Comparative Profile of Rubisco-interacting Proteins From Arabidopsis: Photosynthesis Under Cold Conditions

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Abstract  Rubisco (Ribulose 1, 5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) is crucial in biological circumstance fluctuation. Although disassembly of Rubisco after chill treatment has been reported in previous studies, there is only little known data on Rubisco interactive proteins involved in the disassembly process of Rubisco. Both repression of net photosynthesis rate and disassembly of Rubisco large subunits (Ru-L) have been investigated in the wild type, Arabidopsis thaliana (Col-0), treated at 4°C for 4 h and 24 h together with their 24 h recoveries at 20°C. Co-immunoprecipitation coupled with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and MALDI-TOF/TOF, Matrix-assisted laser desorption/ionization tandem time-of-flight; PMF/MS, Peptide mass fingerprint/mass spectrum identification was used to explore Rubisco-interacting proteins. Five protein candidates were profiled. The identified AAA-type ATPase family and glycosyltransferase were determined crucial for Rubisco activity. It is also strongly correlated to cold acclimation. Results suggest that the disassembly of Rubisco might have been the main cause of photosynthesis rate reduction under chill conditions, rather than photosystem or biogenesis involvement.

Key words  Arabidopsis, cold stress, Rubisco-interacting proteins, photosynthesis
DOI: 10.3724/SP.J.1206.2011.000009

Rubisco, the major stroma protein in higher plants, is oligomeric and consists of eight large and eight small subunits. During photosynthesis, the catalytic site located in large subunit combines CO₂ with ribulose-1, 5-bisphosphate to form glycerate-3-phosphate; this is reduced to triose-phosphate with NADPH and ATP generated by photosynthetic electron transport. In order to catalyze photosynthetic CO₂ fixation at high rates, large amounts of Rubisco are needed to compensate for the slow catalytic rate (3 ~ 10 s⁻¹) of the enzyme. Rubisco accounts for a quarter of leaf nitrogen and up to half of the soluble protein in leaves of C3 plants. Rubisco holoenzyme assembly in chloroplast stroma is an ATP-dependent process requiring the presence of other proteins with chaperone function-Rubisco binding protein (RBP)/cpn 60 [¹], as well as Rubisco activate (RA) possessing ATPase activity [²]. In past years, investigations on Rubisco and its changes under different stress conditions have been reconsidered with special emphasis on the importance of RA and RBP[³-⁶]. These proteins might associate with each other by protein-protein interactions facilitating direct Rubisco...
assembly and activation \[7\]. In consideration of its complex structure and biological function, some interacting proteins might have not been detected to date. Rubisco is an enzyme with very complex and poorly elucidated regulation in terms of activity and quantity\[8\]. Supercomplexes containing variable amounts of Rubisco and other stromal proteins that might allow metabolic channeling and/or regulation have been a recurring topic in Rubisco research. Rubisco itself is a very thermostable enzyme, as revealed by studies with isolated protein \[2\].

The major components of photosynthesis typically affected by short-term light or dark chills in thermophilic species are primarily compromised by interference with carbohydrate metabolism, inhibition of Rubisco activity, and stomatal closure; these are with concurrent increase in energy dissipation (i.e., heat) in the thylakoid antennae \[9-10\]. Although these factors can also be observed during concurrent chilling with incident light, the potential for photodamage to PS I is more apparent, similar to the disruption of redox control of stromal bisphosphatases SBPase and FBPase, and possibly RA \[11-12\]. The effects of growth under chilling conditions on PS I have already been investigated \[13-14\]. Evidences showing PS I activity declining at a greater rate compared with PS II \[15-16\] is insufficient to identify PS I as a primary target of chilling. One reason is that the evidence does not exclude the possibility of downstream chill-susceptible processes (carbon metabolism and stomatal conductance; to be described below) as the primary target; the observed changes in PS I and/or PS II activities act as secondary response. Recent studies with Arabidopsis have shown that a sequence of events could reverse the inhibition of photosynthesis as plants acclimate to low temperatures. Interestingly, some changes in photosynthetic metabolism occurring during cold acclimation are reminiscent of responses to low Pi \[17\]. The recovery involves two important functions: increasing sucrose production and protection against photoinhibition by allowing the increased turnover of the photosynthetic electron chain \[18\].

Carbohydrate metabolism has been reported to have greater instantaneous low temperature sensitivity compared with other components of photosynthesis \[19\]. However, when the oxygen sensitivity of CO₂ assimilation was examined after return to permissive temperatures, the persistent inhibition of photosynthesis following dark chill seemed to be not directly related to end-product inhibition.

Declines in photosynthesis after a chill under both dark and light conditions have been attributed to loss of Rubisco activity by various studies \[20\]. As previously suggested, chilling could damage Rubisco protein \[21\]. However, a general consensus on the significance, composition and function of Rubisco-containing complexes still awaits future research. Reports have also suggested that the activity repression of Rubisco, along with disassembly, might be the dominant reason of photosynthesis decrease under cold conditions \[21\]. Nevertheless, solid evidence supporting this hypothesis still needs to be provided.

In this study, we used Arabidopsis in an attempt to elucidate novel Rubisco-interacting protein candidates under cold acclimation conditions. Co-immunoprecipitation, followed by MALDI-TOF MS, was used. Results suggest that the disassembly of Rubisco might be the main cause of photosynthesis rate reduction, rather than photosystem or biogenesis involvement.

1 Materials and methods

1.1 Materials

Seeds of Arabidopsis thaliana Columbia ecotype were obtained from the Arabidopsis Biological Resource Center (Ohio State University, USA). Seeds were germinated in mixture soil. The seedlings routinely grew in a climate-simulated chamber at 75% humidity with 16 h light (80 \( \mu \)E·s\(^{-1} \)·m\(^{-2} \)) at 22°C and 8 h dark at 19°C. Plants were harvested after 4 weeks of growth and washed with Milli-Q water in order to remove the attached soil. Immediately thereafter, the intact plants were frozen in liquid nitrogen and stored at −80°C prior protein preparation. For the cold treatment, 3-week-old plants were placed in 4°C for a particular time span.

1.2 Photosynthesis measurement

Leaf net photosynthetic rates were measured by a portable gas analysis system, cl-301PS (CID, USA). Eight leaves for each sample were measured.

1.3 Anti-Rubisco immunoprecipitation

All steps were conducted at 4°C. Anti-Rubisco was subjected to IP from 20 g of Arabidopsis cells sampled from the control at 4°C for 4 h; 4°C for 24 h; and 24 h after re-shifting to normal condition. Crude cell extracts were prepared in IP buffer (2 mmol/L
Tris-HCl, pH 7.5, 150 mmol/L NaCl, 10% (v/v) glycerol, 0.07% polyethyleneimine, 10 mmol/L ethylenediaminetetra acetic acid/EDTA, 1% (v/v) NP-40, 5 mmol/L dithiothreitol/DTT, and 1 mmol/L phenylmethylsulfoxylfluoride/PMSF). Clarified extracts were obtained by homogenizing cells and centrifuged for 15 min at 15 000 g. Supernatants were incubated for 90 min with 100 μl of immune serum or pre-immune serum. Antigen-antibody complexes were incubated for 30 min with 500 μl protein G-Sepharose beads (Watson, USA); previously, these were equilibrated in IP buffer and centrifuged for 1 min at 15 000 g. Pellets were washed 5 times with 2 ml IP buffer. Immunoprecipitates were eluted by 1 mol/L NaCl, processed by organic extraction, and boiled for 2.5 min in SDS sample buffer without DTT. The immunoprecipitates were then immediately analyzed by SDS-PAGE.

1.4 SDS-PAGE and two-dimensional gel electrophoresis

The protocol was conducted following those by Xi, et al. (2006)[22].

1.5 Western blotting

Following electrophoresis, either gels were stained with silver (Bio-Rad protocol)/Brilliant Blue or proteins were transferred to NC membranes (Hybond-C Extra, Amersham Biosciences) using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 0.45 A for 30 min. Proteins separated from membranes were blocked in freshly prepared TBS containing 5% nonfat dry milk (Bio-Rad) for 1 h at room temperature with constant agitation, and then incubated in anti-Rubisco antibodies diluted in freshly prepared TBS containing 5% nonfat dry milk for 2 h with agitation at room temperature. Membranes were subsequently washed twice with TBS, incubated in goat anti-rat horseradish peroxidase-conjugated IgG (upstate) diluted in freshly prepared TBS containing 5% nonfat dry milk for 1 h with agitation at room temperature, and washed three times with TBS. Membrane were developed using an ECL system (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

1.6 Image analysis

Image analysis was accomplished using PDQuest 7.3 software (Bio-Rad). After automated detection and matching, manual editing was conducted. Three well-separated gels of each sample were used to create "replicate groups". Statistic, quantitative, and qualitative "analysis sets" were created between control group and each treated group. In the statistic sets, student's t-test at a significance level of 95% was chosen. Only spots displaying reproducible change patterns were considered as differentially expressed proteins.

1.7 Protein identification based on PMF/MS spectra

Protein identification based on PMF/MS spectra was conducted following those by Sun (2009)[23].

1.8 Chilling injury indexes assessment of cold responsive gene mutants

For cold responsive gene candidates, chilling injury indexes between Arabidopsis col-0 (control) and cold responsive gene T-DNA insertion mutants were investigated as described by Semeniuk (1986)[24]. Seeds of T-DNA insertion mutants were ordered from ABRC stock of Tair. These were checked by PCR using primers designed according to manual.

1.9 Protein analysis

Web-based TAIR Patmatch(http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) was used to analyze the obtained proteins. Analysis of hypothetical proteins was conducted using software at servers accessible on the Internet (Blast, Pfam, Prosite, Blocks, Prints, Prodom, and Proclass). Predictions of functions were carried out using BLAST (www.ch.embnet.org/software/BottomBLAST.html) and InterProScan (www.ebi.ac.uk/interpro/scan.html) tools. Predictions for chloroplast localization and chloroplast were conducted using software programs, PSORT (http://psort.nibb.ac.jp:8800/), ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), and SignalP(http://www.cbs.dtu.dk/services/SignalP/).

The structure of PSAD-1 (e.g., Amunts et al., 2007) and large subunit of Rubisco (e.g., Duff et al., 2000) from X-ray diffraction were obtained from RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). Structures were then subjected to the online Z-dock server (http://zdock.bu.edu/) for Molecular modeling.

2 Results and discussion

2.1 Large subunits of Rubisco disassemble when net photosynthetic rate decreases under cold conditions

Photosynthesis in tropical warm climate plants
is affected by chilling stress substantially. This process is called low-temperature photoinhibition [25]. To evaluate the adverse effects of chilling stress quantitatively, net photosynthetic rate ($P_n$) was determined. Net photosynthetic rate decreased from 12.1 to 9.5 and 1.96 $\mu$mol·m$^{-2}$·s$^{-1}$ after 4 h and 24 h of chill treatment, respectively, and returned to almost basal level (11.7 $\mu$mol·m$^{-2}$·s$^{-1}$) after 24 h recovery (Figure 1a). Previously, it was suggested that stomatal conductance (Gs) was not the limiting factor for $P_n$ in this condition. The decreased $P_n$ probably resulted from other factors, such as Rubisco activity and ATP availability. The disassembly and even degradation of Rubisco were observed as well [26].

Then, the assembly of Rubisco was investigated by 2-DE (Figure 1b). Ru-Ls were found smeared under conditions of 24 h chill. This suggests that disassembly has occurred with a change in proteins-Rubisco interaction.

![Fig. 1 Disassembly of Ru-L when Arabidopsis net photosynthesis rate ($P_n$) repression at 24 h chill](image)

(a) Net photosynthesis rates are repressed at 4 h chill for 24 h and recovered after 24 h shift to normal incubate condition. (b) Disassembly of Ru-L at 24 h chill. Total protein extraction from control and 24 h chill were subjected to two-dimensional gel electrophoresis; the smear of Ru-L increased, indicating disassembly of Ru-L.

### 2.2 Effects of chill acclimation on Rubisco interactive proteins

Given that Rubisco disassembles and its activity declines, the effects of chill acclimation on Rubisco complex were examined. Co-immunoprecipitation techniques were used to study protein-protein interactions of plant proteins with Rubisco. Compared with conventional yeast two-hybrid system that use domains as bait for screening proteins, this study uses the full-length Rubisco as bait for screening for proteins interacting directly with Rubisco, as well as with second messenger proteins of Rubisco acting as a protein complex. It has been previously reported that Rubisco undergoes disassembly under sugar gradient ultracentrifugation, and as such, plastid isolation of Arabidopsis for IP probably lose some of Rubisco-interacting proteins. The specificity of the antibody employed for immunoprecipitation was examined using dot blotting and Western blotting (data not shown). Rubisco was recognized strongly by the antibody.

Immunoprecipitated proteins were explored using SDS-PAGE. Subsequent gel scanning and image analysis identified five protein spots that appeared to be expressed differentially compared with the controls, while antibody and Rubisco acting as controls did not change in the immunoprecipitation profile (Figure 2a). Reproducible maps were obtained from more than three independent experiments. In mock immunoprecipitations using preserum, neither Rubisco nor Rubisco-interacting proteins was adsorbed to the antibody (Figure 2a).

The quantity of the particular band was evaluated as a tense ratio to its lane (Figure 2b, c). The five proteins could be classified into two groups: First, bands 1 and 3 decreased along with chill time course and recovered to normal quantities after 24 h recovery (Figure 2b); their correlations to time course under chill and $P_n$ were observed as well. Second, bands 2, 4 and 5 seemed to increase at 4 h chill and increased largely at 24 h recovery (Figure 2c).
2.3 The identification of Rubisco-interacting proteins using PMF/MS

To identify the five co-immunoprecipitated proteins, trypsin-digested peptides of protein were analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The summary is presented in Table 1. AAA-type ATPase family protein AT4G24860, α1,4-glycosyltransferase family protein/glycosyltransferase sugar-binding DXD motif-containing protein AT5G01250 were strongly correlated to chill time course and \( P_n \). The details are shown in Figure 2b. By using database searches and multiple sequence alignments, Neuwald [27] reported that RA is related to an AAA family of proteins, a class of chaperone-like ATPases associated with a variety of cellular activities such as assembly, operation, and disassembly of protein complexes. As for α1, 4-glycosyltransferase family protein, at optimal temperatures, it has an essential function in releasing inhibitory sugar phosphates from the active site of Rubisco, thereby allowing continuous \( \text{CO}_2 \) fixation [3].

A previous report has shown that heat treatment of thylakoid membranes would induce rapid dephosphorylation of the PS II reaction center protein D1 [28], and that this is followed by rapid degradation of damaged D1 copies. However, as a direct NADPH donor of Rubisco \( \text{CO}_2 \) fixation, PS I was poorly investigated and thus with limited information [29]. The present work found that PSAD-I (photosystem I subunit D-1) interacting with Rubisco elucidate further α helix of PSAD-1, directly binding to 60 ~ 80 and 100 ~ 120 AAs of Ru-L using Z-dock (Figure 3). The α helix of PSAD-1 was originally bound to the PS I activity center; initially, it seemed that it was reversely interacting with Ru-L, resulting in the feedback inhibition of PS I at 4 h chill. Along with degradation of Ru-L, the photoinhibition by Ru-L might have shifted to other photoprotective mechanisms at 24 h chill. With prolonged heat stress and upon stress repetition, the actual de novo synthesized heat shock proteins were obtained to rescue thylakoid functions; less RA was found associated with thylakoid protein synthesis machinery [30]. At 24 h recovery, Ru-L was synthesized and it up-tensed the interaction, avoiding any X0 access. To guarantee constant repair of photosynthesis complexes, thereby avoiding damage, it is crucial to maintain active protein synthesis in chloroplasts. Hypothetical protein gi|7523687, homologue to protein that is related to translation machinery, was more active at 4 h chill and 24 h recovery. Hence, specific association of Rubisco with thylakoid-bound ribosome nascent chain complexes could have exposed a chaperone-like function of Rubisco; this was crucial in maintaining

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**Fig. 2** Profiles of Rubisco-interacting proteins from *Arabidopsis* under chill conditions

(a) Immunoprecipitates from anti-Rubisco of *Arabidopsis* total protein extracts under particular conditions subjected to SDS-PAGE. Bands 1 ~ 5 were sliced and investigated using PMF/MS. Ru-L (56 ku), Ru-S (15 ku), Rubisco activase (43 ku), IgG large subunits (50 ku) and small subunits (26 ku) are arrowed. (b) Changes in bands 1 and 2 ratios to lane quantities are negatively correlated to time course under chill conditions and positively correlated to \( P_n \). (c) In contrast to control, bands 2, 4, and 5 ratios to lane quantities increased at 4 h chill, recovered to normal quantities at 24 h chill, and increased markedly at 24 h recovery after chill.
Fig. 3 Molecular modeling between PSAD-1 (blue) and Ru-L (green).

The interface covered the $\alpha$ helix of PSAD-1, 60 ~ 80, and 100 ~ 120 AAs of Ru-L.

Table 1 Identification of Rubisco-interacting proteins under chill conditions

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Identity</th>
<th>Accession number</th>
<th>Mascot score</th>
<th>Queries matched</th>
<th>Sequence coverage</th>
<th>Mass(amu)/ theoretical</th>
<th>ChloroP v1.1 prediction</th>
<th>Function prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAA-type ATPase family protein</td>
<td>AT4G24860</td>
<td>69</td>
<td>10</td>
<td>10%</td>
<td>10/124871</td>
<td>0.512</td>
<td>Expected to ATP binding, nucleoside-triphosphatase and to localize in chloroplast.</td>
</tr>
<tr>
<td>2</td>
<td>SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein</td>
<td>AT1G55690</td>
<td>65</td>
<td>10</td>
<td>14%</td>
<td>7.0/72041</td>
<td>0.443</td>
<td>Involved in coordinating actin remodeling guanine, nucleotide exchange, transport of secretory proteins.</td>
</tr>
<tr>
<td>3</td>
<td>Alpha 1,4-glycosyltransferase family protein/glycosyltransferase sugar-binding DXD motif-containing protein</td>
<td>AT5G01250</td>
<td>67</td>
<td>9</td>
<td>24%</td>
<td>3.5/46594</td>
<td>0.512</td>
<td>Expected to transferase activity, transferring glycosyl groups, galactosyltransferase activity.</td>
</tr>
<tr>
<td>4</td>
<td>Hypothetical protein</td>
<td>gi</td>
<td>7523687</td>
<td>62</td>
<td>6</td>
<td>15%</td>
<td>2.5/31768</td>
<td>0.436</td>
</tr>
<tr>
<td>5</td>
<td>PSAD-1 (Photosystem I subunit D-1)</td>
<td>AT4G02770</td>
<td>92</td>
<td>8</td>
<td>33%</td>
<td>2.0/22641</td>
<td>0.570</td>
<td>Thylakoid electron transfer chain component</td>
</tr>
</tbody>
</table>

The translation activity at transiently low temperatures. Meanwhile, SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein AT1G55690 involved in lipid synthesis cooperated in this process. Taken together, our results suggest a temperature-dependent dual function for Rubisco. A switch of protein from one function to another according to the needs of cell under fluctuating environmental conditions represents a great design, not only for cell protection, but also for saving metabolic energy, particularly if such environmental fluctuations are only at short term. The first function involved feedback
photoinhibition to PS I through competitive binding to PSAD-1; the second function, deduced from observed association of Rubisco with thylakoid-bound polysomes upon chill stress, probably occurred to maintain translation of essential thylakoid proteins during sudden exposure and lagging recovery of plants to chill stress. The fast spatial segregation of Rubisco to thylakoid membrane upon chill stress strongly suggests the role of Rubisco as chaperone in maintaining and protecting the thylakoid-associated translation apparatus. An analogous situation has been shown recently for HSP DegP, whose function switches from chaperone at low temperature to protease with increase in temperature [31]. The present results indicate that Rubisco may form complexes with thylakoid proteins in plant cells. In E. coli, the capability of major cytosolic chaperones to cope with protein misfolding and aggregation during heat shock stress in vivo and in cell extracts has been demonstrated [32].

2.4 Gene confirmation using T-DNA insertion mutant

For chilling injury index assessment, only up-regulated gene mutants are accessible for gene chilling function. The PSAD-1 mutant has been searched and ordered from ABRC stock, then the T-DNA insertion mutants of PSAD-1 (SALK_090959C) were selected for further phenotype observation under 4°C cold conditions. The severity of symptoms of wild type (control) and T-DNA insertion mutants was assessed visually according to the five-stage scale. The average extent of chilling-injury damage was investigated. The chilling injury indexes are summarized in Figure 4. The chilling endurances of gene mutants sharply decreased, supporting the idea that the gene has imposed a positive influence on the chilling resistance of Arabidopsis.

![Fig. 4 Chilling injury index of Arabidopsis col-0 and chill responsive gene T-DNA insertion mutants under 4°C chilling stress](image)

(a) The chilling injury indexes of the germplasm lines of Col-0 (control) and SALK_090959C. (b) The pictures of the germplasm lines of Col-0 (control) and SALK_090959C after 15 days chilling treatment.

After incubation under normal conditions for 4 weeks, the chilling injury indexes of Arabidopsis col-0 and cold responsive gene T-DNA insertion mutants were measured after transferring the plants to 4°C for 15 days. The experiment was repeated five times. SD (±) is indicated.

2.5 Localization prediction

Majority of the chloroplast proteins are encoded by the nuclear genome. The presequences of these nuclear-encoded chloroplast proteins share common features; these can be used to predict localization with moderate confidence [33].

All proteins identified on the gels were used for test predictions on chloroplast localization and transit peptides. The software program, ChloroP (see in Table 1) was utilized to evaluate chloroplast localization. The ChloroP server predicts the presence of chloroplast transit peptides (cTP) in the 5 protein sequences and suggest them located in the chloroplast.

3 Conclusion

Co-immunoprecipitation coupled with SDS-PAGE analysis and MALDI-TOF MS identification was used in this study to explore Rubisco-interacting proteins under Ru-L disassembly and Pn repression under chill conditions. Cold acclimation had a marked effect on respiration in A. thaliana. This suggests that the effects of cold acclimation on Rubisco activity have not been initiated by tight control (i.e., upstream or downstream regulation); rather, regulation of Rubisco by
disassembly in cold-treated plants may be more crucial, as shown by our results. It is Rubisco disassembly, rather than Rubisco genesis or degradation and feedback of photosystem, that resulted in Rubisco activity repression, thus net photosynthesis decreased under chill condition. The further experiments using these chilling responsive gene mutants should be explored for solidating the potential relationship between Rubisco disassembly and net photosynthesis reduction.

References

冷胁迫条件下拟南芥 Rubisco 相互作用蛋白质的比较分析

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摘要 1, 5-二磷酸核酮糖羧化酶 / 加氧酶 (Rubisco，EC 4.1.1.39) 在生物适应环境变化的过程中起到重要的作用。位于叶绿体中与冷胁迫密切相关的非常重要的复合酶——Rubisco，其相互作用的蛋白质至今没有系统的研究。对拟南芥进行 4 种处理：a. 持续在 20℃生长(对照)；b. 4℃ 4h 冷胁迫；c. 4℃ 24h 冷胁迫；d. 4℃ 24h 冷胁迫后放入 20℃恢复 24h。然后利用免疫共沉淀、十二烷基硫酸钠聚丙烯酰胺凝胶电泳及质谱辅助激光解析电离飞行时间质谱技术，在冷胁迫条件下研究了拟南芥光合抑制与 Rubisco 相互作用蛋白质解聚之间的关系。在鉴定出的 5 个与冷胁迫相关的 Rubisco 相互作用蛋白质中，AAA-型 ATP 酶家族蛋白和糖基转移酶对 Rubisco 活性及植物适应冷胁迫起着重要的作用。研究结果表明，Rubisco 复合酶体系的解聚可能是低温胁迫下拟南芥光合速率降低的主要原因。

关键词 拟南芥，冷胁迫，Rubisco 相互作用蛋白质，光合作用

学科分类号 Q71

DOI: 10.3724/SP.J.1206.2011.00009