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Probing molecular events associated with early development of thylakoid membranes by comparative proteomics and low temperature fluorescence

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ABSTRACT

A comparison of protein profiles between prolamellar bodies from dark-grown etioplasts and thylakoid membranes from de-etioplasts illuminated respectively for 1, 5 and 9 h revealed 155 differentially expressed CBB-stained spots. Clear results showed that the nonphototransformable $Pchlide_{627–632}$ was the dominant pigment form in the PLBs of rice etioplasts during plant development in dark and transformed slowly to chlorophyllide in rice etioplasts when exposed to light. The light-induced accumulation of ACC oxidase, which catalyzes the final step of ethylene synthesis using ACC as substrate, would facilitate chlorophyll synthesis by inducing PORa/b expression via ethylene signaling. It could be also suggested that cyclic electron transport might play an important role in generation of ATP for carbon fixation and photoprotection of photosystems from excessive light in prothylakoid. Furthermore, the overproduction of ClpC1, which targets proteins to the ClpPR core complex for degradation, was observed only in Stage 1, during which period PLBs disrupted and converted into prothylakoids, suggesting that ClpC1 was of particular importance for disassembly of PLBs of etioplasts when exposed to light. This study revealed the possible biochemical and physiological processes lead to the formation of functional thylakoid membranes.

Biological significance: In this study, we monitored the light-induced transformation of prolamellar bodies into thylakoid membranes, which is correlated to the biogenesis of photosynthetic apparatus involving a complex cascade of biochemical and structural events. Three stages of thylakoid development classified according to the thylakoid development status (Adam et al., 2011) were studied for biogenesis of photosynthetic apparatus: Stage 1, prothylakoids emerge from the disrupted PLBs; Stage 2, prothylakoids converted into primary thylakoids which were dispersed in the stroma; Stage 3, the continuous grana and stroma thylakoids are formed. The development stage-dependent changes in the proteomic profile of the thylakoids were analyzed by two-dimensional electrophoresis (2-DE). This information was complemented with the steady-state 77 K chlorophyll fluorescence of thylakoids at the corresponding development stage. Together, these analyses allowed us to further understand the molecular processes connected to the formation of functional thylakoid membranes.

1. Introduction

When plants grow in dark, proplastid develops into etioplast containing membranous structure called prolamellar body (PLB). Upon exposure to light, the prolamellar bodies (PLBs) disrupt and prothylakoids - perforated lamellae emerge from PLBs, prothylakoids are converted to primary thylakoids, and flattened primary thylakoids transform into the continuous grana and stroma thylakoids [1]. The thylakoid membrane (TM) serves as a lipid matrix for the assembly of large pigmented (PS I and PS II core complexes, light-harvesting complexes of PS I and PS II) and nonpigmented (cytochrome b_6/f complex, ATP synthase) complexes, which are made of proteins, pigments, and other cofactors attached and embedded in the lipid bilayer. Structural transformation is accompanied with protochlorophyllide (Pchlide) conversion to chlorophyll (Chl) and in addition the synthesis of proteins involved in photosynthesis, especially thylakoid proteins, gradually increases upon illumination [2,3,4].

Biogenesis of photosynthetic apparatus in TM depends on multifarious and tightly coordinated processes involving the concerted transactions of two genomes. These include expression of both nuclear and plastid genes; synthesis of pigments and incorporation of them into proteins; import of nuclear gene products into chloroplast via a dedicated import machinery; insertion of proteins into TM, and their assembly into functional complexes. Etioplasts do not contain chlorophyll-protein complexes (CPCs). The synthesis of CPCs occurs during the light-induced greening of etiolated seedlings. It was reported that CP1 (P700-Chl *a* protein complex) appears 45 to 60 min after illumination together with P700 activity, and light-harvesting complex (LHC) begins to accumulate at 2.5 h with the beginning of Chl *b* synthesis [5]. Chl *a*-

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binding proteins of PS II core and light-harvesting chlorophyll *a*/*b* proteins could be detected at 3 to 4 h after illumination [5,6]. However, a large set of the photosynthetic proteins, such as the subunit F0 of the ATP synthase and subunits W and X of the PS II reaction center, are believed to use a spontaneous mechanism for membrane insertion [7,8,9], which can be considered as a means of rapid initiation of thylakoid membrane formation at the stage when thylakoid protein transport pathways have not yet been assembled.

Thylakoids become photochemically competent very early in their development [10]. The level of transcription, which is quite low in proplastid, increases drastically when the chloroplast begins to mature [11]. However, the events and steps lead to the formation of functional TMs are still poorly understood. It is likely that conventional molecular-genetic studies may be difficult in this case, since fundamental defects in thylakoid formation would likely be detrimental to plant survival. In the last years, proteomic approaches combining two dimensional gel electrophoresis and mass spectrometry, were used to identify a great number of proteins in etioplast and chloroplast [12,3,13]. Von Zychlinski and coworkers [3] examined the global state of protein expression in etioplast, by which they suggested etioplast-specific metabolic functions and potential mechanisms in the regulation of plastid gene expression. Plöscher et al. [13] reported that the inner membranes from etioplasts and chloroplasts share a number of membrane protein complexes characteristic for electron transport, chlorophyll and protein synthesis as well as fatty acid biosynthesis. Although the increasing list of proteins reported to exist in etioplast or chloroplast, the ways of photosynthetic apparatus assembly and the adaptive strategies of plastids during chloroplast development are still unclear.

In this study, we monitored the light-induced transformation of prolamellar bodies into thylakoid membranes (TMs), which is correlated to the biogenesis of photosynthetic apparatus involving a complex cascade of biochemical and structural events. Three stages of thylakoid development classified according to the thylakoid development status [1] were studied for biogenesis of photosynthetic apparatus: Stage 1, prothylakoids emerge from the disrupted PLBs; Stage 2, prothylakoids converted into primary thylakoids (PTs) which were dispersed in the stroma; Stage 3, the continuous grana and stroma thylakoids are formed. The stage-dependent development changes in the proteomic profile of the thylakoids were analyzed by two-dimensional electrophoresis (2-DE). This information was complemented with the steady-state 77 K chlorophyll fluorescence of thylakoids at the corresponding development stages. Together, these analyses allowed us to further understand the molecular processes connected to the formation of functional thylakoid membranes.

2. Materials and methods

2.1. Plant growth

Rice (*Oryza sativa* ssp. *Japonica*) seeds were germinated in Kimura solution B and grown in dark with a day/night temperature regime of 28 °C/25 °C. The 14 d old etiolated seedlings were exposed to white light (120 μmol photons m^{-2} s^{-1}) and illuminated for 0, 1, 5 and 9 h, respectively. All manipulations were carried out in the darkness with dim green light, considered as safe and photomorphogenetically inactive.

2.2. Electron microscopy

The rice etiolated leaves grown in dark and de-etiolated leaves exposed to light for 1, 5 and 9 h were used for observation with electron microscopy. Several 1 mm^2 pieces were cut from rice leaves, using a razor blade, fixed with 3% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.2) overnight. Washed with 100 mM potassium phosphate buffer at pH 7.2, and post-fixed in 1% osmium tetroxide overnight. The leaf samples were then dehydrated by means of a graded series of ethanol-water solutions. Uranyl acetate was employed in the final dehydration step at a concentration of 2% in 100% ethanol. Infiltration in propylene oxide was allowed to proceed for 2 h during which time the solution was changed three times. Ultrathin transverse sections were cut on a Leica ultracut R microtome at a thickness of 70 nm. The leaf sections were then double stained with 2% uranyl acetate and lead citrate, and examined with a JEM-1230 (JEOL, Japan) transmission electron microscope.

2.3. Preparation of prolamellar bodies and thylakoid membranes

Intact plastids were isolated essentially as described by Nishio and Whitmarsh [14]. All procedures were done at 4 °C. The leaf blade tip were cut with a single-edged razor blade into 1 cm slices; and homogenized in ice-cold isolation buffer containing 50 mM HEPES-KOH (pH 7.5), 330 mM sucrose, 2 mM EDTA-Na_2, 1 mM $MnCl_2 \cdot 4H_2O$, 5 mM $MgCl_2$, 2 mM DTT, 0.5 mM Phenylmethanesulfonyl fluoride (PMSF) followed by filtration through 10 layers of Miracloth. The homogenates were first centrifuged at 200 ×g for 4 min to remove the cell debris. The plastid pellets were obtained by centrifugation at 2000 ×*g* for 5 min, and fractioned by 40%, 80% Percoll step gradient. Plastids from the etioplasts grown in dark and the de-etioplasts exposed to light for 1, 5 and 9 h, were isolated separately in three independent replicates for each developmental stage. For preparation of PLBs or TMs, the intact plastids were ruptured in 25 mM MES (pH 6.0), 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM PMSF at 4 °C. The broken membranes were washed twice with 100 mM Na_2CO_3 in order to strip peripheral membrane proteins [15], collected by centrifugation at 25,000 ×*g* for 30 min, and stored at −80 °C for further use.

2.4. Two dimensional electrophoresis

PLBs from the etioplasts grown in dark and TMs from the de-etioplasts exposure to light for 1, 5 and 9 h were solubilized, respectively, in IPG buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% Ampholine (pH 3–10), 1% DTT and 0.5 mM PMSF) containing 0.5% n-dodecyl-β-D-maltoside (DM). The samples were centrifuged at 20,000 ×*g* for 20 min to remove insoluble materials. The supernatant was transferred to 2-DE. For the first dimension, the samples were loaded by in-gel rehydration onto 11 cm strips with an immobilized linear pH gradient from 4 to 7 (GE Healthcare, USA). Rehydration was performed overnight. The isoelectric focusing (IEF) was carried out using Ettan-IPGphor-II(GE Healthcare, USA)as follows, 300 V for 1 h, 600 V for 1 h, 1000 for 1 h, and 8000 V until at least 32,000 Vh was reached. Prior to the second dimension, the immobilized pH gradient strips were equilibrated for the reduction/alkylation steps, first for 15 min in equilibration buffer (6 M urea, 2.5% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol) containing 1% Dithiothreitol (DTT), and next for 15 min in equilibration buffer containing 2.5% iodacetamide. The second dimension was performed in laboratory-made homogeneous 15% polyacrylamide gels.

2.5. Gel scanning and image analysis

Proteins were detected by in-gel staining with Coomassie brilliant blue (CBB). All gels were scanned at a 300 dpi and 16-bit grayscale pixel depth with UMAX Power Look 2100XL scanner (Maxium Tech, Taipei, China). The spot detection and gel comparison were made with ImageMasterTM 2D Platinum, version 5.01 (GE Healthcare Bio-Science, Little Chalfont, UK), which allows spot detection, quantification, back ground subtraction, and spot matching among multiple gels. The gels of PLBs from the etioplasts and TMs from the de-etioplasts exposed to light for 1, 5 and 9 h were assigned to different groups. The gels of PLBs from the etioplasts were used as the reference group. Each group of PLBs and TMs was analyzed in five biological replicates. Protein spots were selected for profile analysis only if they were confirmed in three biological replicates. We quantified spots by determining the ratio of

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