Biochimica et Biophysica Acta 1807 (2011) 391–403

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Characterization of photosystem II in transgenic tobacco plants with decreased iron superoxide dismutase

Yan Zhang, Shunhua Ding, Qingtao Lu, Zhipan Yang, Xiaogang Wen, Lixin Zhang, Congming Lu *

Photosynthesis Research Center, Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China

ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 7 January 2011
Accepted 14 January 2011
Available online 20 January 2011

Keywords:
Chlorophyll fluorescence
Iron superoxide dismutase
Photosystem II
Photoinhibition
Transformed tobacco (*Nicotiana tabacum*)
Thermoluminescence

ABSTRACT

Iron superoxide dismutases (FeSODs) play an important role in preventing the oxidative damage associated with photosynthesis. To investigate the mechanisms of FeSOD in protection against photooxidative stress, we obtained transgenic tobacco (*Nicotiana tabacum*) plants with severely decreased FeSOD by using a gene encoding tobacco chloroplastic FeSOD for the RNAi construct. Transgenic plants were highly sensitive to photooxidative stress and accumulated increased levels of $O_2^{\bullet-}$ under normal light conditions. Spectroscopic analysis and electron transport measurements showed that PSII activity was significantly reduced in transgenic plants. Flash-induced fluorescence relaxation and thermoluminescence measurements revealed 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 showed that PSII protein accumulation was also decreased in transgenic plants. PSII photodamage and D1 protein degradation under high light treatment was increased in transgenic plants, whereas the PSII repair was not affected, indicating that the stability of the PSII complex was decreased in transgenic plants. The results in this study suggest that FeSOD plays an important role in maintaining PSII function by stabilizing PSII complexes in tobacco plants.

1. Introduction

For photosynthetic blue-green algae and higher plants, various reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), are inevitable by-products of photosynthesis even under normal light conditions. When the absorption of light energy exceeds its utilization in photosynthesis, production of ROS is dramatically enhanced. It is generally thought that ROS are involved in light-induced decline of photosynthetic activity termed as 'photoinhibition' [1]. Photosystem II (PSII) complex, in particular, the PSII reaction center D1 protein, is the main target of photoinhibition. ROS can induce the specific cleavage of the D1 protein in vitro [2–6]. Based on in vivo studies of cyanobacteria, it has been suggested that ROS act primarily by inhibiting the synthesis of the D1 protein and not by damaging PSII directly [7–9].

ROS is detoxified via both enzymatic and non-enzymatic mechanisms. Superoxide dismutases (SODs) constitute the first line of defense against $O_2^{\bullet-}$ by rapidly converting $O_2^{\bullet-}$ to H_2O_2 and O_2 [10]. In chloroplasts, there are two types of SOD isozymes, iron SOD (FeSOD) and copper–zinc SOD (Cu/ZnSOD). Comparison of deduced amino acid sequences from the two types of SODs suggests that FeSOD has no sequence similarity to Cu/ZnSODs and probably has evolved separately in eukaryotes [11]. In addition, the functions of FeSODs are specific: the induction of chloroplastic Cu/ZnSOD has been observed in *fsd* *Arabidopsis* mutant, whereas the induction cannot compensate for the mutation and cannot reverse the phenotype [12]. This functional specialization of FeSODs may be related to their different localizations in the chloroplast [12]. Chloroplastic Cu/ZnSOD is thought to be attached to the thylakoid membranes at the vicinity of photosystem I (PSI) [13,14]. Although FeSODs are attached to the stromal side of the thylakoid membranes [12,15], their subplastidial localization may be different. Immunoblot analysis shows the presence of a FeSOD in the PSII membrane in wheat chloroplasts [16]. FeSODs from *Arabidopsis* and mustard are identified as components of the plastid-encoded polymerase (PEP) complex and are located at thylakoid membrane-associated nucleoids in plastids [12,17,18].

Indeed, there are several lines of evidence suggesting that FeSODs play important roles in protecting chloroplasts from photooxidative damage. Exposure to high light stress results in an increase in FeSOD transcripts in *Arabidopsis* [12]. Proteomic analysis of the response of *Arabidopsis* to high light also revealed that FeSODs are up-regulated [19]. Moreover, knockout FeSOD *Arabidopsis* plants show a typical photooxidative stress symptom even at non-photoinhibitory conditions, as manifested by pale green phenotypes and suppressed growth and abnormal chloroplasts [12]. However, it has been reported that little protection from photoinhibition was observed for overexpression of FeSOD in tobacco [15] or in poplars [20]. This little protective effect may be due to the following reasons: (1) the localization of overexpressed

* Corresponding author. Tel./fax: +86 10 62595516.
E-mail address: lucm@ibcas.ac.cn (C. Lu).

0005-2728/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2011.01.006

FeSOD may be different from membrane-attached chloroplastic SOD, resulting in a failure of overexpressed FeSOD to be accessible to $O_2^{\bullet-}$ produced in PSI or PSII and to protect chloroplasts from photoinhibition [15,20]; and (2) the overexpression of FeSOD may result in a more rapid production of H_2O_2, which can be equally damaging [15].

In addition to chloroplastic Cu/ZnSOD and FeSOD, non-heme iron and heme-iron of cyt b_{559} have been considered to exhibit superoxide dismutase activity [21,22]. The intermediate potential (IP) form of cyt b_{559} serves as superoxide oxidase that catalyze $O_2^{\bullet-}$ to O_2, and the reduced high potential (HP^{red}) form acts as superoxide reductase to catalyze $O_2^{\bullet-}$ to H_2O_2 [22].

Although it has been shown that FeSOD plays an important role in chloroplast development [12], it is unknown if and how FeSODs protect PSII against photooxidative stress. In addition, the role of increased chloroplastical $O_2^{\bullet-}$ in photoinhibition has not been investigated in vivo. To address the above questions, we generated transgenic tobacco plants with severely decreased chloroplastic FeSOD by the RNA interference technique (RNAi). Our results suggest that FeSOD plays an important role in maintaining the stability of PSII complex and high levels of $O_2^{\bullet-}$ accumulated in transgenic plants result in accelerated PSII photodamage and increased D1 degradation.

2. Materials and methods

2.1. Vectors and plant transformation

The partial coding region for tobacco FeSOD gene (A09032, gi: 411893) was cloned into pKANNIBAL vector between the *Xho*I–*Kpn*I sites in sense orientation and the *Cla*I–*Xba*I sites in antisense orientation [23]. The primers used were: 5′-AAA CTC GAG ATT TGA ACT CCA GCC TCC-3′ and 5′-ATA GGT ACC GAC ACG AGC TTC TCC ATA-3′ (iFSD sense primers) and 5′-TA TCT AGA ATT TGA ACT CCA GCC TCC-3′ and 5′-TAG ATC GAT GAC ACG AGC TTC TCC ATA-3′ (iFSD antisense primers). Construct made in pKANNIBAL was subcloned as *Not*I fragment into pART27, then introduced into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating [24]. *Nicotiana tabacum* (Wisconsin 38) were transformed by the standard *Agrobacterium*-mediated transformation [25]. 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 12/12-h light/dark.

2.2. Plant materials and growth conditions

The independent transgenic lines were screened by Southern blot and immunoblot analyses. Three independent lines of transgenic tobacco plants (i4, i29, and i34) were selected in the present study. The seeds of these transgenic lines were allowed to germinate on agar in the presence of 50 μg l^{-1} kanamycin. After growth for 2 weeks, plants were transferred to vermiculite which was soaked with Hoagland solution. The transplanted plants were then grown for 2 weeks in a growth chamber at 25 ± 1 °C with PPFD of 100 or alternatively 20 μmol m^{-2} s^{-1}, a relative humidity of 75–80%, and a photoperiod of 12/12-h light/dark. Unless otherwise stated, tobacco plants grown under normal light conditions (100 μmol m^{-2} s^{-1}) were used for most experiments. All the measurements on physiological and biochemical parameters were carried out on the second leaves from the top.

2.3. RT-PCR and real-time RT-PCR

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). RT-PCR was performed using the iFSD sense primers and amplified PCR products were collected and analyzed following different numbers (25 cycles, 30 cycles) of amplification cycles. The expression level of tobacco actin was used as an internal control as described previously [26].

The real-time RT-PCR reactions were performed using TaKaRa SYBR Premix ExTaq in an Mx3000P real-time PCR instrument (Stratagene). The amplification of actin was used as an internal control for normalization. The primers for analysis of FeSOD transcript levels were: sense, 5′-ATCAATGAAGCCCAACGG-3′ and antisense, 5′-GCCCAACCAGAGCCAAAT-3′.

2.4. SOD activity gels

Tobacco leaves (0.1 g) were homogenized in 200 μl extraction buffer (100 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM PMSF, 2% (w/v) PVP) and the homogenate was centrifuged at 12,000 × *g* at 4 °C for 10 min. Total protein (80 μg) was separated on 15% nondenaturing polyacrylamide gel in Tris–Gly buffer (pH 8.3) at 200 V for 30 min. The gel was soaked in 36 mM phosphate buffer (pH 7.8) containing 2 mM nitroblue tetrazolium for 30 min, rinsed with distilled water, and then transferred to 36 mM phosphate buffer (pH 7.8) containing 0.028 mM riboflavin and 28 mM TEMED (*N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine) for another 30 min. After being washed with distilled water, the gel was illuminated until white bands appeared. The SOD activity was verified by KCN and H_2O_2 as described by Kurepa et al. [27].

2.5. Detection and measurement of ROS in leaves

In situ detection of $O_2^{\bullet-}$ was performed by using the nitroblue tetrazolium (NBT) staining method as described by Kawai-Yamada et al. [28]. Detached leaves were vacuum-infiltrated with 10 mM NaN_3 in 10 mM potassium phosphate buffer (pH 7.8) for 1 min, and incubated in 1 mg ml^{-1} nitroblue tetrazolium (in 10 mM potassium phosphate buffer, pH 7.8) for 20 min in the dark at room temperature. In situ detection of H_2O_2 was performed by using the 3,3′-diaminobenzidine (DAB) staining method as described by Thordal-Christensen et al. [29]. Detached leaves were vacuum-infiltrated with 1 mg ml^{-1} DAB solution (pH 3.8) for 1 min and incubated in the dark at room temperature for 6 h. Stained leaves were cleared by boiling in acetic acid/glycerol/ethanol (1:1:3 [v/v/v]) solution before photographs were taken.

Total leaf H_2O_2 content was determined according to the method of Veljovic-Jovanovic et al. [30] as described previously [26].

2.6. Enzyme measurements

Total superoxide dismutase (SOD, EC1.15.1.1) activity was estimated by its ability to inhibit photoreduction of nitroblue tetrazolium as described previously [26]. Activities of the different SOD isoforms were determined by using 3 mM KCN or 5 mM H_2O_2 as the final concentration in the reaction mixture. KCN inhibits Cu/ZnSOD and H_2O_2 inhibits both FeSOD and Cu/ZnSOD activities. In order to determine the chloroplastic Cu/ZnSOD activity, the intact chloroplast was isolated [26].

Peroxidase activity (POX, EC1.11.1.7) was analyzed according to Ros-Barceló [31]. The activities of ascorbate peroxidase (APX, EC1.11.1.11) and catalase (CAT, EC1.11.1.6) were determined as described previously [26]. Glutathione peroxidase activity (GPX, EC1.11.1.9) was assayed spectrophotometrically as described by Yoshimura et al. [32].

2.7. Chlorophyll (Chl) fluorescence analysis

Chl fluorescence was measured using a PAM-2000 portable chlorophyll fluorometer (Heinz Walz, Germany). After a dark adaptation period of 30 min, minimum fluorescence (Fo) was determined by a weak red light. Maximum fluorescence of dark-adapted state (Fm) was measured during a subsequent saturating pulse of white light