



Enhanced sensitivity and characterization of photosystem II in transgenic tobacco plants with decreased chloroplast glutathione reductase under chilling stress

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ARTICLE INFO

Article history:

Received 6 April 2012

Received in revised form 3 June 2012

Accepted 8 June 2012

Available online 15 June 2012

Keywords:

Chilling stress

Chlorophyll fluorescence

Glutathione reductase

Photosystem II

Transformed tobacco (*Nicotiana tabacum*)

Thermoluminescence

ABSTRACT

Chloroplast glutathione reductase (GR) plays an important role in protecting photosynthesis against oxidative stress. We used transgenic tobacco (*Nicotiana tabacum*) plants with severely decreased GR activities by using a gene encoding tobacco chloroplast GR for the RNAi construct to investigate the possible mechanisms of chloroplast GR in protecting photosynthesis against chilling stress. Transgenic plants were highly sensitive to chilling stress and accumulated high levels of H₂O₂ in chloroplasts. Spectroscopic analysis and electron transport measurements show that PSII activity was significantly reduced in transgenic plants. Flash-induced fluorescence relaxation and thermoluminescence measurements demonstrate that there was a slow electron transfer between Q_A and Q_B and decreased redox potential of Q_B in transgenic plants, whereas the donor side function of PSII was not affected. Immunoblot and blue native gel analyses illustrate that PSII protein accumulation was decreased greatly in transgenic plants. Our results suggest that chloroplast GR plays an important role in protecting PSII function by maintaining the electron transport in PSII acceptor side and stabilizing PSII complexes under chilling stress. Our results also suggest that the recycling of ascorbate from dehydroascorbate in the ascorbate–glutathione cycle in the chloroplast plays an essential role in protecting PSII against chilling stress.

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1. Introduction

Glutathione, a multifunctional thiol tripeptide, has a number of functions in biosynthetic pathways, detoxification, antioxidant biochemistry, and redox homeostasis. These functions are associated with many processes involved in the regulation of gene expression, cell signaling, cell cycle, plant development, and cell death [1,2]. Glutathione exists with two different forms, i.e. the reduced form (c-Glu-Cys-Gly, GSH) and the oxidized form (glutathione disulphide, GSSG). Glutathione is maintained almost exclusively in the reduced form in plants and the proportion of glutathione in the reduced form is normally higher than 0.9 [3]. Moreover, the physiological functions of glutathione have been mainly attributed to its reduced form in plants [4]. Therefore, it is important to maintain a high proportion of glutathione in its reduced form in plants.

Acting as a major antioxidant in plant cells, GSH is oxidized to the disulphide form GSSG. However, plants have evolved some mechanisms to maintain a high proportion of the reduced glutathione [2]. One mechanism is that GSH can be synthesized in the chloroplast and the cytosol in plant cells. Another one is that GSSG can be reduced to GSH by glutathione reductase (GR, EC 1.6.4.2) [5,6].

One of key functions of GR is that it is involved in the ascorbate–glutathione cycle, an important pathway found in almost all cellular compartments to scavenge H₂O₂ [2,7,8]. In addition, several other enzymes are also involved in this cycle, including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) [8]. In this cycle, GSH is oxidized to GSSG, and the reduction of GSSG back to GSH is catalyzed by GR. The efficient recycling of glutathione ensured by GR makes re-reduction of ascorbate, an electron donor used by APX to reduce H₂O₂. Therefore, glutathione acts as a recycled intermediate in the reduction of H₂O₂ using electrons derived ultimately from H₂O [9], indicating that GR may play an important role in the protection of plants against oxidative stress [2,5,10].

Two genes are annotated to encode GR in plants. One is predicted to encode a cytosolic GR. The other is originally predicted to encode a chloroplast GR only but later it has been suggested that it encodes a dual targeting GR protein to chloroplasts and mitochondria, respectively [11,12]. It seems that there are significant differences in the GR activities either in different cellular compartments or in different species. In pea plants, most of the GR activity (about 77%) is in the chloroplast and only about 20% and 3% of total GR activity is detected in the cytosol and mitochondria, respectively [13]. In tobacco plants, GR activity is also localized mainly in the chloroplast in which it represents about 70% of total GR activity [14]. However, in *Arabidopsis*, most of the GR activity (65%) is located in the cytosol and about 35% of the total GR activity

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is contributed by chloroplast/mitochondrion GR [15]. In addition, GR activity is also detected in peroxisomes [16].

The possible roles of GR located in different cellular compartments in protecting plants against oxidative stress have been investigated by many studies. It seems that GR located in cytosol does not play an important role in protecting against oxidative stress although most of GR activity is located in cytosol in *Arabidopsis* [15,17]. Indeed, it has been demonstrated that elevated levels of cytosolic GR activity in transgenic plants have no effect either on the amount of glutathione pool or on the reduction state of glutathione under optimal conditions or under oxidative conditions [18]. On the other hand, several studies seem to suggest that GR located in the chloroplast may play an important role in the protection of plants against oxidative stress. It has been shown that enhanced chloroplast GR activities in transgenic tobacco, poplar, and cotton plants result in increased protection against oxidative stress [19–23] while decreased chloroplast GR activities in tobacco and tomato plants lead to an increased sensitivity to oxidative stress [14,24,25].

Chilling stress, normally referring to non-freezing temperatures (0–12 °C), is an important environmental factor limiting the productivity and geographical distribution of plants. It is common during the growing season in temperate regions. Chilling stress has profound limitations on the various aspects of photosynthesis, in particular on thylakoid electron transport and this is because chilling often exacerbates oxidative stress induced by light [26]. Acclimation to chilling stress often leads to increased GSH contents and GR activities [27,28]. A positive correlation between chilling resistance and GR activities has been demonstrated in some crops, such as rice, maize, and tomato [29–31]. Attempts to increase chilling resistance using transgenic over-expression of GR have made some success [21–23,32]. Elevated chloroplast GR activities can lead to the decrease in the levels of photosystem II (PSII) and photosystem I (PSI) photoinhibition during short term exposure of leaf disks of cotton to 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 10 °C [23,32]. However, it is still unknown how chloroplast GR protects PSII against chilling stress.

We have previously obtained transgenic tobacco (*Nicotiana tabacum*) plants with 30–70% decreased GR activity which was mainly due to the suppression of GR in chloroplasts by using a gene encoding tobacco chloroplast GR for the RNAi construct [14]. In this study, using these transgenic tobacco plants, we investigated the role of GR in the chloroplast in protecting PSII from chilling stress. Our results suggest that chloroplast GR plays an important role in protecting PSII against chilling stress by maintaining the stability of PSII complex. Our results also suggest that the recycling of ascorbate from dehydroascorbate in the ascorbate–glutathione cycle in the chloroplast plays an essential role in protecting PSII against chilling stress.

2. Materials and methods

2.1. Plant growth conditions and chilling treatments

Four independent lines of transgenic tobacco plants (i2, i21, i28, and i42) were established as described in our previous study [14]. These transformed plants were used as the source of plant materials. Plants derived from untransformed seeds were designated as wild type plants. For normal temperature treatments, the seeds of wild type and transgenic i2, i21, i28 and i42 tobacco plants were allowed to germinate and plants were grown in MS medium in a growth chamber at 25 \pm 1 °C for 10 days with PPFD of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a relative humidity of 75%–80%, and a photoperiod of 14/10 h light/dark. For chilling treatments, the seedlings after growth for 7 days at normal temperature were transferred to a growth chamber where temperature was set at 10 \pm 1 °C and grown further for another 7 days with PPFD of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a relative humidity of 75%–80%, and a photoperiod of 14/10 h light/dark.

2.2. Chlorophyll (Chl) fluorescence analysis

A PAM-2100 portable Chl fluorometer (Heinz Walz, Germany) was used to measure Chl fluorescence as described by Zhang et al. [33]. Leaves were dark adapted for 30 min. After that, F_o (minimum Chl fluorescence of dark-adapted state) was measured by a weak red light. F_m (maximum Chl fluorescence of dark-adapted state) was determined during a subsequent saturating pulse of white light (8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s). Then actinic light at an intensity of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (which is equivalent of growth light intensity) was applied continuously for about 7 min. F_s (steady-state Chl fluorescence) was thereafter recorded and a second saturating pulse of white light (8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s) was applied to determine F_m' (maximum Chl fluorescence level in the light-adapted state). Then, the actinic light was removed and F_o' (minimal Chl fluorescence level in the light-adapted state) was recorded by applying a 3 s pulse of far-red light. Using the above fluorescence parameters, we calculated: the maximal efficiency of PSII photochemistry in the dark-adapted state, $F_v/F_m = (F_m - F_o)/F_m$ and the actual PSII efficiency, $\Phi_{\text{PSII}} = (F_m' - F_s)/F_m'$.

2.3. Chl fluorescence relaxation kinetics analysis

The decay of Chl a fluorescence yield after a single turnover flash was measured with a double-modulation fluorescence fluorometer (model FL-200, Photon Systems Instruments, Brno, Czech Republic) as described by Zhang et al. [33]. The instrument contained red LEDs for both actinic (20 μs) and measuring (2.5 μs) flashes, and was used in the time range of 100 μs to 100 s. Before measurements, the tobacco leaves were dark adapted for 30 min in the absence or presence of 50 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU). With this type of measurement, it is important to avoid distortion of the relaxation kinetics due to the actinic effect of measuring flashes. This was carefully checked, and the intensity of the measuring flashes was set at a value that was low enough to avoid reduction of Q_A (primary quinone electron acceptor of PSII) in the presence of DCMU.

2.4. Thermoluminescence (TL) measurements

TL measurements were performed with the thermoluminescence extension of the Double-Modulated Fluorometer FL2000-S/F, consisting of Thermoregulator TR2000 (Photon Systems Instruments, Brno, Czech Republic). According to Zhang et al. [33], after 30 min dark adaptation, the samples were cooled to -5 °C and illuminated with one or multiple number of single-turnover flashes. Then the samples were warmed up to 60 °C at a heating rate of 1 °C s^{-1} and the TL light emission was measured during the heating. To detect period-four oscillation of the B-band, leaves were illuminated with a series of single-turnover flashes. For $S_2Q_A^-$ recombination studies, leaves were measured in the presence of 50 μM DCMU after the flash illumination. Decomposition analysis of the TL glow curves was performed by a non-linear, least squares algorithm that minimizes the χ^2 function using a Microcal™ Origin™ Version 6.0 software package (Microcal Software Inc., Northampton, MA).

2.5. P700 absorbance

The light-induced P700 absorbance changes at 820 nm were measured using the PAM-101 fluorometer connected to an emitter-detector unit ED 800T (Walz) as described by Meurer et al. [34]. Absorbance changes induced by saturating far-red light were used to estimate the photochemical capacity of PSI.

2.6. Measurements of electron transport activity

Thylakoid membranes were isolated as described by Peng et al. [35] and the electron transport activities of PSI and PSII was measured according to Zhang et al. using a Clark-type oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) [33]. PSI electron transport activity was measured in a 1 ml reaction mixture composed of 50 mM Tricine–NaOH (pH 7.5), 400 mM sucrose, 10 mM NaCl, 5 mM MgCl₂, 1 mM sodium azide, 0.5 mM methyl viologen, 10 μM DCMU, 1 mM sodium ascorbate, 200 μM 2,6-dichlorophenol indophenol (DCPIP), and thylakoids corresponding to 10 μg of chlorophyll. PSII electron transport activity was assayed in a 1 ml reaction mixture composed of 50 mM Mes–NaOH (pH 6.5), 400 mM sucrose, 50 mM CaCl₂, 5 mM MgCl₂, 0.2 mM 2,6-dichloro-*p*-benzoquinone (DCBQ), 1 mM ferricyanide, and thylakoids corresponding to 30 μg of chlorophyll.

2.7. Detection and measurement of ROS

Cytochemical detection of H₂O₂ was assayed according to the method of Bestwick et al. [36]. Leaf pieces (approximately 1–3 mm²) were excised and incubated in freshly prepared 5 mM CeCl₃ in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) at pH 7.2 for 1 h. Leaf pieces were then fixed with 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 1 h. After fixation, tissue was washed twice in the same buffer for 10 min, samples were post fixed 45 min in phosphate buffer containing 1% osmium tetroxide, dehydrated in an ethyl-alcohol series, transferred to propylene oxide, embedded in Epon812, and polymerized by heat. Ultrathin sections (70 nm thick) were obtained with a Leica ultramicrotome, stained with uranyl acetate and lead citrate, and observed under a JEM-1230 TEM (JEOL).

In situ detection of O₂^{•−} was determined by using the nitroblue tetrazolium (NBT) staining according to Kawai-Yamada et al. [37]. Whole seedlings were vacuum-infiltrated with 10 mM NaN₃ in 10 mM potassium phosphate buffer (pH 7.8) for 1 min, and incubated in 1 mg ml^{−1} NBT (in 10 mM potassium phosphate buffer, pH 7.8) for 20 min in the dark at room temperature.

Total leaf H₂O₂ content was determined according to the method of Veljovic-Jovanovic et al. [38].

2.8. Isolation of intact chloroplasts

Intact chloroplasts were isolated by centrifugation on a Percoll density gradient according to the method described by Yoshimura et al. [39]. Intactness of the Percoll-purified chloroplasts was found to be 90–95% as determined by the ferricyanide reduction method. Purity of chloroplasts was determined by using the enzymes catalase, cytochrome-c oxidase, and phosphoenolpyruvate carboxylase (PEPCase) as markers for peroxisome, mitochondria, and cytosol, respectively [13,40].

2.9. Enzyme measurements in chloroplasts

Intact chloroplasts were isolated from wild type and transgenic tobacco plants and diluted with 50 mM potassium phosphate buffer (pH 7.6) containing 0.2 mM EDTA and 2% (w/v) polyvinylpyrrolidone (PVP). GR activity was determined by following the rate of GSH formation at 412 nm [41]. DHAR activity was determined by the increase in absorbance at 295 nm due to DHA reduction [42]. MDHAR activity was measured by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH [43]. SOD activity was estimated by its ability to inhibit photoreduction of nitroblue tetrazolium [44]. The activities of stromal (sAPX) and thylakoid membrane-bound (tAPX) were determined by monitoring the decrease in absorbance at 290 nm due to ascorbate oxidation according to Zhou et al. [45].

2.10. Determinations of antioxidants in chloroplasts

Intact chloroplasts isolated from wild type and transgenic tobacco plants were suspended in 10% trichloroacetic acid (TCA) and then centrifuged. The supernatant was used to measure the contents of GSH, GSSG, ASC and DHA according to Ding et al. [14].

2.11. BN-PAGE, SDS-PAGE, and immunoblot analysis

BN-PAGE was performed as described by Peng et al. [35]. The thylakoid membranes were washed in 330 mM sorbitol and 50 mM BisTris–HCl, pH 7.0, and suspended in resuspension buffer (20% glycerol and 25 mM BisTris–HCl, pH 7.0) at 1.0 mg chlorophyll/mL. An equal volume of resuspension buffer containing 2% (w/v) DM was added to the thylakoid suspension in a dropwise manner. After incubation at 4 °C for 5 min, insoluble material was removed by centrifugation at 12,000 g for 10 min. The supernatant was combined with one-tenth volume of 5% Serva blue G in 100 mM BisTris–HCl, pH 7.0, 0.5 M 6-amino-*n*-caproic acid, and 30% (w/v) glycerol and applied to 0.75-mm-thick 6 to 12% acrylamide gradient gels in a Hoefer Mighty Small vertical electrophoresis unit connected to a cooling circulator. For two-dimensional SDS-PAGE analysis, excised BN-PAGE lanes were soaked in SDS sample buffer and 5% β-mercaptoethanol for 30 min and layered onto 1-mm-thick 15% SDS polyacrylamide gels containing 6 M urea [46].

For immunoblot analysis, total leaf proteins were separated using 15% SDS polyacrylamide gels containing 6 M urea [46]. After electrophoresis, the proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Amersham Biosciences, USA), probed with specific primary antibodies, and visualized by the enhanced chemiluminescence method. X-ray films were scanned and analyzed using ImageMaster™ 2D Platinum software.

2.12. Measurements of Chl and protein contents

Chl content was determined according to Arnon [47]. Protein content was determined according to Bradford [48].

3. Results

3.1. Phenotype of the transgenic tobacco plants under chilling stress

We have previously obtained transgenic tobacco plants with 30–70% decreased GR activity which was mainly due to the suppression of GR in chloroplasts by using a gene encoding tobacco chloroplastic GR for the RNAi construct [14]. Four transgenic lines (i2, i21, i28, and i42) that had decreased GR activities were chosen to obtain progeny (T₁ generation) by self-fertilization. The GR activity in transgenic i2 plants was about 70% of GR activity in wild type plants. The activities of GR in transgenic i21, i28, and i42 plants were about 30% of GR activity in wild type plants [14]. In this study, the seedlings of wild type and transgenic plants (i2, i21, i28, and i42) were grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. Under normal temperature conditions, no significant differences in phenotype were observed between wild type and transgenic plants. Under chilling stress conditions, a pale-green phenotype was observed in both wild type and transgenic plants but such pale-green phenotype was much more obvious in transgenic i21, i28, and i42 plants than wild type and transgenic i2 plants (Fig. 1A). Measurements of leaf Chl content show that there were no significant differences between wild type and transgenic plants under normal temperature conditions. There was a decrease in leaf Chl content in both wild type and transgenic plants under chilling stress conditions. However, there were no significant differences in Chl content between wild type and transgenic i2 plants. A greater

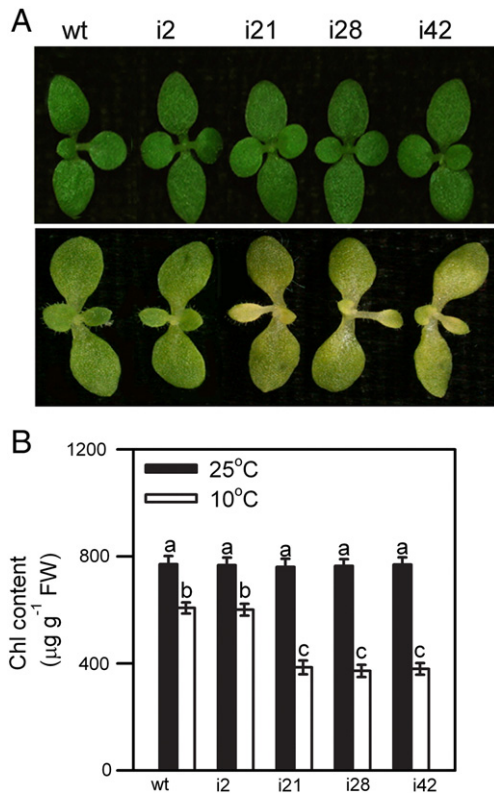


Fig. 1. (A) Phenotypes of wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. (B) Total chlorophyll content in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

decrease in Chl content was observed in transgenic i21, i28, and i42 plants than in wild type and transgenic i2 plants (Fig. 1B).

3.2. ROS accumulation in wild type and transgenic plants under chilling stress

Glutathione acts as a recycled intermediate in the reduction of H_2O_2 and GR ensures an efficient recycling of glutathione [9]. We assumed that the pale-green phenotype of transgenic i21, i28 and i42 plants under chilling conditions may suffer from oxidative stress which may be possibly due to substantial accumulation of H_2O_2 . To assess this possibility, H_2O_2 accumulation was examined in wild type and transgenic plants. To determine in vivo H_2O_2 levels more accurately, we quantified leaf H_2O_2 using an improved spectrophotometric assay [38]. Under normal temperature conditions, H_2O_2 accumulated at low levels and no obvious differences were observed between wild type and transgenic plants. However, under chilling stress conditions, more accumulation of H_2O_2 was observed in both wild type and transgenic plants. As compared to that in wild type plants, there was substantial accumulation of H_2O_2 in transgenic i21, i28 and i42 plants (Fig. 2A).

The cytochemical assay demonstrates that under normal temperature conditions, H_2O_2 accumulation, indicated by $CeCl_3$ deposits, was not observed in the mesophyll cells in both wild type and transgenic plants. Under chilling stress conditions, H_2O_2 accumulation was consistently visible in the cell walls of mesophyll cells and in the chloroplast in wild type and transgenic i28 plants. However, H_2O_2 accumulation in the chloroplast was greater in transgenic i28 plants than in wild type plants (Fig. 2B). Similar results were also observed in transgenic i21 and i42 plants (data not shown).

NBT staining was also performed in wild type and transgenic plants to detect $O_2^{\bullet-}$, another ROS that produce largely in the chloroplast stroma. NBT staining shows that there were no significant differences in the accumulation of $O_2^{\bullet-}$ between wild type and transgenic plants

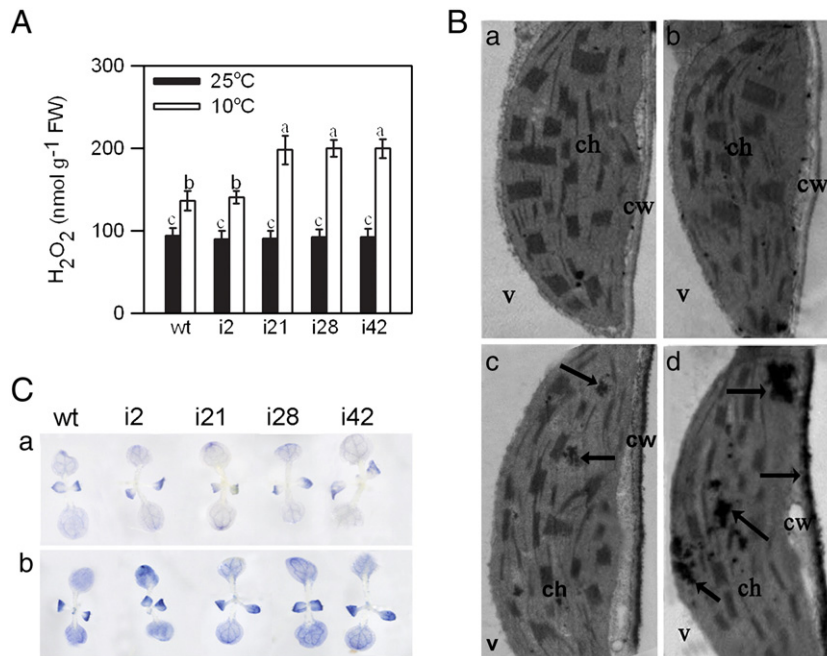


Fig. 2. (A) Leaf H_2O_2 content, (B) H_2O_2 cytochemical localization, and (C) NBT staining for $O_2^{\bullet-}$ in wild type (wt) and transgenic i2, i21, i28 and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. B(a) and (c): wild type tobacco plants grown under normal temperature (25 °C) and chilling stress (10 °C) conditions, respectively; B(b) and (d): transgenic i28 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C). Arrows indicate $CeCl_3$ precipitates. cw, cell wall; v, vacuolar; and ch, chloroplast. Bar = 1 μ m. C(a) and (b): NBT staining for $O_2^{\bullet-}$ in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. Three independent experiments were carried out with similar results. The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

although chilling stress resulted in an increase in the accumulation of $O_2^{\bullet-}$ in both wild and transgenic plants (Fig. 2C).

3.3. Functions of PSI and PSII in wild type and transgenic plants under chilling stress

The pale-green phenotype observed under chilling stress in wild type and transgenic plants suggests that the functions of PSI and PSII may be severely affected. We thus investigated the changes in some parameters related to the functions of PSI and PSII in wild type and transgenic plants (Fig. 3). Under normal temperature conditions, there were no significant differences in functions of the PSI and

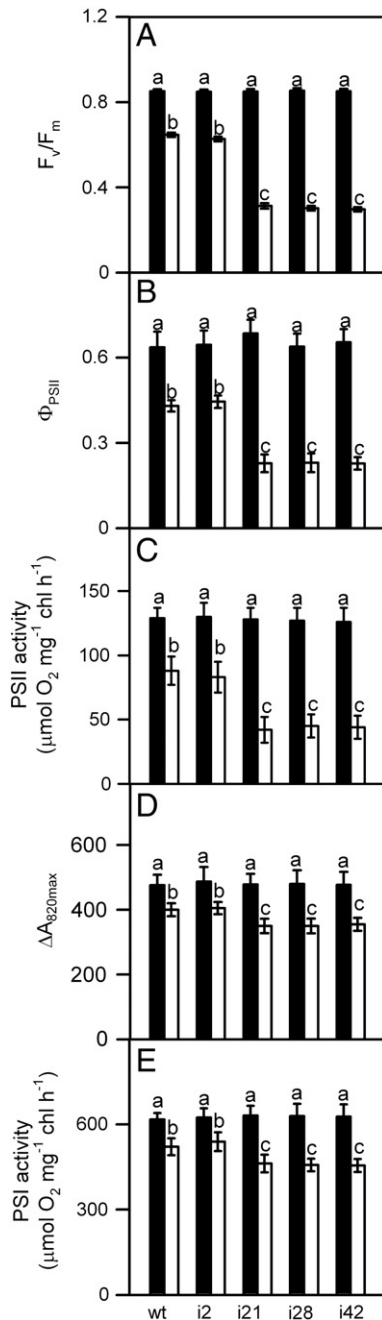


Fig. 3. (A) Maximal efficiency of PSII photochemistry (F_v/F_m), (B) the actual PSII efficiency (Φ_{PSII}), (C) PSII activity, (D) P700 absorbance changes ($\Delta A_{820\text{max}}$), and (E) PSI activity in wild type (wt) and transgenic i2, i21, i28 and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

PSII between wild type and transgenic plants since there were no significant differences in some parameters related to the functions of PSI and PSII between wild type and transgenic plants, such as F_v/F_m , Φ_{PSII} , ΔA_{820} , and the activities of PSI and PSII. Under chilling stress conditions, we observed a significant decrease in F_v/F_m , Φ_{PSII} , and PSII electron transport activity in wild type and transgenic plants but such a decrease was much more considerable in transgenic i21, i28, and i42 plants as compared to that in wild type plants under chilling stress conditions (Fig. 3A, B, C). The values of F_v/F_m , Φ_{PSII} and PSII electron transport activity in transgenic i21, i28, and i42 plants under chilling stress conditions were about 49%, 52% and 50% of those in wild type plants, respectively. We also observed a decrease in ΔA_{820} and PSI electron transport activity in wild type and transgenic plants under chilling stress conditions and this decrease was more pronounced in transgenic i21, i28, and i42 plants than in wild type plants (Fig. 3D, E). ΔA_{820} and PSI electron transport activity in transgenic i21, i28 and i42 plants under chilling stress were about 85% and 86% of that in wild type plants, respectively. It should be noted that all these parameters related to PSI and PSII functions in i2 plants were similar to those of wild type plants under chilling stress conditions. The above results suggest that the PSII function was more profoundly affected than the PSI function in transgenic i21, i28 and i42 plants under chilling stress conditions.

In order to investigate whether the decreased PSII function in transgenic i21, i28 and i42 plants under chilling stress was due to an increased rate of degradation or/and a decreased rate of protein synthesis, we examined the effects of protein synthesis inhibitor on the PSII function in wild type and transgenic plants during chilling stress (see supplemental Figure S1). In the absence of lincomycin, there was a slight decrease in F_v/F_m in transgenic i28 but a significant decrease in F_v/F_m in wild type plants during chilling stress. However, in the presence of lincomycin, the decrease in F_v/F_m was more rapid and the decrease in F_v/F_m in transgenic i28 plants was similar to that in wild type plants. Since lincomycin blocks PSII repair process by inhibiting de novo protein synthesis in the chloroplast, the decrease in F_v/F_m reflects the rate of PSII damage. Thus, the above results suggest that the decrease in PSII activity in transgenic plants under chilling stress could be mainly due to the decreased rate of protein synthesis but not due to the rate of PSII damage as compared with that in wild type plants.

3.4. Properties of both the donor and acceptor sides of PSII in wild type and transgenic plants under chilling stress conditions

In order to examine how the PSII function is affected in transgenic i21, i28, and i42 plants under chilling stress conditions, we investigated the functional status of the donor and acceptor sides of the PSII complex in wild type and transgenic plants by the analyses of Chl fluorescence relaxation kinetics.

In the absence of DCMU, the relaxation of the flash induced increase in variable Chl fluorescence yield in wild type and transgenic plants can be resolved into three distinct phases: the fast phase, the middle phase, and the slow phase (Fig. 4A–B, Supplemental Fig. S2A–B, Table 1). The fast phase is associated with the electron transfer from Q_A^- to Q_B in PSII reaction centers having an occupied Q_B pocket at the time of flashing. The middle phase is associated with the electron transfer from Q_A^- to Q_B in PSII reaction centers that have an empty Q_B pocket at the time of flashing and have to bind plastoquinone to the Q_B site from the pool before Q_A^- reoxidation. The slow phase is associated with the reoxidation of Q_B^- with the S_2 state of the OEC via $Q_A^- Q_B \leftrightarrow Q_A Q_B^-$ equilibrium [49]. Under normal temperature conditions, the modulated fluorescence intensity relaxation in wild type plants is dominated by the fast phase ($t_{1/2} = 285 \mu\text{s}$), whose relative amplitude is about 66%. The contribution of the middle phase ($t_{1/2} = 7.1 \text{ ms}$) was about 19%, and that of the slow phase ($t_{1/2} = 7.7 \text{ s}$) was about 15%. Compared to wild type plants, no significant changes in the kinetics of three phases

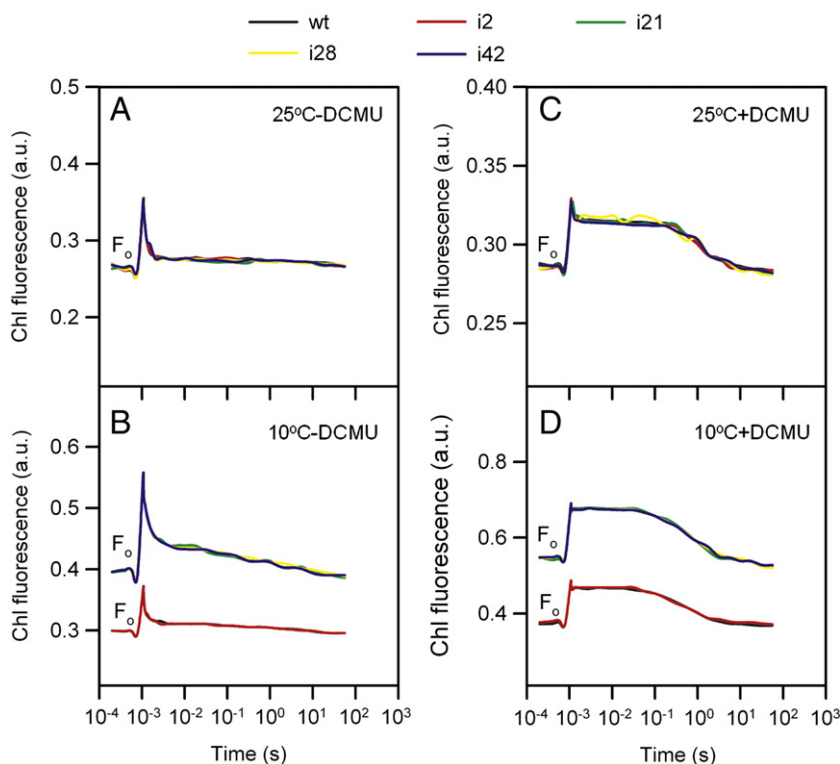


Fig. 4. Relaxation of flash-induced fluorescence yield in leaves of wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. (A) and (B), the curves were the actual data of the fluorescence signals in the absence of 50 μM DCMU under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. (C) and (D), the curves were the actual data of the fluorescence signals in the presence of 50 μM DCMU under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively.

Table 1
Parameters of decay kinetics of flash-induced variable fluorescence in wild type (wt) and transgenic tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively.

	Total amplitude	Fast phase	Middle phase	Slow phase
	(%)	$t_{1/2}$ (μs) [A (%)]	$t_{1/2}$ (ms) [A (%)]	$t_{1/2}$ (s) [A (%)]
25 °C				
Without DCMU				
wt	100 ^a	285 ± 30 (66.4 ± 3.7) ^b	7.1 ± 0.4 (18.5 ± 0.6)	7.7 ± 0.4 (15.1 ± 0.4)
i2	98.9 ± 1.4	290 ± 27 (65.6 ± 2.4)	6.8 ± 0.4 (18.7 ± 0.7)	7.4 ± 0.5 (15.7 ± 0.5)
i21	99.2 ± 1.3	293 ± 42 (64.9 ± 3.6)	7.2 ± 0.5 (19.5 ± 0.6)	7.9 ± 0.5 (15.6 ± 0.4)
i28	98.9 ± 0.9	288 ± 37 (65.4 ± 2.6)	6.9 ± 0.5 (19.1 ± 0.8)	7.6 ± 0.2 (15.5 ± 0.6)
i42	99.6 ± 1.0	287 ± 40 (66.4 ± 3.3)	6.8 ± 0.5 (18.7 ± 0.5)	7.3 ± 0.4 (14.9 ± 0.6)
With DCMU				
wt	100	– (0)	– (0)	1.8 ± 0.4 (100)
i2	100	– (0)	– (0)	1.8 ± 0.2 (100)
i21	100	– (0)	– (0)	1.9 ± 0.3 (100)
i28	100	– (0)	– (0)	1.7 ± 0.3 (100)
i42	100	– (0)	– (0)	1.8 ± 0.4 (100)
10 °C				
Without DCMU				
wt	100 ^a	336 ± 30 (59.2 ± 3.7) ^b	10.8 ± 0.4 (21.7 ± 0.6)	5.2 ± 0.4 (19.1 ± 0.6)
i2	100 ± 0.4	340 ± 27 (58.3 ± 2.4)	10.4 ± 0.4 (21.6 ± 0.7)	5.6 ± 0.4 (20.1 ± 0.5)
i21	92 ± 1.3	443 ± 42 (48.1 ± 3.6)	14.7 ± 0.4 (25.2 ± 0.6)	3.3 ± 0.4 (26.7 ± 0.5)
i28	93 ± 1.4	430 ± 37 (48.4 ± 2.6)	14.3 ± 0.4 (25.1 ± 0.5)	3.5 ± 0.4 (26.5 ± 0.4)
i42	93 ± 1.6	428 ± 40 (48.8 ± 3.3)	14.1 ± 0.4 (25.1 ± 0.7)	3.7 ± 0.4 (26.1 ± 0.6)
With DCMU				
wt	100	– (0)	– (0)	1.1 ± 0.3 (100)
i2	100	– (0)	– (0)	1.2 ± 0.2 (100)
i21	100	– (0)	– (0)	1.3 ± 0.1 (100)
i28	100	– (0)	– (0)	1.2 ± 0.2 (100)
i42	100	– (0)	– (0)	1.1 ± 0.2 (100)

The relaxation of the flashed-induced fluorescence yield was measured without or with 50 μM DCMU. Mean ± SD values were calculated from four to six independent experiments.

^a Values represent the amplitude of total variable fluorescence as a percentage of that in wild type plants.

^b Values in parentheses are relative amplitude as a percentage of total variable fluorescence obtained from wild type and transgenic tobacco plants.

were observed in transgenic plants. Under chilling stress conditions, for the fast phase, there was a greater increase in the decay half-time and a greater decrease in the amplitude in transgenic i21, i28, and i42 plants than in wild type plants. The decay half-time of the fast phase increased from 336 μ s in wild type plants to about 430 μ s in transgenic i21, i28, and i42 plants. The amplitude of the fast phase decreased from 59% in wild type plants to about 48% in transgenic i21, i28 and i42 plants. For the middle phase, there was greater increase in the decay half-time and the amplitude in transgenic i21, i28, and i42 than in wild type plants. The decay half-time of the middle phase increased from 10.8 ms in wild type plants to about 14.0 ms in transgenic i21, i28, and i42 plants. The amplitude of the middle phase increased from 21.7% in wild type plants to about 25% in transgenic i21, i28, and i42 plants. For the slow phase, a greater decrease in the decay half-time but a greater increase in the amplitude in transgenic i21, i28, and i42 plants than in wild type were observed. The decay half-time of the slow phase decreased from 5.2 s in wild type plants to 3.5 s in transgenic i21, i28, and i42 plants. The amplitude of the slow phase increased from 19.1% in wild type plants to about 26% in transgenic i21, i28, and i42 plants.

When Chl fluorescence induction kinetics is determined in the presence of DCMU, which prevents the electron transfer from Q_A^- to Q_B , the fluorescence relaxation reflects the reoxidation of Q_A^- via recombination with donor side components [49]. In the presence of DCMU, the fluorescence relaxation was dominated by a slow component in wild type and transgenic plants, and the slow phase arose from the reoxidation of Q_A^- with the S_2 state of the OEC (Fig. 4C–D, Supplemental Fig. S2C–D, Table 1). There were no significant differences in the fluorescence relaxation kinetics between wild type and transgenic plants either under normal temperature conditions or under chilling stress conditions.

TL was further used to investigate the redox properties of the acceptor and donor sides of PSII in wild type and transgenic plants. Recombination of positive charges stored in the S_2 and S_3 oxidation states of the water-oxidizing complex with electrons stabilized on the reduced Q_A and Q_B acceptors of PSII results in characteristic TL emissions [50,51]. The TL intensity is indicative of the amount of recombining charges, whereas the peak temperature reflects the energetic stabilization of the separated charge pair [52]. Illumination of single turnover flash with leaves after a short dark adaptation induces a major TL band, called the B-band which appears at around 30 °C and arises from $S_2/S_3Q_B^-$ recombination. If the electron transfer between Q_A and Q_B is blocked by DCMU, the B-band is replaced by the so-called Q-band arising from the $S_2Q_A^-$ recombination at around 10 °C [51,53,54].

We thus investigated the effects of chilling stress on the TL glow curves in wild type and transgenic plants. In the absence of DCMU, wild type plants under normal temperature conditions exhibited TL emission maxima for the $S_2Q_B^-$ charge recombination at approximately 34 °C and there were no significant changes in transgenic plants compared to wild type plants (Fig. 5A, Table 2). Under chilling stress conditions, there was a downshift in the peak temperatures for the $S_2Q_B^-$ charge recombination in both wild type and transgenic plants, indicating that chilling stress led to decrease in the stabilization of the $S_2Q_B^-$ charge pair. However, such a downshift was greater in transgenic i21, i28, and i42 plant than in wild type plants. The peak temperature in wild type plants was about 30 °C but the peak temperatures in transgenic i21, i28 and i42 plants were about 26 °C (Fig. 5B, Table 2). These results indicate that the stabilization of the $S_2Q_B^-$ charge pair was decreased in transgenic i21, i28, and i42 plants as compared to that of wild type plants under chilling stress conditions.

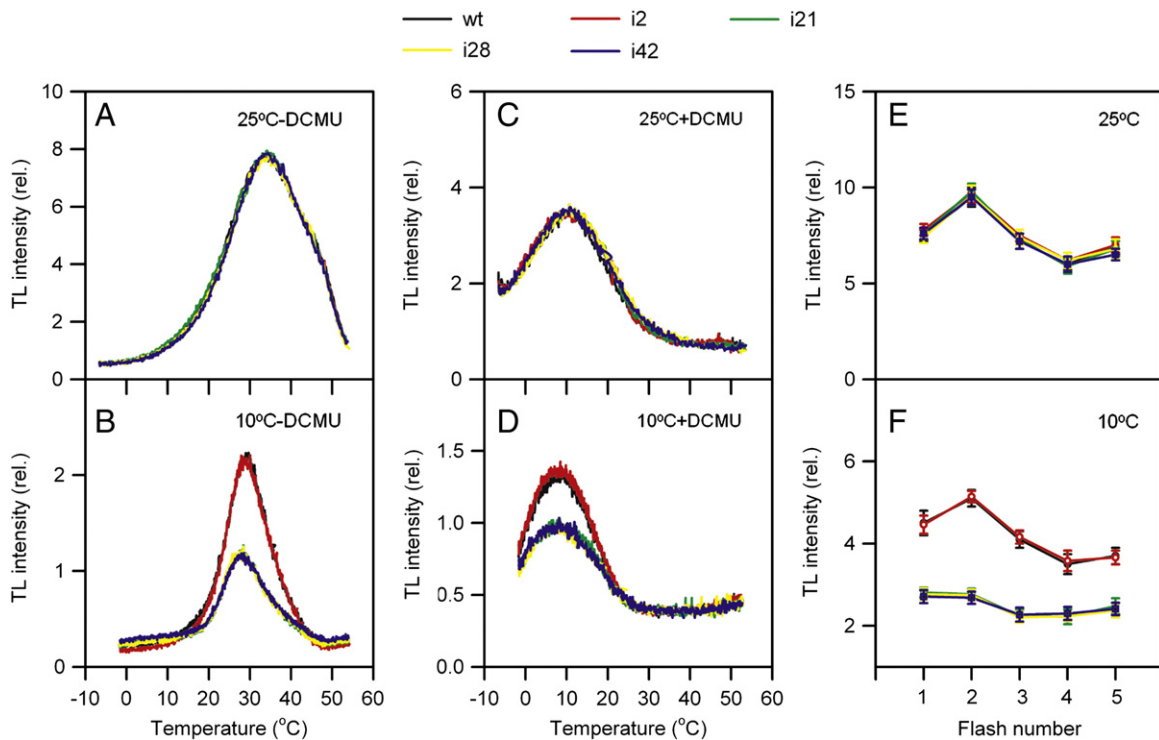


Fig. 5. (A) and (B) Thermoluminescence glow curves in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. The leaves were excited with a single flash in the absence of 50 μ M DCMU. (C) and (D) Thermoluminescence glow curves in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. The leaves were excited with a single flash in the presence of 50 μ M DCMU. (E) and (F), The flash-induced oscillation of the B thermoluminescence band in the leaves of wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively. The values are means \pm SD of three independent experiments.

Table 2

Peak emission temperatures of the TL curves in wild type (wt) and transgenic tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C), respectively.

	Peak temperature (°C)	
	Without DCMU	With DCMU
25 °C		
wt	34.4 ± 0.2	13.7 ± 0.2
i2	34.3 ± 0.3	13.8 ± 0.2
i21	34.6 ± 0.2	13.7 ± 0.1
i28	34.4 ± 0.2	13.8 ± 0.3
i42	34.2 ± 0.3	13.6 ± 0.1
10 °C		
wt	29.8 ± 0.1	8.4 ± 0.1
i2	29.6 ± 0.2	8.6 ± 0.2
i21	26.4 ± 0.1	8.3 ± 0.2
i28	26.1 ± 0.1	8.6 ± 0.3
i42	26.3 ± 0.3	8.4 ± 0.3

The measurements were performed in the absence and presence of 50 μM DCMU. Mean ± SD values were calculated from four to six independent experiments.

In the presence of DCMU, under normal temperature conditions, wild type plants and transgenic plants exhibited very similar TL emission maxima for the $S_2Q_A^-$ charge recombination at approximately 14 °C (Fig. 5C, Table 2). Under chilling stress conditions, there was a downshift in the peak temperatures for the $S_2Q_A^-$ charge recombination in both wild type and transgenic plants. However, there were no significant differences in the peak temperature for the $S_2Q_A^-$ charge recombination between wild type and transgenic plants and their peak temperatures for the $S_2Q_A^-$ charge recombination were at approximately 8 °C (Fig. 5D, Table 2). Since the $S_2Q_B^-$ recombination was destabilized in transgenic i21, i28 and i42 plants but the $S_2Q_A^-$ recombination in transgenic i21, i28 and i42 plants was similar to that in wild type plants, the change in TL emission maxima for the $S_2Q_B^-$ in transgenic i21, i28 and i42 plants may be due to a modification in the redox properties of Q_B and the redox properties of the S_2 state were not affected in transgenic i21, i28 and i42 plants.

We also investigated the effects of chilling stress on the oscillation of the intensity of the B-band in wild type and transgenic plants (Fig. 5E–F). Under normal temperature conditions, both wild type and transgenic plants exhibited typical oscillation with a periodicity of four with the maximum emission occurring on the second flash (Fig. 5E). Under chilling stress conditions, the oscillatory pattern was damped greatly in transgenic i21, i28, and i42 plants and the period-four oscillations almost could not be observed (Fig. 5F).

3.5. Contents of PSII proteins in wild type and transgenic plants under chilling stress

We further investigated the effects of chilling stress on the contents of PSII proteins (D1, D2, CP43, LHCI and PsbO) and other photosynthetic membrane protein contents (PsaA/B, Lhca3 for PSI, Cyt f for cytochrome b_6/f , and CF1 β for ATP synthase) in wild type and transgenic plants (Fig. 6). Under normal temperature conditions, there were no significant differences in the contents of PSII proteins and other proteins related to PSI, cytochrome b_6/f , and ATP synthase between wild type and transgenic plants (Fig. 6A). Under chilling stress conditions, the levels of D1, D2 and CP43 in transgenic i21, i28, and i42 were about 40–50% of those in wild type plants. The levels of LHCI and PsbO in transgenic i21, i28, and i42 were about 80% of those in wild type plants. The contents of PsaA/B, Lhca3, and CF1 β in transgenic i21, i28 and i42 plants were reduced to about 90% of those of wild type plants. However, the contents of Cyt f in transgenic i21, i28, and i42 plants were very similar to those of wild type plants (Fig. 6B).

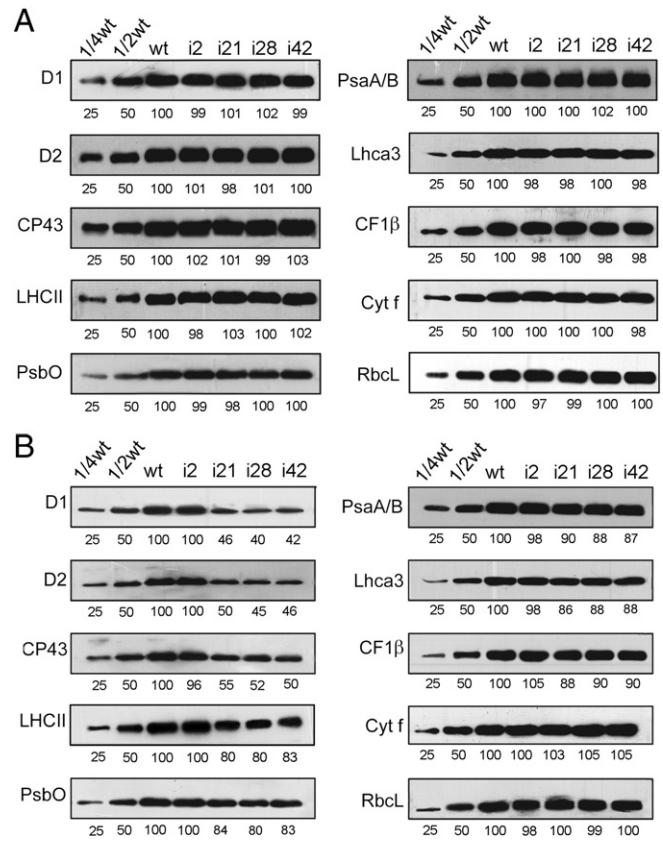


Fig. 6. (A) Immunodetection of thylakoid membrane proteins in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature conditions (25 °C). (B) Immunodetection of thylakoid membrane proteins in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under chilling stress conditions (10 °C). The percentage protein levels shown below the lanes were estimated by comparison with levels of corresponding thylakoid proteins of wild type plants.

It should be pointed out that we observed a down-shift of peak temperatures for the B-band and the Q-band by about 5 °C upon chilling in wild type and transgenic i2 plants (Table 2). This indicates that the S_2 state is generally destabilized upon chilling. We thus further investigated the difference in the amount of PsbO in wild type and transgenic i2 plants at the two temperatures. Our results show that as compared with the temperature at 25 °C, there was a small decrease (about 13%) in the amount of PsbO in wild type and transgenic i2 plants at 10 °C (see supplemental Figure S3).

We also examined the effects of chilling stress on thylakoid membrane complexes in wild type and transgenic plants by BN-PAGE and subsequent SDS-PAGE (Fig. 7). In the first dimension, the native thylakoid membrane complexes were separated into six major bands which represent PSII supercomplexes (band I), monomeric PSI and dimeric PSII (band II), monomeric PSII (band III), CP43-free PSII (band IV), trimeric LHCI (band V), and monomeric LHCI (band VI) in wild type plants [55]. After BN-PAGE, the second-dimensional SDS-PAGE was performed to separate proteins in different fractions. Under normal temperature conditions, the first-dimensional BN-PAGE analysis shows that there were no significant differences in the amounts of chlorophyll-containing protein complexes between wild type and transgenic plants. Analyses of the SDS-PAGE gels followed by the BN-PAGE confirmed that no significant differences in the amounts of the PSII core subunits D1, D2, CP47, and CP43 were observed between wild type and transgenic plants (Fig. 7A). Under chilling stress conditions, there were significant decreases in the amounts of bands I, II, III, and IV in the BN-PAGE in transgenic i21, i28, and i42 plants as compared to wild type plants. Analyses of the SDS-PAGE gels followed by the BN-PAGE further

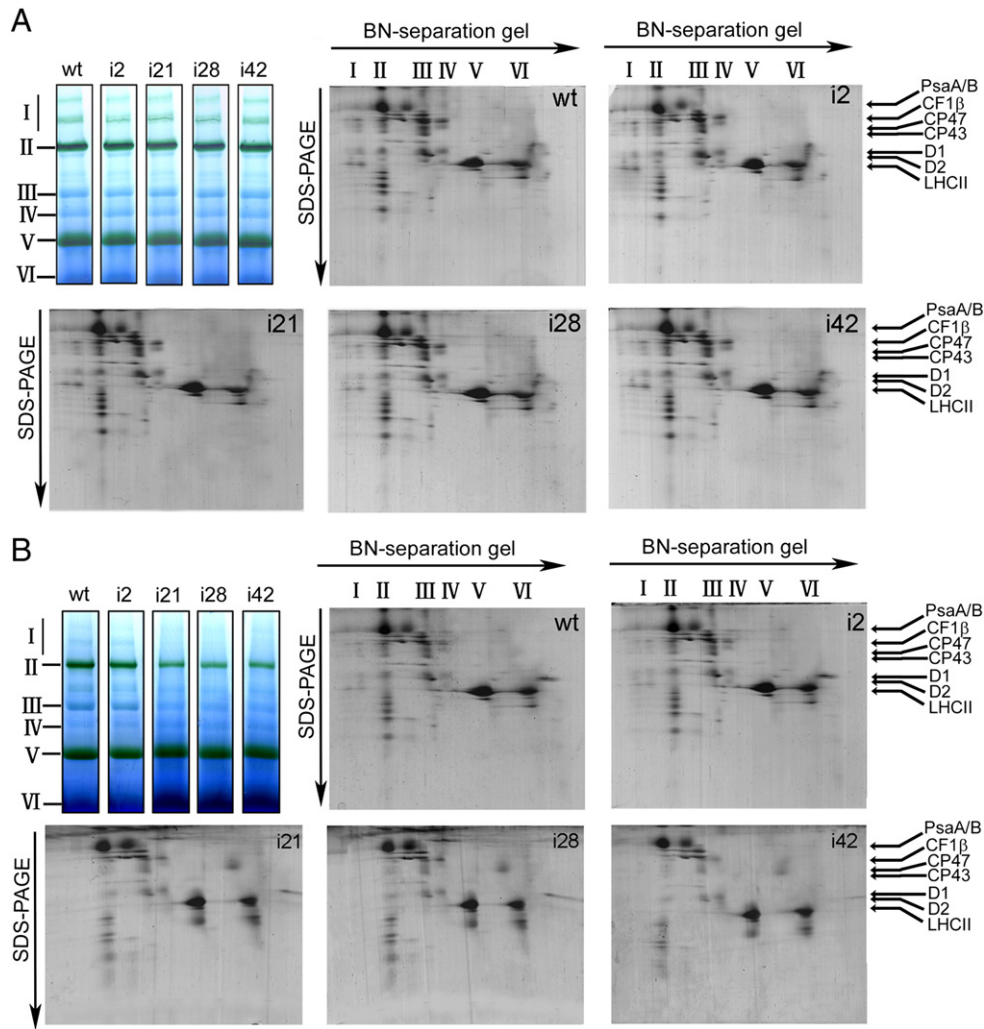


Fig. 7. (A) BN gel analysis and two-dimensional separation of protein complexes in the thylakoid membrane protein complexes (10 μ g chlorophyll) in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature conditions (25 °C). (B) BN gel analysis and two-dimensional separation of protein complexes in the thylakoid membrane protein complexes (10 μ g chlorophyll) in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under chilling stress conditions (10 °C).

confirmed that there were significant decreases in the amounts of the PSII core subunits D1, D2 and CP43 (Fig. 7B).

3.6. Changes in the ascorbate–glutathione cycle in the chloroplast in wild type and transgenic plants under chilling stress

Since GR is involved in the ascorbate–glutathione cycle to scavenge H_2O_2 in the chloroplast and the above results show that an accumulation of H_2O_2 in the chloroplast in transgenic i21, i28, and i42 plants, we thus investigated the effects of decreased chloroplast GR on the ascorbate–glutathione cycle in the chloroplast under normal temperature or chilling stress conditions. Fig. 8 shows the effects of chilling stress on glutathione and ascorbate pools in wild type and transgenic plants. Under normal temperature condition, there were no significant differences in glutathione and ascorbate pools assessed by the contents of GSH and GSSG (for glutathione pools) and the contents of ASC and DHA (for ascorbate pools) between wild type and transgenic plants. Under chilling stress conditions, as compared to wild type plants, there was a significant decrease in the content of GSH and a significant increase in the content of GSSG, thus resulting in a decrease in the ratio of GSH/GSSG in transgenic i21, i28, and i42 plants (Fig. 8A–C). In addition, there was a significant decrease in the contents of ASC and DHA as well as in the ratio of ASC/DHA in transgenic i21, i28, and i42 plants as compared to those in wild type plants (Fig. 8D–F).

Fig. 9 shows the effects of chilling stress on the activities of enzymes involved in the ascorbate–glutathione cycle as well as the activity of SOD in the chloroplast in wild type and transgenic plants. There were no significant differences in the activities of sAPX, tAPX, MDHAR, DHAR, and SOD between wild type and transgenic plants either under normal temperature or chilling stress conditions although chilling stress resulted in an increase in the activities of these enzymes.

In plants, the major substrate for reductive detoxification of H_2O_2 by APX is ASC in the chloroplast [5,6]. Low ASC content in the chloroplast leads to inactivation of sAPX and tAPX [56–58]. Our results show that there was a significant decrease in the content of ASC in the chloroplast in transgenic i21, i28, and i42 plants as compared to that in wild type plants under chilling stress conditions (Fig. 8D). However, we did not observe any differences in the activities of sAPX and tAPX in wild type and transgenic plants under chilling stress conditions (Fig. 9B, C). These conflicting results may be due to the possibility that the activities of sAPX and tAPX in Fig. 9B and C were determined in the reaction mixture with high ASC concentration (2 mM) according to Zhou et al. [45] but not using *in vivo* ASC concentrations in both wild type and transgenic plants. To investigate this possibility, we determined the activities of sAPX and tAPX in wild type and transgenic plants by using their *in vivo* ASC concentrations in the reaction mixture according to the data from Fig. 8D. Under normal temperature conditions, there were no significant differences in the activities of sAPX

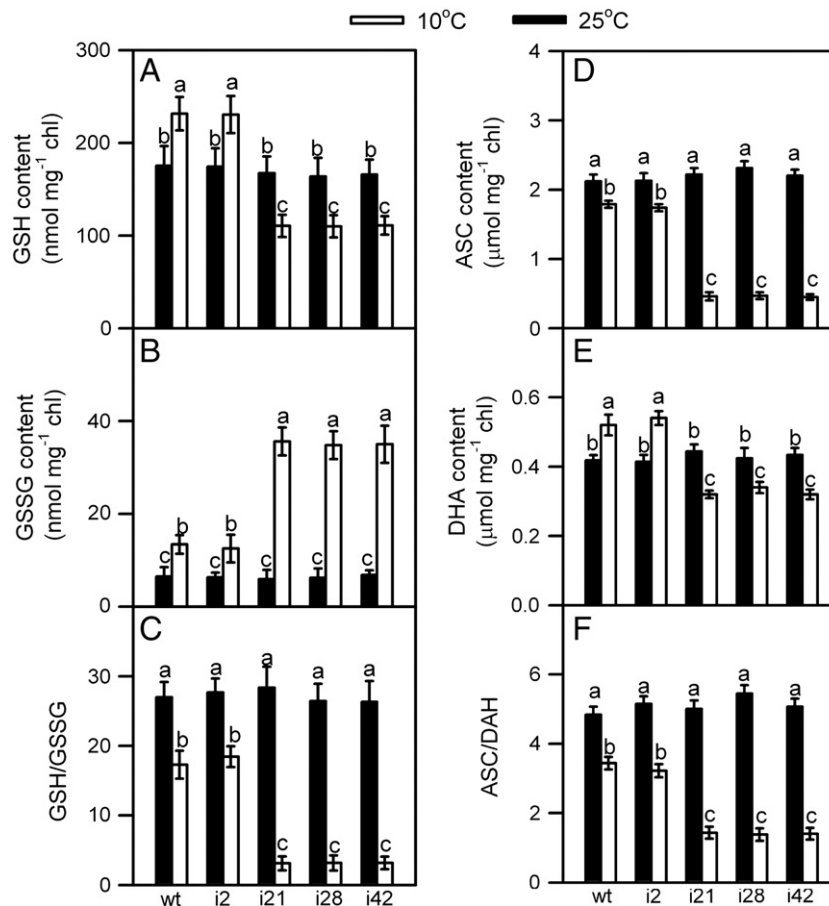


Fig. 8. (A) GSH content, (B) GSSG content and (C) the ratio GSH/GSSG in the chloroplast in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C). (D) ASC content, (E) DHA content and (F) the ratio ASC/DHA in the chloroplast in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C). The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

and tAPX between wild type and transgenic plants. Under chilling stress conditions, however, we observed a significant decrease in the activities of sAPX and tAPX in transgenic i21, i28, and i42 plants as compared to those in wild type plants (Fig. 10). These results suggest that the decrease in the contents of ASC in the chloroplast in transgenic i21, i28, and i42 plants under chilling stress conditions may be a factor that inactivated the activities of sAPX and tAPX, thus resulting in an accumulation of H₂O₂ in transgenic plants under chilling stress conditions.

4. Discussion

It has been reported that GR located in the chloroplast plays an important role in maintaining photosynthetic function under chilling stress [21–23,32]. However, the protecting mechanisms are not fully understood. In this study, we used the transgenic tobacco plants with severely decreased chloroplast GR by RNAi to investigate the role of GR in protecting PSI and PSII against chilling stress. Our results demonstrate that PSII was severely impaired in transgenic tobacco plants, whereas the PSI was less affected under chilling stress conditions when exposed to low light (Fig. 3). Immunoblot and BN-PAGE analysis further confirmed these results (Figs. 6–7). Under chilling stress conditions, the amounts of the PSII core subunits were significantly reduced in transgenic tobacco plants, whereas the reduction in the contents of PSI reaction center PsaA/B protein and PSI light harvesting chlorophyll protein Lhca3 as well as component of ATP synthase (CF1 β) was much less. In addition, component of the cytochrome b₆/f complex accumulated to the same levels as to wild type plants. Taken together, these data suggest that PSII in transgenic plants

is primarily affected by chilling stress and chloroplast GR plays an important role in protecting PSII under chilling stress.

In this study, we observed that chilling stress resulted in the low accumulation of PSII proteins in transgenic plants (Fig. 6). Our BN-PAGE and subsequent SDS-PAGE analysis show that the levels of PSII dimers and PSII–LHCII supercomplexes were severely reduced in transgenic plants under chilling stress (Fig. 7). These results suggest that stability of higher order PSII complexes is affected in transgenic plants under chilling stress. It has been shown that H₂O₂ produced at the acceptor side of PSI leads to damage to PSII [59,60]. ROS such as H₂O₂ acts exclusively by inhibiting the repair of PSII and not by damaging PSII directly [61,62]. In the model for the action of ROS in PSII repair, elongation factors appear to be the primary targets of H₂O₂ in cyanobacteria, and H₂O₂ might inhibit the synthesis de novo of the D1 protein by inactivating the elongation factors [63,64]. The results in this study suggest that the decrease in the amount of the PSII complexes in transgenic plants under chilling stress was mainly due to the decreased rate of protein synthesis but not due to the damage to PSII (see Supplemental Fig. S1). In addition, there was a significant accumulation of H₂O₂ in transgenic plants under chilling stress (Fig. 2). Thus, we propose that the low accumulation of PSII proteins in transgenic plants under chilling stress may be associated with the inhibition of the synthesis de novo of the D1 protein induced by the accumulation of H₂O₂.

In this study, we further investigated how the PSII function is affected in transgenic plants under chilling stress conditions. Analyses of flash-induced chlorophyll fluorescence kinetics in the absence of DCMU show that there was a significant increase in the decay half-time of the fast phase but a decrease in its amplitude in transgenic plants as

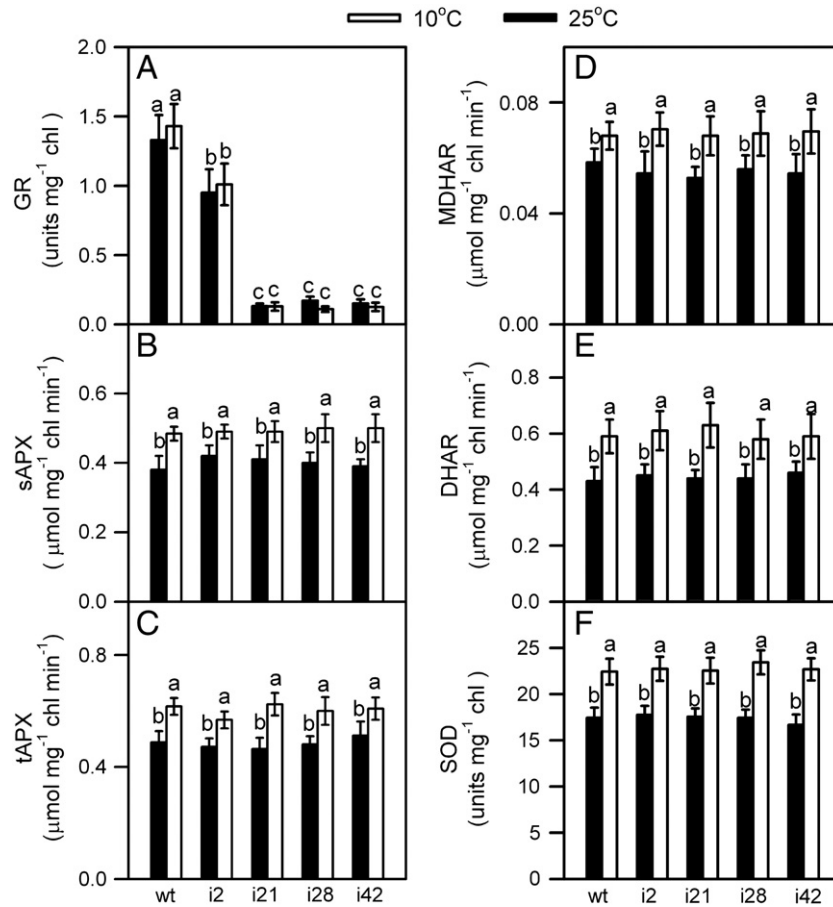


Fig. 9. (A) GR activity, (B) sAPX activity, (C) tAPX activity, (D) MDHAR activity, (E) DHAR activity, and (F) SOD activity in the chloroplast in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C). The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

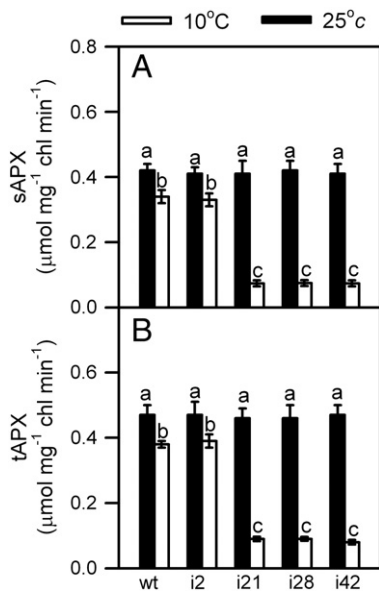


Fig. 10. (A) sAPX activity and (B) tAPX activity in the chloroplast in wild type (wt) and transgenic i2, i21, i28, and i42 tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C). The activities of sAPX and tAPX in the chloroplast in wild type and transgenic tobacco plants grown under normal temperature (25 °C) and chilling stress conditions (10 °C) were determined by adding their respective *in vivo* ASC concentrations according to the data of Fig. 8D. The values are means \pm SD of three independent experiments. Different letters indicate significant difference ($P < 0.05$).

compared to that in wild type plants under chilling stress (Table 1), indicating that decreased chloroplast GR leads to an inhibition of electron transfer from Q_A^- to Q_B (Q_B^-). This inhibition may be associated with a decrease in the apparent equilibrium constant for sharing the electron between Q_A^- and Q_B since we observed a decrease in the $t_{1/2}$ of the slow phase in the absence of DCMU ($t_{1/2}(S_2Q_AQ_B^-)$) but no changes in the $t_{1/2}$ of the slow phase in the presence of DCMU ($t_{1/2}(S_2Q_A^-)$) (Table 1). The decreased apparent equilibrium may be due to a decreased affinity of Q_B binding pocket, since we observed an increased decay half time of the middle phase under chilling stress (Table 1). In addition, we observed a decrease in the stability of the $S_2Q_B^-$ charge recombination in transgenic plants, whereas the stability of the $S_2Q_A^-$ was not affected under chilling stress conditions (Fig. 5, Table 2). These results suggest that the redox potential of Q_B is decreased in transgenic plants, while the redox potential of the S_2 state in transgenic plants is similar to that in wild type plants under chilling stress. The decrease in the affinity of Q_B binding pocket and the redox potential of Q_B in transgenic plants under chilling stress conditions may result from modification of the Q_B niche. The inhibition of the synthesis *de novo* in transgenic plants under chilling stress (see Supplemental Fig. S1) may lead to the decrease in the amounts of the functional PSII complexes which results in the changes in occupancy of the Q_B site plastoquinone species. Therefore, modification of the Q_B niche in transgenic plants under chilling stress could be explained by the decrease in the amounts of the functional PSII complexes due to the inhibition of the synthesis *de novo*.

We observed that in the presence of DCMU, the overall fluorescence relaxation kinetics in transgenic plants were not changed compared with wild type plants under chilling stress conditions (Table 1), suggesting that decreased chloroplast GR may have no effect on the

donor side of PSII under chilling stress. As discussed above, the downshift of the peak temperature for the B-band may be due to the decrease in the redox potential of Q_B but not due to the redox potential of the S_2 state in transgenic plants (Table 2). Why the donor side of PSII was not affected in transgenic plants under chilling stress conditions? In spite of the fact that H_2O_2 is thought to be able to diffuse through membranes, possibly through aquaporins [65,66], the amount of H_2O_2 that can be detected outside the chloroplast has been shown to be only up to 5% of the total H_2O_2 produced inside the chloroplast [67], suggesting that H_2O_2 diffusion across membranes is very limited. In addition, it has been suggested that H_2O_2 can be produced on the donor side of PSII [68,69]. However, the production of H_2O_2 at the donor side of PSII is at very low rate and this process can be probably largely neglected under physiological conditions as compared with the Mehler reaction [70–73]. It is reasonable to deduce that H_2O_2 accumulated in stroma or nearby thylakoid membranes cross the thylakoid membrane to the lumen is very limited in transgenic plants under chilling stress conditions. Thus, the donor side of PSII is not easily damaged by the attack of H_2O_2 although accumulated H_2O_2 accumulated in stroma or nearby thylakoid membranes in the stromal side or around PSI in transgenic plants under chilling stress conditions.

APX is an H_2O_2 -scavenging peroxidase that uses ASC as an electron donor which is mainly maintained by the glutathione–ascorbate cycle [6,8]. Two APXs has been found in chloroplasts, one in chloroplast stroma (sAPX) and the other anchored to the thylakoid membrane (tAPX) [74]. tAPX is important for protecting thylakoid membrane proteins by scavenging H_2O_2 nearby thylakoid membrane [58]. It has been reported that sAPX and tAPX can be inactivated at the low concentration of ascorbate [75]. In this study, we observed that there was a significant decrease in the content of ASC in the chloroplast in transgenic plants under chilling stress (Fig. 8). Our results further show that there was a significant decrease in the activities of tAPX and sAPX at *in vivo* ASC concentrations (Fig. 10), although we observed that there was an increase in the activities of MDHAR, DHAR, and SOD in chloroplasts in transgenic plants under chilling stress (Fig. 9). These results suggest that the accumulation of H_2O_2 in transgenic plants under chilling stress may be associated with the decreased activities of tAPX and sAPX. Thus, the results in this study imply that the decreased GR activity in transgenic plants under chilling stress resulted in the decrease in the regeneration of GSH and then ASC, which consequently led to the decrease in the activities of tAPX and sAPX and thereby an accumulation of H_2O_2 . The accumulation of H_2O_2 would inevitably result in enhanced sensitivity of PSII to chilling stress in transgenic plants.

In conclusion, the results in this study suggest that chloroplast GR plays an important role in protecting PSII function against chilling stress by maintaining the electron transport in PSII acceptor side and stabilizing PSII complexes. Our results also suggest that the recycling of ascorbate from dehydroascorbate in the ascorbate–glutathione cycle in the chloroplast plays an essential role in protecting PSII against chilling stress.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30725024), the State Key Basic Research and Development Plan of China (2009CB118503), and Solar Energy Initiative of the Chinese Academy of Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbabi.2012.06.003>.

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