Changes in electron transport, superoxide dismutase and ascorbate peroxidase isoenzymes in chloroplasts and mitochondria of cucumber leaves as influenced by chilling

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Abstract

In order to clarify the relationship between chill-induced disturbance in photosynthetic, respiratory electron transport and the metabolism of reactive oxygen species (ROS), leaf gas exchange, chlorophyll fluorescence quenching, respiration, and activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) were investigated in chloroplasts and mitochondria of cucumber (*Cucumis sativus*) leaves subjected to a chill (8 ^{o}C) for 4 d. Chilling decreased net photosynthetic rate (P_N) and quantum efficiency of photosystem 2 (Φ_{PS2}), but increased the ratio of Φ_{PS2} to the quantum efficiency of CO_2 fixation (Φ_{CO2}) and non-photochemical quenching (NPQ) in cucumber leaves. While chilling inhibited the activity of cytochrome respiration pathway, it induced an increase of alternative respiration pathway activity and the reduction level of Q-pool. Chilling also significantly increased $O_2^\cdot$ production rate, H_2O_2 content, and SOD and APX activities in chloroplasts and mitochondria. There was a more significant increase in SOD and APX activities in chloroplasts than in mitochondria with the increase of membrane-bound Fe-SOD and tAPX in chloroplasts being more significant than other isoenzymes. Taken together, chilling inhibited P_N and cytochrome respiratory pathway but enhanced the photosynthetic electron flux to O_2 and over-reduction of respiratory electron transport chain, resulting in ROS accumulation in cucumber leaves. Meanwhile, chilling resulted in an enhancement of the protective mechanisms such as thermal dissipation, alternative respiratory pathway, and ROS-scavenging mechanisms (SODs and APXs) in chloroplasts and mitochondria.

Additional key words: ascorbate peroxidase; *Cucumis sativus*; net photosynthetic rate; photosynthetic electron transport; quantum efficiency; reactive oxidative metabolism; respiratory electron transport; respiration pathways; superoxide dismutase.

Introduction

Generation of reactive oxygen species (ROS) is inevitable in aerobic organisms and their accumulation is balanced by ROS production and ROS scavenging (Asada 1999, Alscher *et al.* 2002, Mittova *et al.* 2004). When plants are exposed to abiotic and biotic stresses, ROS homeostasis is disturbed, resulting in an oxidative stress in plant cells (Asada 1999, Mittler 2002, Neill *et al.* 2002). In plants, chloroplasts and mitochondria are major sources of ROS production in cells (Mittler *et al.* 2004). In chloroplasts, the primary sources of ROS production are the Mehler

reaction and the antenna pigments, and ROS production is increased under conditions limiting CO_2 fixation (Asada and Takahashi 1987). In mitochondria, over-reduction of the respiratory electron transport chain (RETC) is the main source of ROS production under stress (Møller 2001).

In the long evolution, plants have evolved protection strategies to prevent plant cells from ROS accumulation (Mittler 2002). Energy dissipation mechanisms such as photorespiration, cyclic flow of electron, and xanthophyll cycle prevent the electron flux to oxygen in chloroplasts, then decrease ROS production (Schöner and Krause 1990, Ort and Baker 2002). Similarly, RETC in plant mitochondria can be kept adequately oxidized to minimize ROS production by (*a*) maintaining a balance between substrate availability and ATP requirement; (*b*) activation of alternative oxidase (AOX); (*c*) activation of uncoupler protein; (*d*) activation of rotenone-insensitive NAD(P)H dehydrogenases (Møller 2001).

Plants also possess efficient scavenging systems for ROS to protect them from destructive oxidative stress. Superoxide dismutase (SOD) is the first line of defence against oxidative stress by catalyzing the dismutation of superoxide ($O_2^{\bullet-}$) to molecular oxygen and H_2O_2 (Okamoto *et al.* 2001). Three isoenzymes of SOD have been reported in various plant species: Mn-SOD, Cu/Zn-SOD, and Fe-SOD. Mn-SOD is located in mitochondria whilst Cu/Zn-SOD and Fe-SOD are located in chloroplasts (Alscher *et al.* 2002). Ascorbate peroxidase (APX), which comprises a family of isoenzymes in different cellular compartments, plays a crucial role in the detoxification of H_2O_2, the toxic product of superoxide dismutation (Asada 1992, Jiménez *et al.* 1997). APX isoenzymes are distributed in at least four distinct cellular compartments, the stroma (sAPX) and thylakoid membrane (tAPX) of chloroplasts, microbodies (mAPX) including peroxisomes and glyoxysomes, and the cytosol

(cAPX) (Wada *et al.* 2003). Recent studies showed that APX is also present in mitochondria (Jiménez *et al.* 1998, Mittova *et al.* 2004).

Environmental stresses such as salt, drought, and low temperature lead to increased production of ROS, resulting in oxidative damage at the cellular level (Fadzillah *et al.* 1996, Shigeoka *et al.* 2002, Gómez *et al.* 2004, Luna *et al.* 2004). Although some researchers have studied the avoidance mechanisms of ROS production and/or ROS-scavenging mechanisms in chloroplasts and/or mitochondria, the two major ROS production sources in plant cells (Asada and Takahashi 1987, Alscher *et al.* 1997, Jiménez *et al.* 1997, Mittova *et al.* 2002), the picture is still very fragmentary. Information about ROS generation and the roles of avoidance and scavenging metabolisms in chloroplasts and mitochondria under stresses is still scanty (Møller 2001, Mittler *et al.* 2004). Furthermore, to our best knowledge, there are few studies that simultaneously investigated the response of SOD and APX isoenzymes to a stress (Bowler *et al.* 1992, Mittova *et al.* 2000, Gómez *et al.* 2004).

Recently, we have shown that chill under low irradiance resulted in a loss or inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase, together with an increased proportion of electron influx to O_2 and increased contents of antioxidants in leaves and in isolated chloroplasts of cucumber plants (Zhou *et al.* 2004a,b, 2006). We also found the proportion of mitochondrial alternative respiratory pathway in total respiration apparently increased in chilled cucumber leaves (Hu *et al.* 2006). The relationship between ROS production and scavenging at organelle level, however, was largely unknown. In this study, gas exchange, chlorophyll (Chl) *a* fluorescence, respiration, the ROS accumulation, and activities of SOD and APX isoenzymes in chloroplasts and mitochondria were determined in cucumber leaves exposed to chill.

Materials and methods

Plants: Cucumber (*Cucumis sativus* L. cv. Jinyan No. 4) seeds were sown in a medium containing a mixture of soil : perlite (50 : 50, v : v) in tray in a greenhouse. Seven days later, the seedlings were transferred into a container (40×25×15 cm) filled with half-strength Enshi nutrient solution (Yu and Matsui 1997) in a growth chamber for 10 d. The environmental conditions were as follows: a 12 h photoperiod, temperature of 25/17 °C (day/night), photosynthetic photon flux density (PPFD) of 600 µmol m^{-2} s^{-1}.

Chilling treatment: Half the plants at the 3-leaf stage were exposed to 100 µmol m^{-2} s^{-1} at 8 °C for 4 d and the other half were maintained at 25/17 °C with a PPFD of 600 µmol m^{-2} s^{-1}. The net photosynthetic rate (P_N), Chl *a* fluorescence, respirations (including total, KCN- and

SHAM-resistant respirations), reduction state of the ubiquinone pool, $O_2^{\bullet-}$ producing rate, H_2O_2 content, and activities of SOD and APX isoenzymes in chloroplasts and mitochondria in cucumber leaves were measured 4 d after the chilling.

Leaf gas exchange: Chilled plants were transferred to a growth room at 25 °C under a PPFD of 600 µmol m^{-2} s^{-1} for 2 h before measurements. P_N was measured using an infrared gas analyzer (*LI-6400, LI-COR Biosciences*, USA) on the second fully developed leaf of each plant. The air temperature was 25 °C, air relative humidity 80−90 %, CO_2 concentration 360 µmol mol^{-1}, and PPFD 600 µmol m^{-2} s^{-1}. The quantum efficiency of CO_2 fixation (Φ_{CO2}) was determined simultaneously at 600 µmol m^{-2} s^{-1} PPFD. Φ_{CO2} was calculated by dividing the rate of

P_N at 600 μmol m^{-2} s^{-1} PPFD by the rate at which quanta were absorbed after correction for dark respiration (Zhou *et al*. 2004b).

Chl *a* fluorescence was measured using a portable pulse modulated fluorometer (*FMS-2, Hansatech*, UK) in the same leaves previously used for gas exchange measurements. Before each measurement leaves were dark-adapted for at least 30 min. The minimal fluorescence (F_0) was determined under a weak pulse of modulating radiation over a 0.8 s and maximal fluorescence (F_m) was induced by a saturating pulse (8 000 μmol m^{-2} s^{-1}) applied over 0.8 s. An "actinic light" source (600 μmol m^{-2} s^{-1}) was then applied to achieve a steady state of photosynthesis and to obtain F_s (steady state fluorescence yield), after which a second saturation pulse was applied for 0.8 s to obtain irradiance adapted maximum fluorescence (F_m'). Quantum efficiency of PS2 (Φ_{PS2}) and non-photochemical quenching coefficient (NPQ) were calculated as (F_m' $-$ F_s)/F_m' and F_m/F_m' $-$ 1, respectively (Demmig-Adams 1996).

Respiration was measured as a decrease of the oxygen concentration in a 2 cm^3 closed cuvette using a Clark type oxygen electrode (*Oxygraph-lab, Hansatech*, UK) at 25 ^{o}C. Samples (0.1 g of fresh mass, FM) were kept in the dark for 30 min before respiration measurements were taken. To assess the alternative pathway activity, the cytochrome pathway was inhibited with 10 mM KCN (form a 100 mM stock solution in 20 mM phosphate buffer, pH 8.0), whereas the alternative pathway was inhibited with 20 mM SHAM (form a 100 mM stock solution in ethanol). The respiration measured at 10-15 min after addition of the inhibitors was used to calculate the percentage inhibition from control respiration rates. Measurement temperature was controlled by a water bath set (*2219 Multitemp II Thermostatic Circulator*, Germany), which was connected to the measurement cuvette.

The reduction state of ubiquinone pool: The oxidized forms (UQ) and reduced forms (UQr) of ubiquinone were assayed following the method of Wagner and Wagner (1995) with some modification. All reagents were chilled prior to use and all procedures were performed under dim light. Samples [10 g(FM)] were frozen in liquid N$_2$, ground to a powder. The powder was then dissolved in 3 cm^3 of 0.2 M HClO$_4$ in 80 % (v/v) methanol : water and then extracted with 3 cm^3 petroleum ether for three times. The petroleum ether phase was evaporated to dryness under a flow of nitrogen. Immediately before use, the extracted UQ and UQr were re-suspended in 100 mm^3 of nitrogen-purged methanol (HPLC grade) gently, and analyzed using a HPLC (*LC-10AT* pump system, *Shimadzu*, Japan) with a UV-VIS detector (*SPD-10A* detector, Japan) equipped with a reverse phase *Spherisorb C-18* analytical column (5 μm, 250×4.6 mm).

The column was equilibrated with nitrogen-purged ethanol : methanol (3 : 2, v/v), and this mixture was used as the mobile phase with a flow rate of 1 cm^3 min^{-1}. UQ and UQr were detected at 290 nm. The amounts of UQ and UQr were calculated from the peak areas and quantified using a standard series ranging from 0 to 800 μM. Commercially available UQ$_{10}$ (*Sigma*) was used as a standard. The amounts of total ubiquinone (UQt) were calculated as the sum of UQ and UQr. The reduction level of ubiquinone pool (Q-pool) was calculated as UQr/UQt (Hu *et al*. 2006).

O$_2$$^{•-}$ producing rate and H$_2$O$_2$ content were analysed as described by Zhou *et al*. (2004b). In general, O$_2$$^{•-}$ was measured by monitoring the nitrite formation from hydroxylamine in the presence of O$_2$$^{•-}$. Samples (0.5 g) were homogenized with 3 cm^3 of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5 000×*g* for 10 min. The incubation mixture contained 0.9 cm^3 of 65 mM potassium phosphate buffer (pH 7.8), 0.1 cm^3 of 10 mM hydroxylamine hydrochloride, and 1 cm^3 of the supernatant. After incubation at 25 ^{o}C for 20 min, ethyl ether in the same volume was added and centrifuged at 1 500×*g* for 5 min. The absorbance in the aqueous solution was read at 530 nm. A standard curve with NO$_2^-$ was used to calculate the production rate of O$_2$$^{•-}$ form the chemical reaction of O$_2$$^{•-}$ and hydroxylamine. The H$_2$O$_2$ content was assayed diluted 2.5-fold with acetone and was measured by monitoring the A$_{410}$ of titanium-peroxide complex.

Isolation of chloroplasts and mitochondria from cucumber leaves was done by differential and density gradient centrifugation as described by Mittova *et al*. (2000) with a modification. Briefly, leaves (10 g each) were chopped using a blender (*HR-2826, Philips*, China) with 5 volumes of medium containing 50 mM HEPES [*N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); pH 7.5], 5 mM γ-caproic acid, 0.3 % bovine serum albumin (m/v), 0.4 M sucrose, 10 mM NaCl, 10 mM mercaptoethanol, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 % (m/v) polyvinylpyrrolidone. The homogenates were filtered through four layers of gauze. The crude chloroplasts fraction from leaves was sedimented by centrifugation at 1 000×*g* for 5 min. Chloroplasts in the residues were then purified by a 10, 40, 70, and 90 % *Percoll* discontinuous gradient in the presence of 2 mM ascorbate, and centrifugation at 4 700×*g* for 15 min. Intact chloroplasts layer was obtained in between the 40 and 70 % *Percoll* fractions. The ferricyanide method was used to measure the intactness of chloroplasts (Takeda *et al*. 1995). We found that about 85−90 % of chloroplasts were intact.

The 1 000×*g* supernatant was re-centrifuged at 12 000×*g* for 15 min and the pellet was collected and re-suspended in 20 mM HEPES-KOH (pH 7.5), 330 mM sorbitol, 10 mM NaCl, and 2 mM EDTA. The 12 000×*g*

pellets were fractionated on a 25, 37, 45, and 57 % (m/m) sucrose gradient at 68 000×g for 3.5 h and the intact mitochondria layer between the 37 and 45 % sucrose fractions was removed. The integrity of the mitochondria was estimated from the cytochrome C oxidase activity, as described by Millenaar *et al.* (2002). The purified mitochondria had intactness rates between 75−90 %.

Antioxidant enzyme activities (SOD and APX) were assayed in intact chloroplasts and mitochondria diluted 2−5-fold with 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbic acid (AsA), and 2 % (m/v) polyvinylpyrrolidone, unless otherwise indicated.

Total SOD activity was measured by the photochemical method described by Giannopolitis and Ries (1977). Ascorbate was removed from the enzyme solution, since ascorbate may interact with superoxide and interfere with the SOD assay. One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of the rate of *p*-nitro blue tetrazolium chloride reduction at 560 nm. The activities of Cu/Zn- and Fe-SODs in chloroplasts were subtracted sequentially from the total SOD activity using 3 mM KCN or 5 mM H_2O_2, respectively [Zhou *et al.* 2004a]. APX activity was measured following Amako and Asada (1994) by monitoring the rate of ascorbate oxidation at 290 nm (E = 2.8 mM cm^{-1}).

Activities of cAPX, sAPX, tAPX, and mAPX in the leaf extract were separately determined as reported by Amako and Asada (1994), utilizing the different sensitivities of these isoenzymes to a low AsA (reduced ascorbate) condition established with AsA oxidase. Cucumber leaves were ground to a fine powder in liquid N_2 and then homogenized in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate, 20 % (m/v) sorbitol, 1 mM EDTA, and 0.1 % (m/v) phenylmethanesulfonyl fluoride using a mortar and pestle. The homogenate was squeezed through four layers of cheesecloth and then centrifuged at 120 000×g for 10 min. The obtained soluble fraction contained activities of sAPX and cAPX.

The soluble fraction (10 mm^3) was added to 5.0 cm^3 of N_2-bubbling 50 mM potassium phosphate buffer (pH 7.0) containing 10 μM H_2O_2. At 3, 4, 5, and 6 min after the start of the incubation, the incubated mixture (1.98 cm^3) was sampled and mixed with 10 mm^3 of 100 mM ascorbate to terminate the inactivation. The residual oxidizing activity of ascorbate was then assayed by adding 10 mm^3 of 10 mM H_2O_2. The oxidation of ascorbate was followed by a decrease in the A_{290}, and the results were plotted on the graph. The cAPX and sAPX activities were calculated from the inactivation curve of each isoenzyme. The 120 000×g-membrane fraction was washed and suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM ascorbate. The suspended-membrane fraction contained activities of tAPX and mAPX isoenzyme, which were assayed separately by the same method using each half-inactivation time as measurements of activities of sAPX and cAPX isoenzymes.

Northern blot analysis of cAPX transcript: Total RNA was extracted by grinding the leaf tissues in a mortar in the presence of *TRIzol* reagent (*GIBCO/BRL*), following the manufacturer's instructions. After extraction, total RNA was dissolved in diethyl pyrocarbonate-treated water. RNA (10 μg) was separated in a denaturing 1.2 % (m/v) agarose gel containing 2 % formaldehyde and blotted onto a *Hybond N*$^+$ membrane. Relative loading was confirmed by subsequent running of the samples and using UV fluorescence of the ethidium bromide stain. Probe preparation and visualization of hybridized bands were carried out according to standard procedures. A cAPX cDNA probe prepared in our laboratory for other purpose was used for detection of the transcript level of cAPX, as described by Yoshimura *et al.* (2000).

Statistics: Results were tested with *SPSS 11.5* for windows (*SPSS*, Chicago, IL, USA) by one-way analysis of variance (ANOVA). Significant differences between treatment means were separated by using least-significant difference (LSD) test at the *p*<0.05 level.

Results

Gas exchange, Chl *a* fluorescence quenching, and respirations: Chilling for 4 d at low irradiance significantly decreased P_N and Φ_{PS2}. In comparison to the reduction in Φ_{PS2} (16.5 %), a more significant reduction was found in P_N (67.6 %) after the chilling. Accordingly, the rate of Φ_{PS2}/Φ_{CO2} increased from 9 for the un-chilled plants to 19.8 for the chilled plants. Meanwhile, chilling resulted in a sharp increase in NPQ.

Chilling decreased total respiration (V_t) and cytochome pathway activity (V_{SHAM}) by 12.6 and 38.1 %, respectively. In contrast, alternative pathway activity (V_{KCN}) was increased by 23.1 % after the chilling. In addition, the reduction level of Q-pool (UQr/UQt) increased from 0.79 to 0.87 after the chilling (Table 1).

$O_2^{\bullet-}$ **and** H_2O_2: Chilling increased $O_2^{\bullet-}$ producing rate and H_2O_2 content in cucumber leaves. The producing rate of $O_2^{\bullet-}$ and H_2O_2 content were increased by 94.0 and 65.9 %, respectively, after exposure to chilling for 4 d (Fig. 1).

Activities of SOD in chloroplasts and mitochondria: Chilling significantly increased the activities of SOD both in chloroplasts and in mitochondria. Total SOD activities in chloroplasts and mitochondria increased by 136.9 and 57.6 %, respectively. A close examination of activities of SODs showed that Cu/Zn-SOD and Fe-SOD activities increased by 66.1 and 275.0 %, respectively (Fig. 2).

Table 1. Effects of chilling on net photosynthetic rate (P_N) [μmol m^{-2} s^{-1}], chlorophyll a fluorescence, respiration rate (V_t) [nmol(O_2) kg^{-1}(FM) s^{-1}], activities of alternative (V_{KCN}) and cytochrome pathway activities [nmol(O_2) kg^{-1}(FM) s^{-1}], and reduction level of Q-pool (UQr/UQt) in cucumber leaves. Means of at least three replicates with standard errors. *Different letters* indicate statistically significant differences between treatments at the 5 % level.

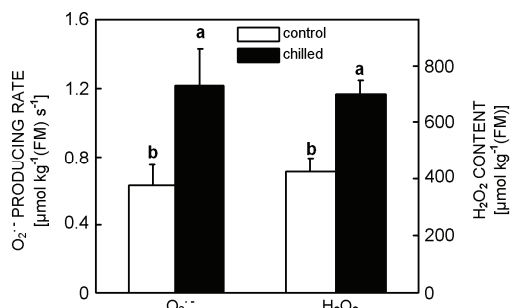| Treatment	P_N	Φ_{PS2}	Φ_{PS2}/Φ_{CO2}	NPQ	Vt	V_{KCN}	V_{SHAM}	UQr/UQt
Control	10.3±1.1a	0.25±0.01a	8.9±1.5b	0.35±0.02b	4.83±0.19a	2.17±0.14b	3.86±0.19a	0.79±0.02b
Chilled	3.3±1.1b	0.21±0.01b	19.8±2.4a	0.85±0.08a	4.22±0.25b	2.67±0.19a	2.39±0.25b	0.87±0.01a

Fig. 1. Effects of chilling on $O_2^{\cdot-}$ production rate and H_2O_2 content in cucumber leaves. Means of at least three replicates with standard errors shown by *vertical bars*. *Different letters* indicate statistically significant differences between treatments at the 5 % level.

Activities of APX isoenzymes in chloroplasts and mitochondria: The activity of APX in chloroplasts and mitochondria was increased by 101.4 and 73.5 %, respectively, after chilling for 4 d. The activities of the four APX isoenzymes were all increased by the chilling. The activities of sAPX and tAPX in chloroplasts increased 2.2- and 14.8-fold, while mAPX and cAPX activities increased 1.8- and 1.6-fold compared with un-chilled control leaves, respectively (Fig. 3). In agreement with the changes in cAPX activity, the transcript level of cAPX was up-regulated after the chilling (Fig. 4).

Discussion

Decrease in P_N under stress conditions such as chilling and drought is well observed and is usually associated with a decrease in the demand for ATP and NADPH, leading to an oxidative stress and photo-damage induced by an overproduction of reactive oxygen species in many cases (Foyer *et al.* 2002, Ort and Baker 2002, Zhou *et al.* 2004b). We also observed that chilling significantly decreased P_N (Table 1) and increased ROS generation (Fig. 1) in cucumber leaves. Our results are in agreement with the early observations. Meanwhile, plants have also developed processes such as xanthophyll cycle-dependent energy dissipation as heat from antenna in PS2, the D1 repair cycle, NPQ, photorespiration, and the operation of water-water cycle to protect the chloroplasts from damage (Chaumont *et al.* 1995, Demmig-Adams 1996, Asada 1999, Ort and Baker 2002, Hendrickson *et al.* 2003). We found that chilling inhibited P_N, but increased Φ_{PS2}/Φ_{CO2} at 2 % O_2 pressure (Table 1), implying that other electron sinks other than CO_2 assimilation were enhanced (Fryer *et al.* 1998). This indirectly supports our early finding that water-water cycle operated at high rates together with increased accumulation of ROS in chloroplasts when CO_2 assimilation was restricted in chilled cucumber leaves (Zhou *et al.* 2004b). Chill decreased Φ_{PS2}, however, it increased NPQ greatly (Table 1). High NPQ implies high ability of thermal energy dissipation through xanthophyll cycle, which would dissipate excessive absorbed photons and mini-mize excessive electron flux in PETC (Demmig-Adams

1996). In addition, the decrease of Φ_{PS2} might be a protective mechanism in order to match the electron transport with the lower demand for ATP and NADPH in Calvin cycle, thus avoiding the accumulation of reductants in PETC.

Mitochondria are another energy metabolism centres in plant cells. Chilling restricted cytochrome pathway activity and induced high reduction level of RETC, but increased alternative pathway activity (Table 1). This is consistent with earlier observations (Purvis and Shewfelt 1993, Luxova and Gasparikova 1999). A constraint of cytochrome pathway after a chill leads to over-reduction of the RETC, which would enhance ROS production in mitochondria (Møller 2001). Alternative pathway, which competes for electrons from the reduced ubiquinone pool with cytochrome pathway, allows the uncoupling of electron transfer from ATP production and prevents the over-reduction of the respiratory electron transport chain (Vanlerberghe and McIntosh 1997). Therefore alternative pathway diminished the risk of electron transfer to O_2 and ROS generation in mitochondria under chilling (Wagner and Moore 1997, Møller 2001). At the same time, up-regulation of mitochondrial alternative pathway would avoid over-reduction of the PETC to diminish the production of ROS in chloroplasts. There are several lines of evidence that inhibition of AOX in mitochondria causes an over-reduction of the photosynthetic electron transport chain (Yoshida *et al.* 2006). Bartoli *et al.* (2005) observed that the up-regulation of the mitochondrial
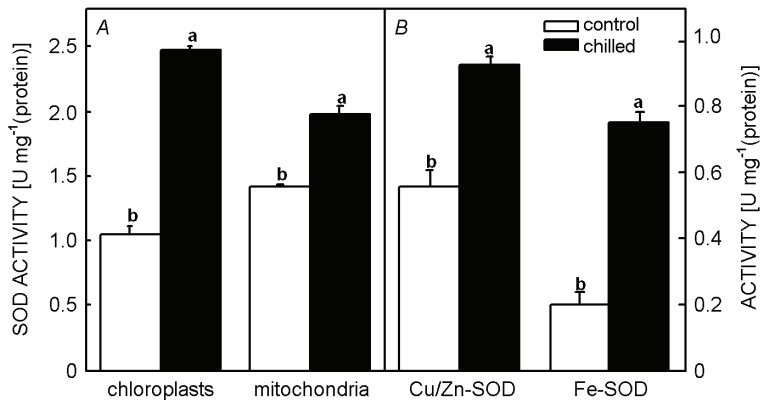
Fig. 2. Effects of chilling on total superoxide dismutase (SOD) activities in chloroplasts and mitochondria (*A*), and the activities of Cu/Zn-SOD and Fe-SOD of chloroplasts (*B*) in cucumber leaves. Means of at least three replicates with standard errors shown by *vertical bars*. *Different letters* indicate statistically significant differences between treatments at the 5 % level.
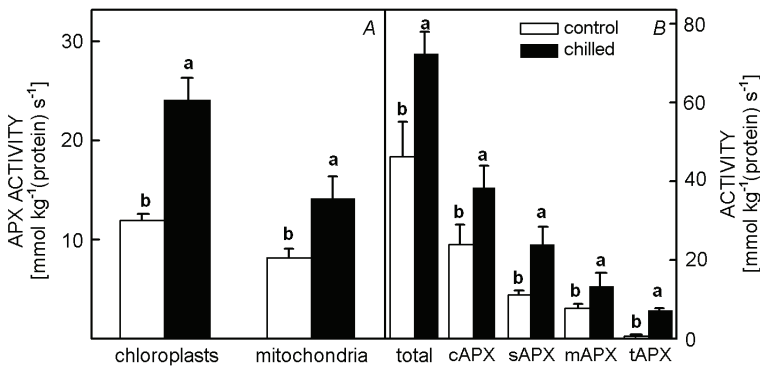

Fig. 3. Effects of chilling on total ascorbate peroxidase (APX) activities in chloroplasts and mitochondria (*A*), and the activities of four APX isoenzymes (cAPX, sAPX, mAPX, tAPX) in cell (*B*) in cucumber leaves. Means of at least three replicates with standard errors shown by *vertical bars*. *Different letters* indicate statistically significant differences between treatments at the 5 % level.
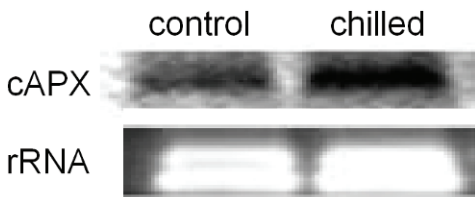

Fig. 4. Northern blot analysis for the expression of cAPX after chilling in cucumber leaves.

alternative pathway enhances photosynthetic electron transport under drought. We also observed that the inhibition of AOX by AOX inhibitor decreased photosynthetic electron transport in chilled cucumber leaves (unpublished data).

The production and accumulation of ROS were apparent in cucumber leaves (Fig. 1), in spite of increased thermal dissipation and alternative pathway activity in cucumber leaves after a chill. Chilling induced an up-regulation of ROS-scavenging enzymes both in chloroplasts and mitochondria, with a more significant increase in chloroplasts than in mitochondria (Figs. 2 and 3). In chloroplasts, there was a more significant increase in Fe-SOD (275.0 %) than in Cu/Zn-SOD (66.1 %) after a chill (Fig. 2*B*). Fe-SOD activity was detected in the chloroplast membrane in the transgenic tobacco plants (van Camp *et al.* 1996). Overproduction of chloroplast Fe-SOD protects the plasma membrane and the PS2 reaction centre against paraquat both in transgenic

tobacco (van Camp *et al.* 1996) and maize plants (van Breusegem *et al.* 1999). Accordingly, the higher increase in membrane-bound Fe-SOD in the vicinity of the site of ROS production might reduce the diffusion of ROS to both PS2 and the cell membranes. Similar results were observed in chloroplasts from chilled, salt and paraquat-treated cucumber and tomato leaves by Mittova *et al.* (2002) and Zhou *et al.* (2006).

Chilling also induced significant increases of APX activity in cucumber leaves. The more significant increases in the activities of Fe-SOD and tAPX, which are located in chloroplast membrane, than Cu/Zn-SOD and sAPX, which are located in chloroplast stroma, suggested that ROS-scavenging enzymes located in chloroplast membrane may play a more important role in ROS-scavenging of chloroplasts (Fig. 3). In our study, increase of cAPX activity was correlated with its transcript level (Fig. 4), suggesting that up-regulation of cAPX activity was partly due to the up-regulation of its expression at transcription level.

In conclusion, we found that chilling inhibited P_N and cytochrome respiratory pathway but enhanced the photosynthetic electron flux to O_2 and over-reduction of RETC, resulting in ROS accumulation in cucumber leaves. Meanwhile, chilling resulted in an enhancement of the protective mechanisms such as thermal dissipation, alternative respiratory pathway, and ROS-scavenging mechanisms (SODs and APXs) in chloroplasts and mitochondria.

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