf protein was extracted from three biological replicate samples for each treatment using a phenol extraction protocol. Protein pellets was dissolved in buffer (8 M urea, 100 mM TEAB, pH 8.0), and the protein concentration in the supernatant was estimated with a 2-D Quant kit (GE Healthcare, USA) according to the manufacturer’s instructions. To ensure adequate coverage, three biological replicates of each group were collected.

**Phosphoproteomics and bioinformatics analysis**

After extraction, proteins were digested into peptides. TMT labeling, HPLC fractionation and LC–MS/MS were then used to analyze and quantify the dynamic changes of the proteome. To ensure adequate coverage, three biological replicates (i.e., six samples) were collected. The MS/MS data were processed using the Mascot search engine (v.2.3.0). Tandem mass spectra were searched against the SwissPort Green Plant database. For protein quantification, the MAS-COT software package in NCBI were used in the present work. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate the protein pathway. Three-dimensional structural models for phosphoproteins were generated using SWISS-MODEL comparative protein modeling server.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06874-0.

**Authors’ contributions** XZ and HX designed the research; YL and HF prepared the plant materials for sequencing. YL carried out bioinformatics analysis of data; YL, HF and JC performed the experiments and statistical analyses; YL and JD collected data and researched literature. YL interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

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