Role of Temperature Stress on Chloroplast Biogenesis and Protein Import in Pea[1][OA]

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Modulation of photosynthesis and chloroplast biogenesis, by low and high temperatures, was studied in 12-d-old pea (*Pisum sativum*) plants grown at 25°C and subsequently exposed to 7°C or 40°C up to 48 h. The decline in variable chlorophyll *a* fluorescence/maximum chlorophyll *a* fluorescence and estimated electron transport rate in temperature-stressed plants was substantially restored when they were transferred to room temperature. The ATP-driven import of precursor of small subunit of Rubisco (pRSS) into plastids was down-regulated by 67% and 49% in heat-stressed and chill-stressed plants, respectively. Reduction in binding of the pRSS to the chloroplast envelope membranes in heat-stressed plants could be due to the down-regulation of Toc159 gene/protein expression. In addition to impaired binding, reduced protein import into chloroplast in heat-stressed plants was likely due to decreased gene/protein expression of certain components of the TOC complex (Toc75), the TIC complex (Tic20, Tic32, Tic55, and Tic62), stromal Hsp93, and stromal processing peptidase. In chill-stressed plants, the gene/protein expression of most of the components of protein import apparatus other than Tic110 and Tic40 were not affected, suggesting the central role of Tic110 and Tic40 in inhibition of protein import at low temperature. Heating of intact chloroplasts at 35°C for 10 min inhibited protein import, implying a low thermal stability of the protein import apparatus. Results demonstrate that in addition to decreased gene and protein expression, down-regulation of photosynthesis in temperature-stressed plants is caused by reduced posttranslational import of plastidic proteins required for the replacement of impaired proteins coded by nuclear genome.

Temperature has a profound effect on plant development (Xin and Browse, 1998; Guy, 1999; Allen and Ort, 2001; Browse and Xin, 2001). Plants exposed to chill stress, or heat stress, have impaired chlorophyll (Chl) biosynthesis due to down-regulation of gene expression and protein abundance of several enzymes involved in tetrapyrrole metabolism (Tewari and Tripathy, 1998, 1999; Mohanty et al., 2006). Impaired Chl biosynthesis and chloroplast development leads to reduced photosynthesis beyond the optimum temperature, resulting in substantial loss of plant productivity. The reduced photosynthesis affected by temperature stress is attributed to decline in PSII, F_v/F_m (variable Chl fluorescence/maximal Chl fluorescence), inhibition of electron transport, perturbation of thylakoid membrane fluidity, and consequent decline in photophosphorylation and CO_2 assimilation (Berry and Björkman, 1980; Wise and Ort, 1989; Havaux, 1993; Pastenes and Horton, 1996; Bukhov et al., 2000; Sharkey, 2000; Allen and Ort, 2001; Sharkey et al., 2001; Govindacharya et al., 2004; Salvucci and Crafts-Brandner, 2004; Wise et al., 2004).

Rubisco, the major photosynthetic enzyme, is sensitive to the variation in environmental temperature. High temperature inhibits Rubisco activity in spinach (*Spinacia oleracea*), wheat (*Triticum aestivum*), rice (*Oryza sativa*), and soybean (*Glycine max*; Weis, 1981; Kobza and Edwards, 1987; Eckhardt and Portis, 1997; Eckhardt et al., 1997; Salvucci et al., 2001; Vu et al., 2001; Gesch et al., 2003; Kim and Portis, 2005; Yamori et al., 2006). The maximum Rubisco activity and consequent carbon assimilation was impaired in chill-stressed maize (*Zea mays*) plants (Kingston-Smith et al., 1999). Reduced Rubisco activity was due to the down-regulation of the gene expression and protein abundance of Rubisco as revealed from its radioimmuno assay (Gesch et al., 2003). Transcript abundance and protein content of Rubisco is sensitive to both high- and low-temperature stress in rice and cucumber (*Cucumis sativus*; Bose et al., 1999; Gesch et al., 2003; Zhou et al., 2006). Both gene expression and the protein abundance of small subunit of Rubisco (SSU) were down-regulated in rapeseed (*Brassica napus*) following 48 h of chill stress (Meza-Basso et al., 1986). Similarly, in highly chlorophyllous soybean cell suspension cultures briefly exposed to high temperature (40°C for 2 h), synthesis of RuBPCase small subunit examined by in vivo labeling and northern and dot hybridization analyses demonstrated a decline in the transcription and translation of SSU by 30%. However,

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Table I. *Impact of temperature-stress on Chl a fluorescence*

Pea plants were grown for 12 d in cool-white fluorescent light (70 μmol photons m^{-2} s^{-1}) and subsequently transferred to heat stress (40°C) or chill stress (7°C) for 24 and 48 h. Leaves were dark adapted for 20 min before the measurement of their minimal fluorescence (F_0) and maximum fluorescence (F_m). For recovery, after 48 h of stress treatment, plants were transferred to 25°C and kept for 24 h in light (70 μmol photons m^{-2} s^{-1}). Values are mean of six measurements, and ± represents SD.

Treatment Temperature	24 h			48 h			Recovery at 25°C		
	F_0	F_m	F_v/F_m	F_0	F_m	F_v/F_m	F_0	F_m	F_v/F_m
7°C	0.253 ± 0.02	1.08 ± 0.8	0.765 ± 0.02	0.262 ± 0.02	1.04 ± 0.06	0.748 ± 0.01	0.270 ± 0.02	1.174 ± 0.04	0.792 ± 0.01
25°C	0.240 ± 0.01	1.18 ± 0.07	0.796 ± 0.02	0.280 ± 0.01	1.09 ± 0.06	0.743 ± 0.01	0.268 ± 0.02	1.136 ± 0.04	0.764 ± 0.01
40°C	0.282 ± 0.01	0.805 ± 0.01	0.649 ± 0.01	0.359 ± 0.01	0.689 ± 0.06	0.478 ± 0.04	0.320 ± 0.02	0.752 ± 0.03	0.574 ± 0.04

synthesis of chloroplast-coded large subunits of Rubisco (LSUs) was only partially affected in high temperature (Vierling and Key, 1985).

Rubisco has chimeric origin with eight LSUs and eight SSUs that are products of chloroplast and cytoplasmic protein synthesis, respectively. The SSU of Rubisco is synthesized in cytosol and posttranslationally imported into chloroplast. The cytosolic synthesized SSU is imported into chloroplast in an energy-dependent manner through protein import machinery, TOC (translocon at the outer envelope of chloroplast), and TIC (translocon at the inner envelope of chloroplast), present on the outer and inner envelope membranes of the chloroplast (Grossman et al., 1980; Kessler and Schnell, 2006; Smith, 2006). In pea (*Pisum sativum*), the TOC complex consists of at least three subunits: Toc159, Toc75, and Toc34 (Waegemann and Soll, 1991; Kessler et al., 1994; Perry and Keegstra,1994; Schnell et al., 1994; Seedorf et al., 1995; Tranel et al., 1995, Davila-Aponte et al., 2003). Electron microscopy and molecular analysis reveal a calculated mass of approximately 550 kD with a stoichiometry of 4:4 to 5:1 for Toc75:Toc34:Toc159, respectively (Schleiff et al., 2003). Toc159 and Toc34 share a highly conserved GTP-binding domain that, in concert, initially recognize the chloroplast targeting peptide (Kouranov and Schnell, 1997; Keegstra and Froehlich, 1999; Chen et al., 2000; Sveshnikova et al., 2000; Kessler and Schnell, 2002, 2006; Becker et al., 2004). The TIC complex may have several components consisting of Tic110, Tic20, Tic22, Tic55, Tic62, Tic40, Tic32, and Tic21 (Wu et al., 1994; Kessler and Blobel, 1996; Lubeck et al., 1996; Caliebe et al., 1997; Kouranov et al., 1998; Stahl et al., 1999; Chen et al., 2002; Kuchler et al., 2002; Hörmann et al., 2004; Teng et al., 2006). Tic22 is postulated to interact with the preprotein at the intermembrane space that probably facilitates its movement from TOC to TIC (Kouranov and Schnell, 1997; Becker et al., 2004). Electrophysiological studies along with computational analysis predict Tic110 and Tic20 to be the putative components of aqueous pores that form the preprotein conducting channel (Reumann and Keegstra, 1999; Heins et al., 2002). Complete translocation of precursor proteins into the chloroplast interior is accomplished via ATP hydrolysis within the stroma (Theg et al., 1989) mediated by stromal molecular chaperones. One of these is Hsp93 (a member of the Hsp100 family of molecular chaperones) that functions in close association with the import apparatus of inner envelope membrane and is proposed to bind the precursor protein as they enter the chloroplast stroma (Akita et al., 1997; Nielsen et al., 1997; Kovacheva et al., 2007). The other associated TIC

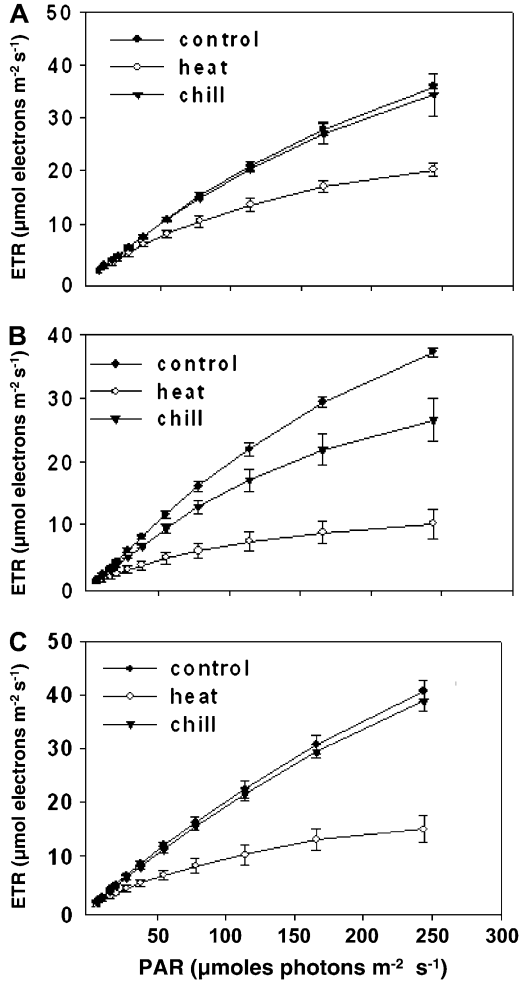

Figure 1. Effects of high (40°C) and low (7°C) temperatures on ETR after 24 h (A) and 48 h (B) of stress treatment. For recovery (C), after 48 h of stress treatment, plants were transferred to 25°C and kept for 24 h in light (70 μmol photons m^{-2} s^{-1}). Values are mean of six measurements and ± represents SD.

components, Tic62, Tic55, and Tic32, are proposed to act as redox regulators for import of precursor proteins (Caliebe et al., 1997; Kuchler et al., 2002; Hörmann et al., 2004). For proper targeting and attaining a conformation for optimal functional activity of nuclear-encoded stromal protein, its transit peptide needs to be readily cleaved, and any defect in this process may result in the loss of biochemical function of that protein (Grossman et al., 1980; Zhong et al., 2003). On entering the chloroplast, the transit peptide is cleaved by stromal processing peptidase (SPP) followed by folding and assembly (Oblong and Lamppa, 1992; VanderVere et al., 1995; Richter and Lamppa, 1998).

Although much work has been done on general protein import pathway, information on the impact of temperature stress on protein import into chloroplast is scanty. The nuclear-encoded plastidic heat shock proteins (HSPs) and other photosynthetic proteins, i.e. OEC33 and precursor of small subunit of Rubisco (pRSS), accumulated in cytosol of plants heat stressed at 43°C. However, it was readily reversible on return to normal growth temperature (Heckathorn et al., 1998). This effect was observed among evolutionary diverse plant species belonging to C_3, C_4, crassulacean acid metabolism, monocot, and dicot groups (Heckathorn et al., 1998). Vierling et al. (1986) compared the protein profile of leaf extract and that of intact chloroplasts, isolated from heat-stressed pea plants, and observed the presence of nuclear-encoded heat shock proteins in both the preparations. Additionally, chloroplast protein import assay using in vitro-translated product of poly(A)-RNA isolated from control and heat shocked pea seedlings showed import of very abundant 21-kD HSPs in the latter that was not present or completely reduced in control samples. However, that of chloroplastic SSU protein was reduced. These led them to conclude that chloroplast envelope transport machinery retains its activity at high temperatures.

In this investigation, we show that although post-translational import of pRSS is retained, it is severely down-regulated in plants exposed to both chill and heat stress due to decreased gene/protein expression of different components of protein import apparatus.

RESULTS

Leaf Chl *a* Fluorescence

To ascertain if chill stress and heat stress had the desired effect on photosynthetic apparatus, Chl *a* fluorescence of leaves of pea plant exposed to 24 and 48 h of low (7°C) and high (40°C) temperatures was measured. In order to understand if prolonged chill or heat stress was lethal, plants after 48 h of stress treatment were transferred to room temperature for 24 h, and the recovery from stress-induced injury was monitored.

Chl *a* fluorescence has been used as a nondestructive and noninvasive signature of photosynthesis (for review, see Krause and Weis, 1991; Govindjee, 1995, 2005). As shown in Table I, exposure of pea plants to low temperature for 24 to 48 h did not significantly affect their F_0 (the minimal fluorescence) and F_m (maximal fluorescence). However, F_o level increased by 18% and 29% after exposure of pea plants to heat stress for 24 and 48 h, respectively. The F_v/F_m (where $F_v = F_m - F_o$) ratio was 0.79 ± 0.02 in the control sample, and it declined to 0.65 ± 0.01 and 0.48 ± 0.04 after 24 and 48 h of heat stress. To study if the damage to the photosyn-

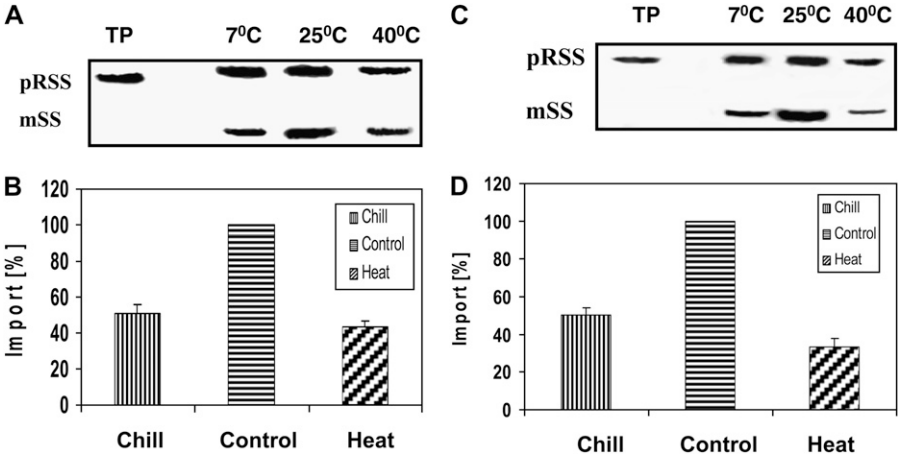

Figure 2. Import of pRSS into chloroplasts isolated from control, chill-, and heat-stressed pea plants. Twelve-day-old pea plants grown under cool-white fluorescent light (70 μmol m^{-2} s^{-1}) at 25°C were exposed to chill and heat stress for 24 h (A and B) and 48 h (C and D) in light (70 μmol m^{-2} s^{-1}). Intact chloroplasts were isolated and import reactions were performed using in vitro-translated radiolabeled pRSS as described in "Materials and Methods." Five percent of in vitro translation product (TP) was loaded to demarcate pRSS. Rate of import is expressed as percentage of control. Number of pRSS molecules imported per chloroplast in control sample average to 100. Data points represent the mean of three independent experiments, and error bars represent SD. mSS, Mature small subunit of Rubisco.

thetic apparatus due to temperature stress was reversible, pea plants after 48 h of stress treatment were transferred to 25°C for 24 h and their Chl *a* fluorescence kinetics was monitored. Heat-stressed plants recovered from temperature-induced damage that resulted in substantial restoration of F_v/F_m ratio. The electron transport rate (ETR; μmol electrons m^{-2} s^{-1}), estimated from Chl *a* fluorescence measurements (see "Materials and Methods"), increased in response to increase in light intensity in control and temperature-stressed plants (Fig. 1). However, after 48 h of stress treatment, the estimated ETR substantially declined by 80% and 25% by heat- and chill-stressed plants, respectively (Fig. 1B). The estimated ETR substantially recovered when stressed plants were transferred to 25°C for 24 h (Fig. 1C). Exposure of plants to 96 h of heat stress resulted in a near-irreversible inactivation of the photosynthetic apparatus, as there was no substantial reversal of photosynthetic functions after transferring the plants to 25°C for 24 h (data not shown). Therefore, further experiments were carried out up to 48 h of heat or chill stress treatment.

Import of pRSS into Chloroplasts Isolated from Pea Plants Exposed to 24 and 48 h of Chill or Heat Stress

As mentioned in the introduction, gene expression and protein abundance of SSU is affected by temperature stress. To understand if decreased gene expression of SSU is accompanied by down-regulation of protein transport into chloroplast, import of pRSS to its destination was studied in pea plants exposed to 24 and 48 h of temperature stress.

Chill or heat stress was applied to pea seedlings for 24 and 48 h as described in "Materials and Methods." Intact chloroplasts were isolated from control, chill-, and heat-stressed seedlings, and import of pRSS into chloroplasts was studied in the dark in the presence of 3 mM ATP at 25°C. Figure 2A shows the autoradiogram demonstrating the import of in vitro-translated pRSS protein into chloroplasts isolated from control plants and those exposed to chill and heat stress for 24 h. The upper band of 20 kD refers to pRSS bound to the chloroplast envelope membrane and not imported into chloroplast. The lower band of 14 kD is the mature subunit after its import into the stroma. The import efficiency of pRSS was reduced by 49% in chill-stressed plants and by 57% in heat-stressed plants (Fig. 2B). After 48 h of exposure to low temperature, the protein import efficiency was similar to that of 24-h chill-stressed samples. However, in plants exposed to heat stress for 48 h, protein import into chloroplast was reduced by 67% (Fig. 2, C and D).

Binding of pRSS to the Envelope Membranes

The inhibition of protein import into chloroplasts isolated from chill- and heat-stressed plants may be due to impairment of binding of pRSS into chloroplast envelope membrane, i.e. reduced efficiency of receptor-pRSS interaction or due to damage to TOC and TIC complexes of the protein import apparatus located in outer or inner envelope membrane, respectively. To distinguish between these possibilities, binding of pRSS with chloroplast envelope membrane was measured.

Intact chloroplasts isolated from control plants and those exposed to chill and heat stress for 48 h were incubated with pRSS in the presence of 0.3 mM ATP at 0°C in dark to prevent protein import and allow binding. As shown in Figure 3, binding of pRSS was almost similar in control and chill-stressed plants. However, in heat-stressed plants, binding of pRSS with the chloroplast envelope membrane was reduced to the extent of 50%.

Thermal Stability of Protein Import Apparatus

When intact plants are exposed to high temperature, the leaves usually do not reach the same temperature as that of ambient temperature as transpiration and other physiological processes keep the leaves cooler. To ascertain the temperature range that causes inactivation of protein import, the leaves harvested from pea plants, grown at 25°C, were incubated for 10 or 40 min at 25°C, 30°C, 35°C, or 40°C in a water bath. Intact chloroplasts were isolated from heat-treated leaves,

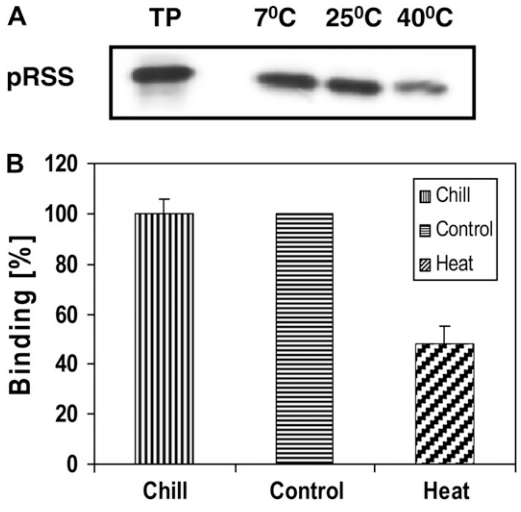

Figure 3. Binding of pRSS to the envelope membranes of chloroplasts isolated from control, chill-, and heat-stressed pea plants. Twelve-day-old pea plants grown under cool-white fluorescent light (70 μmol m^{-2} s^{-1}) at 25°C were exposed to chill (7°C) and heat stress (40°C) for 48 h in light (70 μmol m^{-2} s^{-1}). Intact chloroplasts were isolated, and precursor-binding reactions were performed using in vitro-translated radiolabeled pRSS at 0°C as described in "Materials and Methods." A, Autoradiogram showing the binding of pRSS in to chloroplasts isolated from control, chill-, and heat-stressed pea plants. Five percent of in vitro translation product (TP) was loaded to demarcate pRSS. Number of pRSS molecules bound per chloroplast in control sample average to 100. B, Histogram showing the percentage pRSS bound to each chloroplast. Binding of pRSS to the envelope membrane is expressed as percentage of control. Data points represent the mean of three independent experiments, and error bars represent SD.

and import of pRSS into chloroplasts was measured in the dark in the presence of 3 mM ATP at 25°C. Preprotein import (Fig. 4, A and B) and its binding to the envelope (Fig. 4, C and D) remained almost similar in chloroplasts isolated from leaves pretreated at 25°C, 30°C, 35°C, or 40°C for 10 min. However, protein import was impaired in chloroplasts isolated from leaves heated at 35°C and 40°C for 40 min (Fig. 5, A and B) by 87% and 92%, respectively. Under identical conditions, the binding of pRSS to the envelope membrane substantially declined by 76% and 83%, respectively (Fig. 5, C and D).

To further probe if cytoplasmic factors contribute to the thermal stability of chloroplastic protein import apparatus, preprotein transport was studied in isolated intact chloroplasts preheated at different temperatures. The chloroplasts isolated from pea plants, grown at 25°C, were suspended at a concentration of 1 mg Chl ml^{-1} and incubated for 10 min at 0°C, 25°C, 30°C, 35°C, or 40°C and immediately cooled in an ice bucket. Some chloroplasts might have broken due to heat treatment. Therefore, treated chloroplasts were again passed through Percoll gradient to sediment the intact chloroplasts that were again resuspended at a concentration of 1 mg Chl ml^{-1}, and aliquots of chloroplasts having 35 μg of Chl were taken for import assay at 25°C, as described in "Materials and Methods." In contrast to heating of leaves, that took as long as 40 min for impairment of protein import, heating of isolated intact chloroplasts for 10 min substantially down-regulated protein import. As shown in Figure 6, A and B, protein import almost remained the same in 0°C and 25°C pretreated chloroplasts. Protein import was highly inhibited (90%) in chloroplasts pretreated at 35°C and was completely abolished in those pretreated at 40°C. The binding of pRSS to the envelope membranes was also affected in 35°C or 40°C pretreated chloroplast (Fig. 6, C and D).

Modulation of Protein Import Apparatus in Response to Temperature

Down-regulation of pRSS import into chloroplasts in chill- and heat-stressed plants could be due to modulation of various components of protein import apparatus. Therefore, the gene expression of several components of TOC and TIC complexes, SPP, and the molecular chaperone Hsp93 was studied.

TOC Complex

Semiquantitative reverse transcription (RT)-PCR analysis (Burch-Smith et al., 2006) of the components of TOC complex revealed down-regulation of Toc159, Toc75, and Toc64 in heat- stressed samples (Fig. 7A). However, Toc34 expression remained unaffected at high temperatures. Chill stress did not substantially affect the gene expression of components of the TOC complex. RT-PCR (Burch-Smith et al., 2006) results of three independent experiments revealed that the gene expression of Toc159, Toc75, and Toc64 was reduced by 70%, 50%, and 60%, respectively (Fig. 7D).

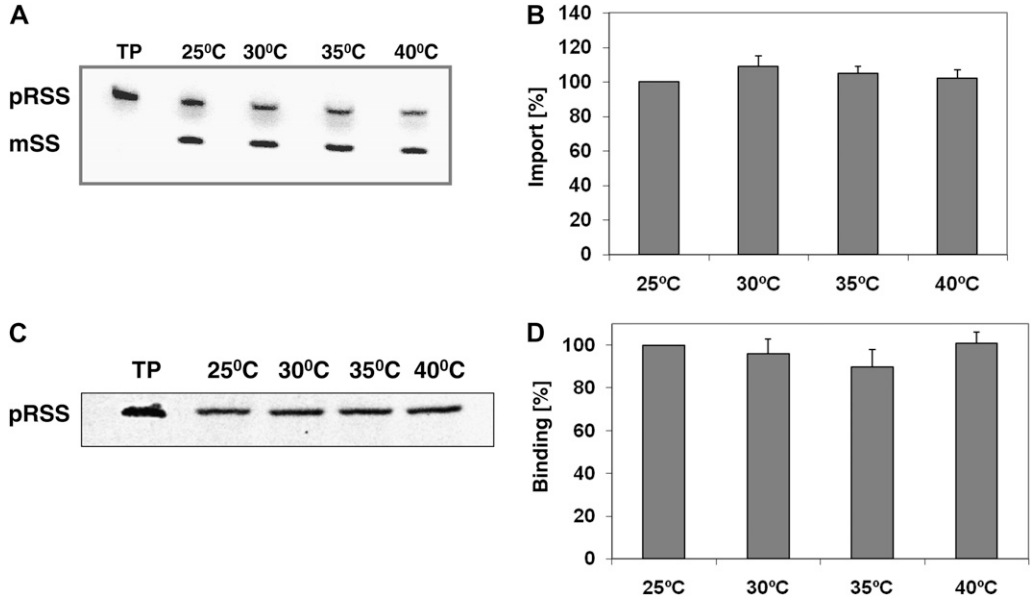

Figure 4. Import (A and B) and binding (C and D) of pRSS into chloroplasts isolated from leaves preheated at different temperatures for 10 min. Pea plants were grown under cool-white fluorescent light (70 μmol m^{-2} s^{-1}) for 12 d at 25°C. Leaves were excised and heated at 25°C, 30°C, 35°C, and 40°C in a water bath for 10 min. Import and binding assay of precursor proteins was performed using in vitro-translated radiolabeled pRSS as described in "Materials and Methods." Rate of binding and import is expressed as percentage of control (25°C). The precursor or mature peptides were quantified using a phosphor imager. Means of at least three independent experiments are given. mSS, Mature small subunit of Rubisco; TP, translation product.

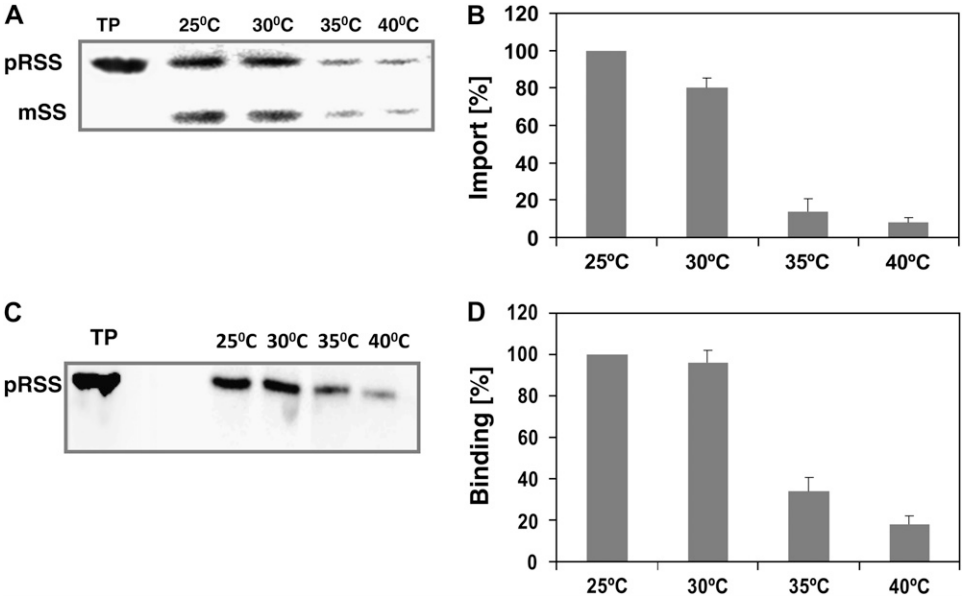

Figure 5. Import (A and B) and binding (C and D) of pRSS into chloroplasts isolated from leaves preheated at different temperatures for 40 min. Pea plants were grown under cool-white fluorescent light (70 μmol m^{-2} s^{-1}) for 12 d at 25°C. Leaves were excised and heated at 25°C, 30°C, 35°C, and 40°C in a water bath for 40 min. Import and binding assay of precursor proteins were performed using in vitro-translated radiolabeled pRSS as described in "Materials and Methods." Five percent of in vitro translation product (TP) was loaded to demarcate pRSS. The precursor or mature peptides were quantified using a phosphor imager. Rate of binding and import is expressed as percentage of control (25°C). Means of at least three independent experiments are given. mSS, Mature small subunit of Rubisco.

TIC Complex

The gene expression of Tic20, Tic32, and Tic62 was reduced in heat-stressed samples (Fig. 7B). Results of gene expression of three independent experiments demonstrated a decline of transcripts of the above components by 35%, 74%, and 56%, respectively (Fig. 7D). The gene expression of Tic22, Tic40, Tic55, and Tic110 were not significantly affected by high temperatures. At low temperature, the expression of Tic110 and Tic40 was down-regulated, whereas that of Tic22, Tic32, Tic62, and Tic55 was unaffected. RT-PCR results of three independent experiments revealed that in chill-stressed plants gene expression of Tic110 and Tic40 was reduced by 33%, and 56%, respectively (Fig. 7D).

Hsp93

The expression of stromal Hsp93 that binds to the inner envelope membrane declined (47%) in heat-stressed plants but was unaffected in chill-stressed samples (Fig. 7, C and D).

SPP

The expression of SPP was down-regulated (61%) in heat-stressed plants but was unaffected in chill-stressed plants (Fig. 7, C and D).

Western-Blot Analysis of Certain Components of Protein Import Apparatus

To study the correlation of gene and protein expression of protein import machinery, certain components were analyzed by immunoblots (Fig. 8). Among the TOC components, the protein abundance of Toc34 was not affected by chill or heat stress, whereas that of the Toc159 was down-regulated by high temperature. Among TIC components, abundance of that of Tic110 and Tic40 was down-regulated in chill-stressed samples, whereas Tic62 was down-regulated in heat-stressed plants. Heat stress partially reduced the protein abundance of Tic40. Hsp93 protein abundance was down-regulated in seedlings treated with high temperature but remained unchanged in chill-stressed samples.

DISCUSSION

Pea plants exposed to higher temperatures (40°C) for 48 h had more extensive damage to their photosynthetic apparatus than those exposed to low temperature (7°C; Fig. 1, Table I). The increase in F_0 in heat-stressed plants could be due to several reasons: inactivation of PSII (e.g. due to formation of non-Q_B centers) and separation of light-harvesting complex II from PSII (Schreiber and Armond, 1978; Bilger et al., 1984, 1987; Ducruet and Lemoine, 1985; Bukhov et al., 1990; Cao and Govindjee, 1990; Havaux, 1993; Yamane et al., 1997). The decrease in F_m could be due to several reasons, including decrease in quantum yield of PSII. The decline in F_v/F_m and the estimated ETR in temperature-stressed plants was substantially restored when temperature-stressed plants were transferred to

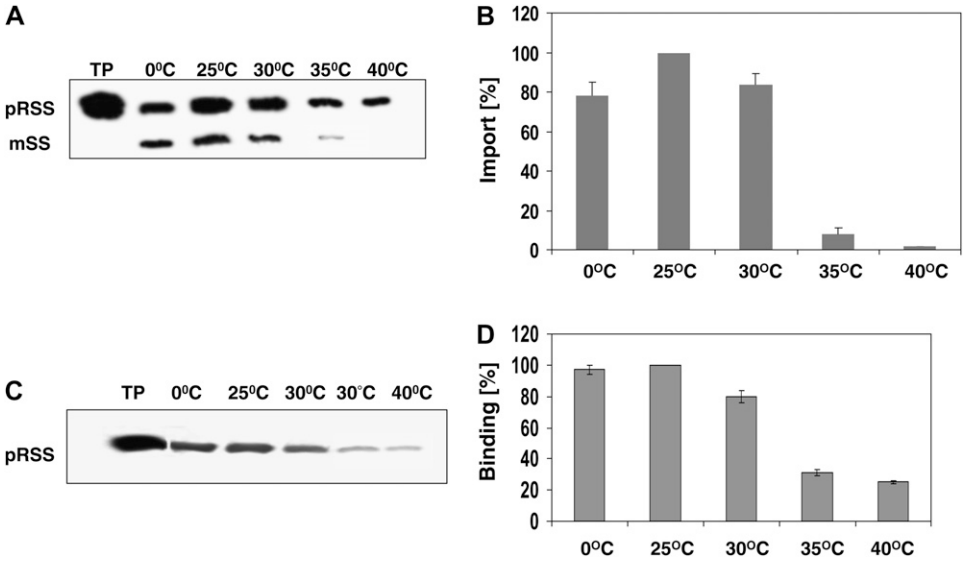

Figure 6. Import (A and B) and binding (C and D) of pRSS to isolated chloroplasts heated at different temperatures. Pea plants were grown under cool-white fluorescent light (70 μmol m^{-2} s^{-1}) for 12 d at 25°C. Intact chloroplasts (1 mg Chl/mL) were heated in vitro at 0°C, 25°C, 30°C, 35°C, and 40°C for 10 min and immediately cooled for 10 min in an ice bucket. Intact chloroplasts were again purified over 40% Percoll gradient and resuspended in import buffer (1 mg Chl/mL). Import and precursor binding reactions were performed using in vitro-translated radiolabeled pRSS as described in "Materials and Methods." Five percent of in vitro translation product (TP) was loaded to demarcate pRSS. The precursor or mature peptides were quantified using a phosphor imager. Rate of binding and import is expressed as percentage of control (25°C). Means of at least three independent experiments are given. mSS, Mature small subunit of Rubisco.

room temperature in light, demonstrating that low or high temperature treatment up to 48 h did not irreversibly inactivate the photosynthetic apparatus. Besides the impairment of electron transport activity, the temperature stress substantially reduces the CO_2 fixation by down-regulating Rubisco gene expression, protein abundance, and activity (Vierling and Key, 1985; Bose et al., 1999; Gesch et al., 2003; Zhou et al., 2006). Although LSU is coded by the chloroplast genome, nuclear-coded pRSS should be optimally imported into the chloroplast and processed into mature peptide for the proper assembly of the Rubisco holoenzyme in the stroma with the apparent involvement of stromal molecular chaperone 60 that often accumulates significantly in response to environmental stresses (Robinson and Ellis, 1984a, 1984b; Holland et al., 1998). After SSU import and assembly in the stroma, the maintenance of Rubisco in active state at high temperature requires faster Rubisco activase reaction to overcome the high- temperature-induced Rubisco inactivation (Crafts-Brandner and Salvucci, 2000; Law and Crafts-Brandner, 2001; Salvucci et al., 2001; Salvucci and Crafts- Brandner, 2004; Kim and Portis, 2005).

The reduced gene expression and protein abundance of nuclear-coded pRSS may also require the stressed plants to down-regulate its protein import efficiency into chloroplasts (Vierling et al., 1986; Heckathorn et al., 1998). Our results demonstrate that ATP-driven import of pRSS into darkened plastid is indeed down-regulated both at low and high temperatures. The nuclear-encoded pRSS is targeted into stroma through TOC-TIC translocon complex present at the chloroplast envelope membrane. Toc159 and Toc34 (Waegemann and Soll, 1991; Perry and Keegstra, 1994; Schnell et al., 1994; Keegstra and Froehlich, 1999; Bauer et al., 2000; Becker et al., 2004; Kessler and Schnell, 2006) are proposed to act as the primary receptors for the binding of preproteins to the chloroplast envelope membrane. Reduction in the binding of the pRSS at the higher temperatures (Fig. 3) could be partially due to down-regulation of Toc159 expression following 48 h of high temperature treatment (Figs. 7 and 8). We did not observe any change in the gene expression or protein abundance of Toc34 in heat-stressed plants; rather, it had constitutive expression both at low and high temperatures (Figs. 7 and 8). In addition to the impaired binding, reduced protein import into chloroplasts in heat-stressed plants was likely due to decreased gene/protein expression of certain components of the TOC complex, the TIC complex, the stromal Hsp93, and the SPP. The expression of Tic20, an essential component of protein import apparatus (Chen et al., 2002; Teng et al., 2006), was down-regulated in heat-stressed plants (Fig. 7), suggesting that protein trafficking through the inner envelope membrane could be severely affected. Other proposed components of the TIC complex, i.e. Tic62, Tic55, and Tic32, that may function as redox sensors (Caliebe et al., 1997; Kuchler et al., 2002; Hörmann et al., 2004)

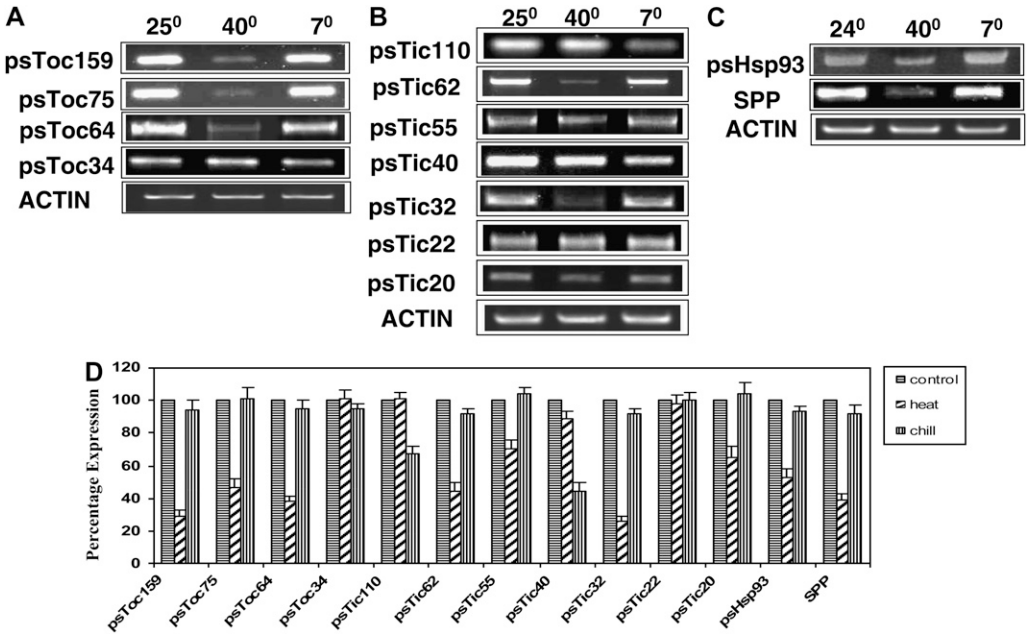

Figure 7. Gene expression of TOC complex components (A), TIC complex components (B), and Hsp93 and SPP (C) in control, chill-, and heat-stressed pea plants. For semiquantitative RT-PCR, cDNAs resulting from reverse transcription of identical amounts of total RNA from leaf tissue of control, chill-, and heat-stressed plants were used as template with primer specific for respective chloroplast protein import components. ACTIN transcript was used as internal control. The number of cycles and annealing temperature were optimized for each specific primer pairs. Ten microliters of the PCR products were loaded and separated on 1% agarose Tris-acetate EDTA gel. Ethidium bromide-stained PCR products were quantified using the Alpha Imager 3400. D, Bar diagram of gene expression (%). Rate of expression is represented as percentage of control (25°C). The data presented are representative of three independent experiments.

had reduced gene/protein expression in high temperature that might have partially contributed to reduced protein translocation across the inner envelope membrane. The observed heat-stress-induced impairment of protein import and preprotein processing to its mature form may also be due to down-regulation of gene/protein expression of Hsp93 and SPP. Our results further demonstrate that although the preprotein binding is affected by 50% due to heat stress, its transport to the stroma via envelope membranes is retarded by 67%. Therefore, impairment of protein import in heat-stressed plants is primarily caused by

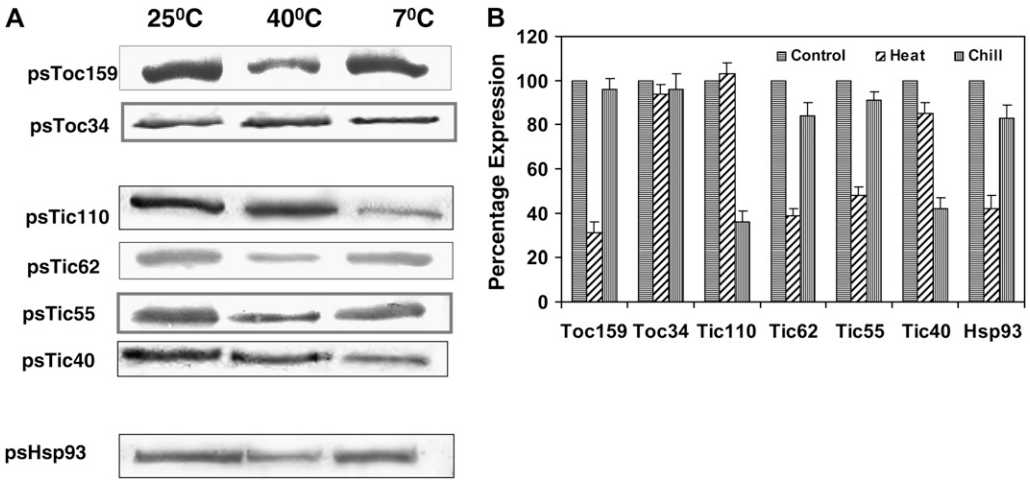

Figure 8. Western-blot analysis of certain components of protein import apparatus. Intact chloroplasts were isolated from control pea plants or those exposed to chill or heat stress for 48 h. A, Western blot of Toc159, Toc34, Tic110, Tic62, Tic55, Tic40, and Hsp93 was performed as described in "Materials and Methods." Each lane was loaded on equal protein basis, i.e. 80 μg of protein per well. B, Bar diagram of protein expression. Rate of expressions is represented as percentage of control (25°C). The data presented are representative of three independent experiments.

abridged preprotein binding owing to reduced expression of Toc159. Furthermore, inhibition of import efficiency to the tune of 67% was probably caused by the down-regulation of gene/protein expression of several other components of TOC/TIC complexes along with Hsp93 and SPP.

The preprotein binding to chloroplast envelope membrane is not affected in chill stress, whereas it is impaired in heat stress. This may be due to differential regulation of the preprotein receptor Toc159 that is unaffected in chill stress and down-regulated in heat stress. The gene or protein expression of stromal molecular chaperone Hsp93 as well as the processing enzyme were not affected by low temperature treatment (Figs. 7 and 8). The only important exceptions were Tic110 and Tic40 where the gene/protein expression was severely down-regulated in chill-stressed plants (Figs. 7 and 8). Tic110 is a putative channel protein involved in protein import through the inner envelope membrane (Heins et al., 2002). Tic 40 functions as a cochaperone that facilitates protein import across the envelope membrane (Chou et al., 2003; Kovacheva et al., 2005). This study demonstrates that although the binding of pRSS is not affected, the preprotein transport across the envelope membranes to the stroma is down-regulated probably due to the suppression of Tic110 and Tic40 gene/protein expression in chill-stressed plants.

The thermal stability of protein import apparatus is quite low. Protein import was 90% abolished upon heating the intact chloroplasts at 35°C for 10 min. Although prolong heat stress to the whole plant down-regulates the gene/protein expression of the TOC/TIC complex, this would not play a role in suppression of the binding and import of preprotein into chloroplasts heated in vitro at different temperatures (35°C–40°C). Inhibition of binding of pRSS to the heat-treated (10 min) intact chloroplasts is probably due to thermal denaturation of the preprotein receptor, Toc159, having GTP binding domain. However, inside the cell cytoplasmic factors, i.e. molecular chaperones protect the protein import apparatus from heat denaturation. This is evident from Figure 5, where protein import remained unaffected in chloroplasts isolated from leaves heated for 10 min at 35°C to 40°C. This protection by cytoplasmic factors was rather short term, i.e. 10 to 20 min as both binding and import were affected if excised leaves are heated a little longer (i.e. 40 min; Fig. 5), implying that protection of the import apparatus is no more effective if heat treatment continues for a longer duration. In spite of the low thermal stability of protein import apparatus, in intact plants exposed to heat stress at 40°C for as long as 24 to 48 h, protein import is only partially down-regulated by 49% to 67%. This is much below 92% and 100% inhibition of protein import observed in heat-treated (40°C) leaves and intact chloroplasts, respectively. This is because the leaves usually do not reach the same high temperature, as that of ambient since transpiration maintains the leaves at much cooler temperature.

Our results suggest that reduced protein import into chloroplasts significantly contribute to the impairment of photosynthetic reactions in temperature-stressed plants. High temperature stress usually results in increased degradation and concomitant reduced synthesis, thereby bringing a shift in the protein turnover (Smrcka and Szarek, 1986). In heat-stressed rice plants, the rate of degradation of SSUs exceeds the rate of their synthesis (Bose et al., 1999). Since Rubisco degradation takes place according to first-order kinetics, control of leaf Rubisco concentration must be exerted by adjustment of Rubisco synthesis and its import into chloroplasts. Compared to chill stress, exposure of plants to high temperature causes rapid degradation of the chloroplast proteins due to its unfolding and its susceptibility to the proteolytic attack. In order to retain their function, these proteins need to be replenished by newly synthesized preproteins that are post-translationally imported into chloroplasts. Thus, under high temperature stress, the net loss in photosynthetic function could be due to degradation of chloroplast proteins coupled with impaired posttranslational targeting of their precursor proteins into the chloroplast. In chill stress (48 h), the chloroplast proteins are not as rapidly degraded as that in high-temperature stress. Therefore, although the gene expression, protein synthesis, and their posttranslational import are impaired in plants exposed to chill stress (48 h), their photosynthetic functions are substantially retained. However, prolonged exposure of plants to chill stress would affect their photosynthetic functions (Yu et al., 2002) due to nonreplenishment of degraded chloroplast proteins.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum*) seeds were obtained from the Indian Agricultural Research Institute, New Delhi. These seeds were first treated with 0.1% $HgCl_2$ solution for 2 min and then washed with tap water several times. They were grown in vermiculite in cool-white fluorescent light (80 μmol photons m^{-2} s^{-1}) at 25°C for 12 d.

Stress Treatment

For temperature stress treatment, plants were transferred to 7°C (for chill stress) and 40°C (for heat stress) in light (80 μmol photons m^{-2} s^{-1}) for 24 or 48 h. One set of plants was kept at 25°C as control.

Recovery from Stress

After 48 h of stress treatment, plants were transferred to 25°C and kept for 24 h in light (70 μmol photons m^{-2} s^{-1}), and their Chl *a* fluorescence was measured as described below.

Chl *a* Fluorescence Measurements

All measurements of Chl *a* fluorescence were performed with a portable PAM-2100 fluorometer (Walz). Before each measurement, the sample leaf was dark-adapted for 20 min (Demmig et al., 1987) in respective temperature regimes with leaf clips provided by Walz. The angle and distance from the leaf surface to the end of the optic fiber cable were kept constant during the experiments. Chl *a* fluorescence was detected by a photodiode (BPY 12;

Siemens) that was shielded by a long-pass far-red filter (RG9; Schott) and a heat filter. To determine the initial fluorescence, F_o, the weak measuring light was turned on and F_o was recorded. Then, the leaf sample was exposed to a 0.8-s saturation flash of approximately 3,000 μmol photons m^{-2} s^{-1} to obtain the maximal fluorescence, F_m. Optimum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_o)/F_m$. The quantum yield of PSII was calculated from Chl *a* fluorescence as $F_v'/F_m' = (F_m' - F)/F_m'$, where F_m' and F are maximum fluorescence yield reached in a pulse of saturating light when the sample is preilluminated and measured fluorescence yield at any given time, respectively (Genty et al., 1989). Using the values of the quantum yield of PSII, the ETR, expressed in μmoles electrons m^{-2} s^{-1}, was estimated using the equation, ETR = calculated yield of PSII × PAR × 0.5 × 0.84, where PAR corresponds to the flux density of the incident photosynthetically active radiation, measured in μmoles photons m^{-2} s^{-1}, 0.5 is based on the assumption that the incident light is equally distributed between PSII and PSI, and 0.84 is for the assumed fractional absorption of light by the leaf. All measurements of F_o were performed with the measuring beam set to a frequency of 0.6 kHz, whereas all measurements of F_m were performed with the saturation flash automatically switching to 20 kHz.

Isolation of Intact Chloroplasts

Intact plastids were isolated as described before (Tewari and Tripathy, 1999). Briefly, pea leaves were homogenized using a kitchen blender in 7 volumes of cold 1× grinding buffer consisting of 0.05 M HEPES, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA, 0.1% BSA, and 0.025% isoascorbate, pH 7.5. The filtrate was centrifuged at 4,000 rpm for 7 min at 4°C in Sorvall RC5C refrigerated centrifuge using a HB4 rotor. Supernatant was discarded, and the pellet was suspended in about 2 mL of 1× grinding buffer using a paintbrush. The suspended pellets were added to 50% Percoll, and the contents were mixed gently by inversion, covering the tube with Parafilm. The contents were centrifuged at 15,000 rpm for 15 min with brake off in a Sorvall RC5C refrigerated centrifuge using a HB4 rotor. After centrifugation, two bands were observed. The lower green band containing intact chloroplasts was collected and diluted with 3-fold 1× import buffer (IB) composed of 0.05 M HEPES and 0.33 M sorbitol, pH 8.0. It was centrifuged at 4,000 rpm for 5 min with brake on. The supernatant was discarded. Pellet was suspended in 1 mL of 1× IB. An aliquot (20 μL) was taken for assaying Chl (Porra et al., 1989). The remaining chloroplasts were pelleted at 3,000 rpm for 5 min and resuspended in 1× IB at a concentration of 1 mg Chl/mL.

Preparation of Precursor Proteins

In vitro ^{35}S-Met-labeled precursor proteins (pRSS) were prepared by the TNT-coupled SP6 polymerase in vitro translation system (Promega). The reaction was set at room temperature. A reaction mixture was prepared containing 25 μL wheat germ extract, 2 μL reaction buffer, 2 μL ^{35}S-Met, 1 μL SP6 polymerase, 1 μL amino acid mix minus Met (1 mM), 1 μL RNAsin (40 U/mL), 2 μL DNA template, and approximately 1 μg RNAse-free water to make the final volume of 50 μL. The mixture was incubated at 25°C for 1 h. The reaction was terminated by adding 50 μL of cold Met (60 mM) in 2× import buffer and was kept in ice for immediate use.

Import/Binding of Precursor Proteins

Chloroplast protein binding and import reactions were performed as described (Olsen and Keegstra, 1992). The reaction mixture was prepared containing 8 μL precursor protein (translation mixture), 3 mM ATP, 35 μg of chloroplast suspension (1 mg Chl/mL), and 1× IB to make final volume of 100 μL. The transport reaction was set at 25°C for 20 min in dark, with gently agitating the reactions every 5 min. For binding, the reaction mixture consisted essentially of the same components except that the amount of ATP was reduced (0.3 mM). The reaction was performed in dark, keeping the tubes in ice. The import and binding reactions were terminated by adding 1 mL of cold 1× IB. Intact plastids were pelleted by layering the reaction mix on the top of 5 mL of 40% Percoll in 1× IB and by centrifuging at 7,000 rpm for 6 min with brake off. The pellet was suspended in 1 mL of cold 1× IB. A 50-μL aliquot was taken for protein estimation by the Bradford method (Bradford, 1976), and the rest was pelleted for 5 min at 6,000 rpm. The pellet was dissolved in 50 μL of 1× sample buffer, boiled for 3 min, and analyzed by SDS-PAGE (Laemmli, 1970). A 15% polyacrylamide gel was run to analyze the Rubisco SSU protein. Gel was run for approximately 5 h at a constant current of 25 mA. After the electrophoresis, the gel was dried onto a filter paper and exposed to x-ray film at room temperature.

Band Elution from the Dried Gel and Radioactivity Counting

After the film was developed, the corresponding band of interest was cut and kept in a scintillation vial. To it 1 mL of 30% H_2O_2 was added and incubated over night at 60°C. The vials were allowed to cool, and 12 mL of scintillation fluid was added to each vial, mixed by vortexing, and further incubated overnight at 60°C. The vials were allowed to cool at room temperature and vortexed, and counts were taken in a Beckman scintillation counter after making appropriate quenching corrections. Alternatively, gels containing radiolabeled proteins were quantified using the Fuji FLA-5000 imaging system (Fujifilm).

RNA Isolation and RT-PCR Analysis

Total RNA from control and stressed pea plant was isolated by guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987). To ensure comparability of the resulting band intensity, spectrophotometric quantification of RNA was confirmed by applying equal amounts of total RNA to an agarose gel. First-strand cDNA was synthesized using 3 μg of total RNA, oligo (dT) primer, and AMV reverse transcriptase (Promega) in a 50-μL reaction. Semiquantitative RT-PCR was performed as described by Burch-Smith et al. (2006). After synthesis, the cDNA was diluted 1:10, and 4 μL of cDNA was used as a template for PCR amplification in a 25-μL reaction mixture. Reaction contained selected couples of the following gene-specific primers: Toc34F, 5′-CGGGATCCATGGCTTCACAACAACAAACT-3′, Toc34R, 5′-GCTCTAGATCACTTCCGATCACCTACATCGCG-3′; Toc64F, 5′-GGGGTACCATGAAATCAATGGCTTCTCCGTCG-3′, Toc64R, 5′-CGGAATTCCTACTGAAATAGTTTCCTCAACCT-3′; Toc159intF, 5′-GCCTGGCATACATTGTTAGAGGT-3′, Toc159R, 5′-CGGGATCCTTAATAGATGGAATAGTTTTC-3′; Toc75F, 5′-CGGGATCCATGCGTACTTCCGTTATTCCC-3′, Toc75R, 5′-GCTCTAGATCAAAATCTTTCTCCAAAACG-3′; Tic55F, 5′-GCTCTAGAATGGCGTTGGCGTTGGCGTCGGCG-3′, Tic55R, 5′-GCTCTAGATCACAATCTCCTATGTACCCT-3′; TicC62F, 5′-CGGGATCCGGTACCATGGAAGGAACTTGTTTTCTC-3′, Tic62R, 5′-CGGGATCCGAATTCCTAATGATTGGTGACTGG-3′; Tic20F, 5′-CGGAATTCATGATTCAAAATGGTGGCACT-3′, Tic20R, 5′-CGGGATCCTTACTCGTGTGGTATTTGAAT-3′; Tic22F, 5′-CGGAATTCATGGAGTCTCAGGGACAGTGG-3′, Tic22R, 5′-CGGGATCCTTAAGCAATAACTCTCTGCAT-3′; Tic110intF, 5′-GCTTGACGAAGGCCAGGGTTGAG-3′, Tic110R, 5′-CGGGATCCCTAGAATACAAACTTCTCTTC-3′; Tic32F, 5′-ATGTGGCCATTCAGCAGCAAG-3′, Tic32R: 5′-CTATTTCTGCTTAACCAAATT-3′; Tic40intF, 5′-CCCGGCCCCAGACATTAATGT-3′, Tic40R, 5′-GAATCAAGGGGGGCCTGAAAC-3′; Hsp93F, 5′-GCATGGCTAGAGTTTTGGCTCAGTCACTT-3′, Hsp93R, 5′-TTATATAGAAAGAGCCTCTGGTAACGACTC-3′; and SPP F, 5′-CGGGATCCATGCCAATGGCTGCTTCAACT-3′, SPP R, 5′-CGGGATCCAGCTTAGGAACTAGAAAACTA-3′.

PCR was performed for 27 to 29 cycles within a linear range of amplification of psToc159, psToc75, psToc64, psToc34, psTic110, psTic62, psTic55, psTic40, psTic32, psTic22, psTic20, psHsp93, psSPP, and psACTIN genes. The number of cycles and annealing temperature were optimized for each specific primer pairs. Ten microliters of the PCR products were loaded and separated on 1% agarose Tris-acetate EDTA gel. Ethidium bromide-stained PCR products were quantified using an Alpha Imager 3400. RT-PCR for each gene was done in triplicate, and the average value was determined using Alpha Ease FC software.

Immunoblot Analysis

Intact plastids were isolated from control, chill-, and heat-stressed seedlings as described earlier (Tewari and Tripathy, 1999). Aliquots of the samples were denatured in the presence of SDS-PAGE sample buffer (15.5 mM Tris-HCl, pH 6.8, 720 mM 2-mercaptoethanol, 10% glycerol, and 3% SDS). Equal amounts (80 μg) of protein samples were resolved using 12.5% SDS-PAGE and subjected to western blotting (Towbin et al., 1979). The blots were probed with anti-psToc34, anti-Toc159, anti-psTic40, anti-psTic110, and anti-psHsp93 antibodies. The rabbit antimouse IgG (1:20,000) conjugated to alkaline phosphatase (Sigma-Aldrich) was used as a secondary antibody. Blots were stained for alkaline phosphatase using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium and quantified using an Alpha Imager 3400.

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