Characterization of photosystem II photochemistry in transgenic tobacco plants with lowered Rubisco activase content

Bin Cai, Aihong Zhang, Zhipan Yang, Qingtao Lu, Xiaogang Wen, Congming Lu*

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.29) is a key regulatory enzyme of photosynthetic carbon assimilation, which catalyzes the first step of both the photosynthetic carbon assimilation and photorespiratory pathways (Woodrow and Berry, 1988). Prior to acting as a catalyst, Rubisco must be activated to become catalytically competent in the photosynthetic carbon reduction cycle by the binding of an ‘activator’ CO₂ and Mg²⁺ to a lysine residue within Rubisco’s catalytic sites (Miziocko and Lorimer, 1983). The Rubisco activation process is catalyzed by the chloroplast enzyme Rubisco activase (Portis, 1990). Rubisco activase does not appear to affect Rubisco activation per se, but it allows uninhibited access to the active site for binding of the activators CO₂ and Mg²⁺ (Portis, 1990, 1992). Rubisco activase requires ATP for activity, and is inhibited by ADP (Streusand and Portis, 1987; Robinson and Portis, 1989). It is thought that Rubisco activase controls the release of ribulose-1,5-bisphosphate (RuBP) and other inhibitors from Rubisco, and thus regulates its activation state (Brooks and Portis, 1988; Portis, 1990). To date, the precise mechanism by which activase acts remains unclear, but most likely involves an interaction between activase and Rubisco–sugar phosphate complexes (Portis, 2003; Portis et al., 2008).

The essential role of activase in regulating photosynthesis is illustrated by the rca mutant of Arabidopsis that lacks this protein and is unable to maintain sufficient activation of Rubisco to grow under ambient CO₂ concentrations (Somerville et al., 1982; Salvucci et al., 1985, 1986). To investigate the role of activase in regulating photosynthesis, a series of studies have examined the effects of different activase contents on CO₂ assimilation rate using transgenic plants with reduced levels of activase compared to wild type plants (Mate et al., 1993, 1996; Jiang et al., 1994; Eckardt et al., 1997; Jin et al., 2006). Although the relatively large amount of protein per leaf is allocated to activase (Fukayama et al., 1996), the level of activase does not proportionally affect CO₂ assimilation rate. Only when activase contents were decreased to about 10–35% of that of the wild type plants was there a significant effect on CO₂ assimila-
tion rate (Mate et al., 1993; Jiang et al., 1994; Eckardt et al., 1997; von Caemmerer et al., 2005; Jin et al., 2006).

Previous studies on the role of activase in photosynthesis have been focused on the regulation of CO2 assimilation (Jiang et al., 1994; Mate et al., 1996; Eckardt et al., 1997; von Caemmerer et al., 2005; Jin et al., 2006). However, to fully examine the control that activase exerts on photosynthesis, there is a need to investigate how activase affects photosystem II (PSII) photochemistry and chloroplast development. In this study, we investigated the role of activase in regulating PSII photochemistry and chloroplast development in transgenic tobacco plants with reduced activase levels. Our results revealed that activase deficiency led to decreased PSII activity and abnormal chloroplast development.

Materials and methods

Vectors and plant transformation

The partial coding region for the tobacco Rubisco activase gene (NTU35111, gi: 1006834) was cloned into the pKANNIBAL vector between the XhoI–KpnI sites in sense orientation (Wesley et al., 2001). The primers used were: 5′-GGT ACC GGA CCA CCC TGG CAT CTT-3′ (irca antisense primers) and 5′-TTC GGA TCC TTA TCT GGG ATC GAT GGA CCC TGG CAT CTT CTT-3′ (irca sense primers). The construct made in pKANNIBAL was subcloned as NotI fragment into pART27, then introduced into Agrobacterium tumefaciens strain LBA4404 by tri-parental mating (An, 1987). Nicotianatobaccum (Wisconsin 38) were transformed by the standard Agrobacterium-mediated transformation (Horsch et al., 1985). Regenerated plants were transplanted into sterilized soil and grown in a greenhouse at 27/20°C (day/night), with maximum PPFD of 1000 μmol m-2 s-1, and a photoperiod of 12/12h light/dark. T1 plants obtained by self-pollination of T0 plants were used in this study.

Plant growth

Three independent lines of transgenic tobacco plants (i7, i28, and i46) were used in the present study. The seeds of these transgenic plants were allowed to germinate on agar in the presence of 50 μg l-1 kanamycin. The seeds of wild type plants were allowed to germinate in the absence of kanamycin. After growth for 2 weeks, plants were transferred to soil. The transplanted plants were then grown for 8 weeks in a growth chamber at 26 ± 1°C with PPFD of 120 μmol m-2 s-1, a relative humidity of 75–80%, and a photoperiod of 12/12 h light/dark. Plants were watered daily and twice weekly with a complete nutrient solution. All the measurements on physiological and biochemical parameters were carried out on the youngest fully expanded leaves.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from 0.1 g fresh leaves using the Trizol reagent (Invitrogen Carlsbad, USA). After DNase I treatment to remove any residual genomic DNA contamination, 2 μg of total RNA from each sample was used to synthesize first-strand cDNA in a 20 μl total volume (SuperScript Pre-amplification System, Promega, USA). The transcript levels in 8-week old plants were examined by semi-quantitative RT-PCR using irca sense primers, and amplified PCR products were collected and analyzed following different numbers (20 cycles, 30 cycles) of amplification cycles. The RT-PCR reactions were repeated twice, with identical results obtained. The expression level of tobacco actin was used as an internal control, as described previously (Ding et al., 2009).

Transmission electron microscopy

For transmission electron microscopy processing, the tobacco leaves from 8-week old plants were collected. The leaf tissue was cut into small pieces and fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in phosphate buffer for 4 h at 4°C. After fixation, tissues were rinsed and post-fixed for 45 min in 1% (v/v) O2O4 overnight at 4°C. After rinsing in phosphate buffer, the samples were dehydrated in an ethanol series, infiltrated with a graded series of epoxy resin in epoxy propane, and embedded in Eponaraldite (Agar Aids, Bishop’s Stortford, UK). After 12 h in pure resin, followed by a change of fresh resin for 4 h, the samples were polymerized at 60°C for 48 h. Ultrathin sections (70 nm thick) were obtained with a Leica ultramicrotome, stained with uranyl acetate, pH 5.0, and lead citrate. Electron micrographs were viewed with a transmission electron microscope JEM-1230 TEM (JEOL) at an accelerating voltage of 80 kV.

Analysis of gas exchange

Gas exchange analysis was performed on a fully expanded attached leaf of tobacco seedlings using an open system (Ciras-1, PP systems, UK). Leaf CO2 assimilation rate was measured in the presence of DCMU (20 μM) before the flash.
Electron transport activities

Photosynthetic electron transport activities were determined as described previously (Yang et al., 2008). The youngest fully expanded leaves were harvested and homogenized in a medium containing 0.4 M sucrose, 50 mM Tricine (pH 7.6). The homogenate was filtered through 16 layers of gauze and the filtrate was then centrifuged at 500 × g for 3 min to remove large debris. The supernatant was further centrifuged at 3000 × g for 10 min. The pellet was washed twice by the buffer (50 mM Tricine, 10 mM NaCl, 5 mM MgCl₂, pH 7.6) at 10,000 × g for 10 min. The resulting washed pellet was thylakoid membranes and resuspended in the same buffer for measuring electron transport activities.

Electron transport activities in the thylakoid membranes were measured with a Clark-type oxygen electrode (Hansatech, King’s Lynn, Norfolk, UK) suspended in the medium (0.4 M sucrose, 50 mM tricine, 10 mM NaCl, 5 mM MgCl₂, pH 7.6) under growth light illumination. The nomenclature of Vass and Govindjee (1996) was used for characterization of the flash-induced TL glow peaks.

Analyses of protein and chlorophyll

Chlorophyll content was determined in 80% (v/v) acetone according to Porra et al. (1989). Soluble protein content was determined by the dye-binding assay according to Bradford (1976).

Statistical analysis

For the comparison of variance of the means, the data were analyzed using analysis of variance (ANOVA) followed by the least significant difference (LSD) test.

Results

Generation of transgenic tobacco plants with decreased Rubisco activase level

In this study, we generated transgenic tobacco plants with the RNAi construct of tobacco Rubisco activase. Thirty independent transgenic lines were obtained, and some of them exhibited low Rubisco activase levels. Three independent lines, i.e., i7, i28, and i46 plants, were chosen to obtain progeny (T1 generation) by self-fertilization. Protein gel blot analysis demonstrated that the level of activase in transgenic i28, i28, and i46 plants was reduced to about 50%, 25%, and 5% of that of wild type plants, respectively. However, there was no significant change in the content of large subunit of Rubisco in the transgenic tobacco plants (Fig. 1A). RT-PCR analysis showed that the mRNA levels of activase in transgenic i7, i28, and i46 plants were suppressed compared to those in wild type plants (Fig. 1B). Compared to wild type plants, transgenic i7 plants did not show significant morphological differences, but transgenic i28 and i46 plants exhibited a considerable decrease in growth (Fig. 1C). There were no differences in the contents of leaf chlorophyll between wild type and transgenic i7 plants. The contents of leaf chlorophyll were 447.4 ± 23.1 and 447.2 ± 19.8 mg m⁻² in wild type and transgenic i7 plants, respectively. The leaves in transgenic i28 and i46 plants appeared green-yellowish. The contents of leaf chlorophyll in transgenic i28 and i46 plants were 391.9 ± 17.5 and 145.8 ± 13.2 mg m⁻², respectively.

CO₂ assimilation

Table 1 shows CO₂ assimilation rates in wild type and transgenic plants. There was no significant change in CO₂ assimilation rate between wild type and transgenic i7 plants. The CO₂ assimilation rates were 8.7 and 8.5 µmol m⁻² s⁻¹ in wild type and transgenic i7 plants, respectively. However, compared to wild type plants, there was a significant decrease in CO₂ assimilation rate in transgenic i28 and i46 plants. The CO₂ assimilation rates in transgenic i28 and i46 plants were 6.9 and −0.12 µmol m⁻² s⁻¹, respectively.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>CO₂ assimilation rate (µmol m⁻² s⁻¹)</th>
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<tbody>
<tr>
<td>WT</td>
<td>8.71 ± 0.23a</td>
</tr>
<tr>
<td>i7</td>
<td>8.52 ± 0.23a</td>
</tr>
<tr>
<td>i28</td>
<td>6.90 ± 0.23a</td>
</tr>
<tr>
<td>i46</td>
<td>−0.12 ± 0.20</td>
</tr>
</tbody>
</table>

Values in the table are means ± SE from three independent experiments. Values indicated with different letters were significantly different at P<0.05.
Table 2
PSI and PSII electron transport activities in wild type (WT) and transgenic i7, i28, and i46 tobacco plants.

<table>
<thead>
<tr>
<th></th>
<th>PSI activity (µmol O₂ mg⁻¹ Chl h⁻¹)</th>
<th>PSII activity (µmol O₂ mg⁻¹ Chl h⁻¹)</th>
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<tbody>
<tr>
<td>WT</td>
<td>236 ± 4a</td>
<td>122 ± 2a</td>
</tr>
<tr>
<td>i7</td>
<td>240 ± 2a</td>
<td>125 ± 4a</td>
</tr>
<tr>
<td>i28</td>
<td>243 ± 4a</td>
<td>95 ± 4b</td>
</tr>
<tr>
<td>i46</td>
<td>246 ± 2a</td>
<td>76 ± 4c</td>
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</table>

Values in the table are means ± SE from three independent experiments. Values indicated with different letters were significantly different at \( P = 0.05 \).

**PSI and PSII electron transport activities**

Table 2 shows PSI and PSII electron transport activities in wild type and transgenic plants. There were no significant differences in PSI electron transport activities between wild type and transgenic plants. Compared to wild type plants, transgenic i7 plants showed no changes in PSII electron transport activity, whereas transgenic i28 and i46 plants demonstrated a significant decrease in PSII electron transport activity.

**PSII photochemistry**

In order to investigate how levels of activase affect PSII electron transport, we examined \( F_v/F_m \) in wild type and transgenic plants (Table 3). Compared to wild type plants, there were no changes in \( F_v/F_m \) in transgenic i7 plants, while there was a decrease in \( F_v/F_m \) in transgenic i28 and i46 plants.

The decrease in \( F_v/F_m \) in transgenic i28 and i46 plants suggests that PSII function is affected. In order to elucidate the functional status of the donor and acceptor sides of the PSII complex in these transgenic plants, the kinetics of the flash-induced chlorophyll fluorescence yield were compared between wild type plants and transgenic plants. The relaxation of the flashed-induced increase in variable chlorophyll fluorescence yield monitors the oxidation of QA⁻, which reflects the reoxidation of QA via forward electron transport to QB (and QB⁻) and back reaction with donor side components (Crofts and Wraight, 1983; Dau, 1994). Thus, through the measurements of the relaxation of the variable fluorescence after single flash excitation (Trtilek et al., 1997), it is possible to obtain simultaneous information for the donor and acceptor side modifications of PSII (Vass et al., 1999, 2002).

In the absence of DCMU, the typical modulated fluorescence decay of wild type plants after a single saturating flash could be resolved into three distinct phases (Fig. 2A and B, Table 4). The modulated fluorescence intensity relaxation in wild type plants is dominated by the fast phase (\( t_{1/2} = 286 \mu s \)), whose relative

Fig. 2. The flash-induced decay of modulated chlorophyll fluorescence in wild type (WT) and transgenic i7, i28, and i46 tobacco plants. (A) The curves were the actual data of the fluorescence signals in the absence of 20 µM DCMU. (B) The curves were normalized relative to the total variable fluorescence in the absence of 20 µM DCMU. (C) The curves were the actual data of the fluorescence signals in the presence of 20 µM DCMU. (D) The curves were normalized relative to the total variable fluorescence in the presence of 20 µM DCMU.

Table 3
Maximal efficiency of PSII photochemistry (\( F_v/F_m \)) in wild type (WT) and transgenic i7, i28, and i46 tobacco plants.

<table>
<thead>
<tr>
<th></th>
<th>( F_v/F_m )</th>
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<tbody>
<tr>
<td>WT</td>
<td>0.83 ± 0.02a</td>
</tr>
<tr>
<td>i7</td>
<td>0.82 ± 0.04a</td>
</tr>
<tr>
<td>i28</td>
<td>0.71 ± 0.05a</td>
</tr>
<tr>
<td>i46</td>
<td>0.64 ± 0.04a</td>
</tr>
</tbody>
</table>

Values in the table are means ± SE from three independent experiments. Values indicated with different letters were significantly different at \( P = 0.05 \).
amplitude is about 60%. The contribution of the middle phase ($t_{1/2} = 8.2$ ms) was about 25%, and that of the slow phase ($t_{1/2} = 7.8$ s) was about 15%. Compared to wild type plants, the fluorescence decay kinetics after a single saturating flash showed no changes in transgenic i7 plants, but there were significant changes in transgenic i28 and i46 plants. There was a gradual decrease in the total fluorescence amplitude in transgenic i28 and i46 plants, suggesting that there was a loss of QA$^-$ reduction. In addition, there was a significant increase in the decay half-time of the fast phase, but a significant decrease in the amplitude of the slow phase. The $t_{1/2}$ of the fast phase increased from 286 μs in wild type plants to 386 and 410 μs in transgenic i28 and i46 plants, respectively. The amplitude of the fast phase decreased from 60% in wild type plants to 52% and 48% in transgenic i28 and i46 plants, respectively. For the slow phase, there was a significant increase in the decay half-time and a decrease in the amplitude. The $t_{1/2}$ of the middle phase increased from 8.2 μs in wild type plants to 11.3 and 15.4 μs in transgenic i28 and i46 plants, respectively. The amplitude of the middle phase decreased from 25% in wild type plants to 20.3% and 19.5% in transgenic i28 and i46 plants, respectively. For the slow phase, there was a significant decrease in the decay half-time and a significant increase in the amplitude of the slow phase. The $t_{1/2}$ of the slow phase decreased from 7.8 s in wild type plants to 6.7 and 5.8 s in transgenic i28 and i46 plants, respectively. The amplitude of the slow phase increased from 15% in wild type plants to 27% and 31% in transgenic i28 and i46 plants, respectively. These results suggest that there was a slow down in the middle phase, but an acceleration in the slow phase in transgenic i28 and i46 plants.

When Chl fluorescence induction kinetics is determined in the presence of DCMU, the fluorescence relaxation reflects the reoxidation of QA$^-$ via recombination with donor side components. Compared to wild type plants, there was no significant change in the modulated fluorescence decay kinetics in transgenic i7 plants, while there were significant changes in the modulated fluorescence decay kinetics in transgenic i28 and i46 plants (Fig. 2C and D, Table 4). The analyses of these fluorescence relaxation curves showed that, in wild type plants, the decay was composed of a fast phase and a slow phase (Table 4). There was no significant change in the time constant for the slow component, but its relative amplitude decreased to 84% and 78% in transgenic i28 and i46 plants, respectively. On the other hand, there was an increase in both time constant and the relative amplitude in these transgenic plants for the fast phase. Its relative amplitude increased from 11% in wild type plants to 16% and 22% in transgenic i28 and i46 plants, respectively. Also, $t_{1/2}$ increased from 120 ms in wild type plants to 173 and 324 ms in transgenic i28 and i46 plants, respectively. These results suggest that, compared to wild type plants, the changes in the fast phase in transgenic i28 and i46 plants may arise from the recombination reactions of QA$^-$ with PSII donor side component(s). It should be noted that the dynamics of the state transition S1 to S2 was generally not influenced in the range from 5% to 100% of activator concentration in the transgenic plants relative to the wild type plants.

TL was further used to investigate the redox properties of the acceptor and donor sides of PSII in wild type plants and transgenic plants, as it is a useful tool to study charge stabilization and subsequent recombination in PSII in higher plants. Recombination of positive charges stored in the S2 and S3 oxidation states of the water-oxidizing complex with electrons stabilized on the reduced Qa and Qb acceptors of PSII results in characteristic TL emissions (Vass and Govindjee, 1996; Inoue, 1996). The TL intensity reflects the amount of recombining charges, whereas the peak temperature is indicative of the energetic stabilization of the separated charge pair; the higher the peak temperature, the greater the stabilization (Vass et al., 1981). Illumination of a single-turnover flash with the plant sample after a short dark adaptation induces a major TL band called the B-band, which appears at around 30°C and arises from S2/S2QA$^-$ recombination (Inoue, 1996; Demeter and Vass, 1984; Rutherford et al., 1982). TL emission following single flash excitation of dark-adapted plants results largely from the recombination of the S2Qa$^-$ charge pair. If electron transfer between QA and QB is blocked by DCMU, the B-band is replaced by the so-called Q-band arising from S2Qa$^-$ recombination at around 10°C (Rutherford et al., 1982).

Fig. 3 shows the TL glow curves in wild type and transgenic plants. The wild type plants exhibited TL emission maxima for the S2Qb$^-$ and S2QA$^-$ charge recombination at approximately 35 and 9°C, respectively. Compared to wild type plants, there was an upshift in the peak temperatures for the S2Qb$^-$ and S2QA$^-$ charge recombinations in transgenic i46 plants. In transgenic i46 plants, the peak temperatures for the S2QA$^-$ charge recombination were at approximately 39 and 14°C, respectively (Fig. 3).

<table>
<thead>
<tr>
<th>Peak temperature (°C)</th>
<th>Without DCMU</th>
<th>With DCMU</th>
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<tbody>
<tr>
<td>WT</td>
<td>34.5 ± 0.1°</td>
<td>9.0 ± 0.2°</td>
</tr>
<tr>
<td>i7</td>
<td>34.8 ± 0.2°</td>
<td>9.2 ± 0.3°</td>
</tr>
<tr>
<td>i46</td>
<td>37.1 ± 0.4°</td>
<td>13.7 ± 0.2°</td>
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</table>

The measurements were performed in the absence and presence of 10 μM DCMU. Mean ± SE values were calculated from four to six independent experiments. Values indicated with different letters were significantly different at $p<0.05$.

The relaxation of the flashed-induced fluorescence yield was measured without or with 20 μM DCMU. Exponential analysis yielded either three or two phasic kinetics with different half-times ($t_{1/2}$) and amplitudes (A). Mean ± SE values were calculated from four 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 different transgenic tobacco plants.
Table 5). Since the temperature at which maximal luminescence occurs is a function of the free energy of stabilization of the charge-separated state, the upshifts in the peak temperatures of the $S_2Q_B^-$ and $S_2Q_A^-$ recombination observed in transgenic i28 and i46 plants indicate that there was an increase in the stabilities of both charge-separated states. Moreover, since the increased stabilizations of the $S_2Q_B^-$ and $S_2Q_A^-$ recombination occurred approximately in parallel, it seems likely that the changes in TL emission maxima for the $S_2Q_B^-$ and $S_2Q_A^-$ were due largely to a modification of the oxygen-evolving complex specifically affecting the charge transfer characteristics of the $S_2$ state.

Fig. 4 shows the oscillation of the B-band in wild type and transgenic plants as a function of the number of flashes given prior to recording the TL curves. In wild type plants, the intensity of the B-band oscillated with a periodicity of four, with the maximum emission occurring on the second flash. This is the same type of oscillation observed for whole leaf tissue (Rutherford et al., 1984). The oscillatory pattern was not changed in transgenic i7 plants. On the other hand, the oscillatory pattern in transgenic i28 plants was gradually damped compared to wild type plants. In transgenic i46 plants, the period-four oscillation could almost not be observed.

**Thylakoid peptide composition**

Fig. 5A shows the effects of lowered activase content on thylakoid peptide composition in wild type and transgenic plants. Compared with wild type plants, the amount of D1 protein did not show a significant change in transgenic i7 plants, but showed a decrease in transgenic i28 and i46 plants, which were about 86% and 74% of wild type plants, respectively. The amount of D2, CP43, and CP47 showed a significant decrease only in transgenic i46 plants, but not in transgenic i7 and i28 plants. There was no significant change in the amount of PsbO protein in transgenic i7 plants, while the amount of PsbO in transgenic i28 and i46 plants was 83% and 68% of that of wild type plant, respectively. There was a slight increase in the level of LHCI in transgenic i46 plants. The levels of PSI-Psa A/B proteins, Cytochrome (Cyt) f and $\gamma$-subunit of the ATP synthase (CF1$\gamma$) did not show significant changes between wild type and transgenic plants.

To investigate structural alterations of thylakoid proteins, chlorophyll–protein complexes were solubilized from thylakoid membranes using dodecyl-$\beta$-$\delta$-maltopyranoside (DM) and separated by blue native PAGE (BN-PAGE) (Fig. 5B). After the first-dimensional separation in the presence of Coomassie blue G 250 dye, five major bands were resolved, apparently representing monomeric PSI and dimeric PSII (band I), monomeric PSII (band II), CP43 minus PSII (band III), trimeric (band IV), and monomeric LHCI (band V) (Peng et al., 2006). The BN-PAGE analysis showed that the monomeric PSI and dimeric PSII (bands I) decreased slightly in transgenic i28 plants but significantly in transgenic i46 plants as compared with wild type plants. Analyses of the two-dimensional SDS-urea-PAGE gels after Coomassie blue staining confirmed similar results, as shown in Fig. 5A.

**Chloroplast development**

The results showed that the lowered content of activase resulted in a decrease in the activity of PSII electron transport, so we investigated whether the lowered activase content affects thylakoid membrane development. Fig. 6 shows electron micrographs of chloroplasts of wild type and transgenic plants. There was no significant difference in chloroplast development between wild type and transgenic i7 and i28 plants. However, compared to wild type plants, transgenic i46 plants showed a significant decrease in the number of grana stacks per chloroplast and discs per grana stack (Table 6).
Fig. 5. (A) Immunological analysis of peptide composition of the thylakoid membranes (5 μg chlorophyll) in wild type (WT) and transgenic i7, i28, and i46 tobacco plants. (B) BN gel analysis of thylakoid membranes (10 μg chlorophyll) in wild type (WT) and transgenic i7, i28, and i46 tobacco plants. (C) Two-dimensional separation of protein complexes in the thylakoid membranes in wild type (WT) and transgenic i7, i28, and i46 tobacco plants.

Discussion

In this study, we obtained transgenic tobacco plants with decreased levels of Rubisco activase compared to wild type plants. Our results show that when the level of activase was decreased to 25% of wild type plants, there was a decrease in CO2 assimilation rate (Table 1), which is similar to the results reported in previous studies (Jiang et al., 1994; Mate et al., 1996; Eckardt et al., 1997; Jin et al., 2006). However, there is no report on how lowered activase content regulates photosynthetic electron transport. Our results show that the lowered activase content had no effect on PSI electron transport, but resulted in a significant decrease in PSII electron transport, which is reflected in a decrease in PSII activity when the level of activase was decreased to 25% of wild type plants (Tables 2 and 3). We further investigated how PSII photochemistry is regulated in these transgenic tobacco plants.

Flash-induced chlorophyll fluorescence kinetics is useful to study the electron transfer at the donor and acceptor sides of PSII. The double-modulation technique makes it possible to study the reoxidation processes of QA− by both forward and back reactions (Trtilek et al., 1997). Analyses of modulated fluorescence decay kinetics show that a significant increase in the decay half-time of the fast phase and a significant decrease in its amplitude in transgenic i28 and i46 plants (Fig. 2, Table 4), indicating clearly that lowered activase content results in an inhibition of electron transfer from QA− to QB. In order to analyze whether the inhibition of

Table 6
Parameters of chloroplast development in wild type (WT) and transgenic i7, i28, and i46 tobacco plants.

<table>
<thead>
<tr>
<th></th>
<th>Number of grana stacks per chloroplast</th>
<th>Number of discs per grana stack</th>
<th>Length of the chloroplast (μm)</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>18.3 ± 1.8a</td>
<td>22.3 ± 2.1a</td>
<td>5.9 ± 0.28a</td>
</tr>
<tr>
<td>i7</td>
<td>17.9 ± 2.3a</td>
<td>22.1 ± 2.3a</td>
<td>5.9 ± 0.36a</td>
</tr>
<tr>
<td>i28</td>
<td>16.7 ± 3.5a</td>
<td>18.1 ± 2.8a</td>
<td>5.8 ± 0.24a</td>
</tr>
<tr>
<td>i46</td>
<td>13.3 ± 2.1b</td>
<td>11.6 ± 1.1r</td>
<td>4.9 ± 0.19b</td>
</tr>
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</table>

Values in the table are means ± SE from three independent experiments. To quantify the differences between chloroplasts of wild type and different transgenic plants, totally 64 chloroplast sections were analyzed from the youngest fully expanded leaves. Values indicated with different letters were significantly different at P=0.05.
electron transfer from QA\(^{-}\) to QB is associated with the decrease in the apparent equilibrium constant for sharing the electron between QA\(^{-}\) and QB in transgenic i26 and i48 plants, we compared the t\(_{1/2}\) of the slow phase in the absence or presence of DCMU. In the absence of DCMU, the t\(_{1/2}\) of the slow phase in wild type plants was about 7.8 s. However, in the presence of DCMU, the t\(_{1/2}\) of the slow phase decreased to about 1.7 s in wild type plants (Table 4). The slow phase reflects the reoxidation of QA\(^{-}\) via charge recombination with the S2 state. In the presence of DCMU, the slow phase occurs from the S\(_2\)QA\(^{-}\) recombination in PSII centers. Thus, the differences in the t\(_{1/2}\) indicate that, in the absence of DCMU, the recombination should occur from the QAQB\(^{-}\) state, which is in charge equilibrium with the QAQB state. Therefore, the inhibition of QA\(^{-}\) to QB in transgenic i28 and i46 plants may be associated with the decrease in the apparent equilibrium constant for sharing the electron between QA\(^{-}\) and QB, which may be due to a decreased affinity of QB binding pocket. Indeed, we observed that there was an increase in the t\(_{1/2}\) of the middle phase in the absence of DCMU in transgenic i28 and i46 plants (Table 4). This decay component is assigned to reoxidation of QA\(^{-}\) in the centers in which a vacant QB site has to be reoccupied by a PQ molecule before the QA\(^{-}\) to QB electron transfer can take place. Thus, an increase in the t\(_{1/2}\) of the middle phase in transgenic i28 and i46 plants suggests that there were modifications of the QB niche by which PQ binding is slowed down.

In this study, we observed that the time rate constant of the middle phase of the flash fluorescence relaxation recorded in the presence of DCMU increased significantly in transgenic i28 and i46 plants (Table 4), suggesting that electron transport at the donor side of PSII is affected. To investigate how electron transport at the donor side is regulated in transgenic plants, we examined the characteristics of TL in wild type and transgenic plants. Our results show that there was a parallel increase in the stability of the S\(_2\)QA\(^{-}\) and S\(_2\)QB\(^{-}\) charge recombination in transgenic i48 plants compared with wild type plants (Fig. 3, Table 5). Our results also show that the period-four oscillation was significantly dampened in transgenic i46 plants (Fig. 4). These results suggest that there is an increase in the redox stability of the S\(_2\) state in transgenic i46 plants. It has been shown that the loss of the PsbO protein is accompanied by an increase in the stability of the S\(_2\) state by the removal of the PsbO protein from PSII preparations in vitro by measuring TL profiles and by monitoring deactivation kinetics of the S states (Miyao et al., 1987; Vass et al., 1987). Moreover, the mutant lacking the PsbO protein results in upshifts in the peak temperatures of S\(_2\)QA\(^{-}\) and S\(_2\)QB\(^{-}\) recombination by determining TL profiles and thus leads to an increase in the stability of the S\(_2\) state in a cyanobacterium Synechocystis sp. PCC 6803 (Burnap et al., 1992). Based on these reports and our results, it is suggested that the increased stability of the S\(_2\) state in transgenic i46 plants may be due to the decreased PsbO protein in the thylakoid membranes. To investigate this possibility, we examined the content of the PsbO protein in wild type and transgenic plants. We observed that there was a significant decrease in the content of the PsbO protein in the thylakoid membranes on a chlorophyll basis in wild and transgenic plants suggest that the increased stability of the S\(_2\) state in transgenic i46 plants is associated with the decrease in the content of the PsbO protein in the thylakoid membranes.

In this study, we observed the pale-green phenotype in transgenic i28 and i46 plants (Fig. 1). We thus investigated chloroplast structure in wild type and transgenic plants. Our results show that chloroplast development in transgenic i28 and i46 plants was defective, with less grana compared to wild type plants (Fig. 6). It seems that the defective nature of the chloroplast was not due to the change in PSI function since there was no difference in PSI electron transport activity between wild type and transgenic plants (Table 2). On the other hand, the defected chloroplast in transgenic i28 and i46 plants may be associated with the PSII function, as
we observed that there was a significant decrease in PSII electron transport activity and the maximal efficiency of PSII photochemistry (Tables 2 and 3). In addition, we observed that there was a decrease in the content of D1 and PsbO proteins in transgenic i28 and i46 plants (Fig. 5). Overall, the ultrastructural defects observed in chloroplasts of transgenic i28 and i46 plants suggest that activase may be required for chloroplast biogenesis.

It should be noted that the levels of the D1 protein were significantly reduced in transgenic i28 and i46 plants, while the levels of other PSII proteins were not decreased in these transgenic plants (Fig. 5). These results suggest that the D1 protein is more sensitive to activase deficiency as compared to other PSII proteins. The significant decrease in D1 protein transgenic i7 and i28 plants can be explained by aggravated photoinhibition due to the significant decrease in CO2 assimilation rate in these transgenic plants.

In conclusion, the results in this study demonstrate that activase plays an important role not only in CO2 assimilation, but also in PSII function. Activase deficiency led to a decrease in PSII activity that was associated with an inhibition of electron transfer at the acceptor side and the donor side. The decrease in PSII function resulted in the defects in the development of chloroplasts. Our results suggest that activase plays an important role in maintaining PSII function and chloroplast development. The results in this study also suggest that there is a homeostasis in respect to photosynthetic electron transport and CO2 fixation upon a genetic down regulation of Rubisco activase to below 50% of the control. The molecular mechanisms involved in how activase regulates the PSII function remains to be investigated further.

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References