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Shotgun proteomics analysis on maize chloroplast thylakoid membrane

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TABLE OF CONTENTS

1. ABSTRACT

In this study we initiated a proteomic investigation of the maize thylakoid membrane by using a shotgun proteomic approach based on LC-MS^E. A total of 34 maize thylakoid membrane proteins were identified, the majority of which are primarily involved in photosynthesis, including the light-reaction and carbon assimilation. It is noteworthy that all of the core subunits of the Photosystem II were identified in our search. Proteins involved in other processes, such as iron storage, were also detected in our study. The quantity of each identified protein was also determined. Of interest, we discovered that the amount of the three ATP synthase subunits were not equivalent, suggesting that these proteins perform other functions in addition to ATP synthesis. To our knowledge this is the first extensive proteomic investigation of the maize thylakoid membrane, and will likely enable further study of maize photosynthesis and chloroplast development.

2. INTRODUCTION

Photosynthesis occurs primarily in the chloroplast of plant cells and eukaryotic algae. The chloroplast absorbs light and uses it in conjunction with water and carbon dioxide to produce carbohydrates. It is generally accepted that a large number of proteins are involved in this process. Four multisubunit protein complexes, including photosystem I (PSI), PSII, the ATP-synthase complex, and the cytochrome b6f complex, which together comprise 75 to 100 proteins, perform the photosynthetic reactions (1). The thylakoid membranes within the chloroplast are the subcompartments in which the primary, light-dependent reactions occur, though many other functions are also performed here. For example, several nonphotosynthetic thylakoid membrane proteins have been identified that assemble, maintain, and regulate the four multiprotein complexes described above. These include thylakoid kinases, which are regulatory proteins

that facilitate the ability of the thylakoid membrane system to adjust to changes in ambiotic conditions (2, 3) superoxide dismutases and peroxiredoxins, which play a role in the antioxidative defense system on the thylakoid membrane to prevent and respond to oxidative stress (4, 5) and some metal cofactor transporters (6, 7). To better understand its function, biogenesis, and involvement in many biosynthetic pathways, we characterized the thylakoid membrane proteome in chloroplasts.

Currently, two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) or tandem MS (MS/MS) identification of selected protein spots is considered to be the most powerful tool for protein separation and identification. However, the shortcoming of this gel-based approach is that only protein fractions of limited complexity can be analyzed. In 2-DE, for example, highly abundant proteins dominate the stained gel while low-abundant proteins are difficult to detect, rendering it limited in sensitivity and inefficient in analyzing proteins with extreme p*I* values or with very high or low molecular weights. An alternative strategy, termed "shotgun proteomics", reduces reliance on protein fractionation and instead digests protein mixtures to produce a collection of peptides that can be analyzed by on-line mass spectrometry. The combination of HPLC separation of complex peptide mixtures with subsequent tandem mass spectrometry (LC/MS/MS) provides an alternative tool for proteome and subproteome analysis (8). The advantages of this technology are the capacity for high throughput and limited bias for or against particular protein classes (9). This technique has been used to study the algae *Chlamydomonas reinhardti* (10) and several higher plant species, including *Oryza sativa* (11), *Arabidopsis thaliana* (12) and *Spinacia oleracea* (13). Important to this study, liquid chromatography coupled with electrospray-ionization MS/MS has been successfully employed to characterize thylakoid membrane proteins of *Arabidopsis thaliana* (5), and although other studies have been performed on maize chloroplasts (14, 15), few proteomic investigations have been performed on the maize thylakoid membrane.

Additional, low/high-collision energy MS analysis (MS^E)-based data acquisition permits one to collect sufficient data points in low-collision mode to quantify peak ion intensities and, at the same time, obtain fragmentation data in high-collision mode for protein identification. Here we employed the method described above to identify and quantify a total of 34 thylakoid membrane proteins in maize. These proteins were classified into four groups based on annotation in the Swiss-Prot database, and are primarily involved in chlorophyll synthesis, the light-reaction, and the Calvin cycle. These findings will contribute a better understanding of the identity of chloroplastic proteins in maize and their functions in metabolic networks.

3. MATERIALS AND METHODS

3.1. Plant material and isolation of thylakoid membrane

The seeds of *Zea mays* L. ecotype B73 were used in these experiments. The seeds were disinfected for 15 min with 0.1% $HgCl_2$, rinsed thoroughly, and dried with absorbent paper. The seeds were moistened with 15 mL distilled water prior to incubation in climate incubators at 29C, then were transferred into the soil after the radicle penetrated the seed coat. Seedlings were allowed to grow for seven days prior to culling. 0.2 g of fresh leaf slices were used to extract thylakoid membranes as previously described (16). Briefly, leaf slices were homogenized in 200 mL of buffer containing 400 mM NaCl, 20 mM Tricine, 2 mM $MgCl_2$, and 0.2% BSA, pH 8.0. The brei was filtered through two Miracloth layers, and the filtrate was centrifuged for 2 min at 300*xg*. The supernatant was centrifuged for 10 min at 12,000*xg*, and the pellet was washed in 40 mL of buffer containing 150 mM NaCl, 20 mM Tricine, 5 mM $MgCl_2$, and 0.2% BSA, pH 8.0, and was subsequently centrifuged for 10 min at 9000*xg*. The pellet was suspended in buffer containing 10 mM NaCl, 5 mM $MgCl_2$, 50 mM MES, and 400 mM sucrose, pH 6.0, and was frozen in liquid nitrogen and stored at –80C until use.

3.2. Thylakoid membrane protein extraction and digestion

Thylakoid membrane proteins were extracted following the method of Lu (17). Briefly, the samples were precipitated in a 10% (w/v) TCA/acetone solution containing 0.07% (v/v) β-mercaptoethanol at -20C for 2 h. The sample was centrifuged for 1 h at 40,000*xg,* and the pellet was rinsed with -20C cold acetone containing 0.07% (v/v) β-mercaptoethanol. The pellet was vacuum-dried and solubilized in 3 mL of buffer containing 7 M urea, 2 M thiourea, 40 mM DTT, 1% (v/v) protease-inhibitor mixture (Sigma), 0.2 mM Na_2VO_3 and 1 mM NaF on ice for 1 h. Insoluble material was removed by centrifugation for 1 h at 100,000×*g*. The concentration of proteins in the sample was determined using the 2-D Quant Kit (AmBic) with BSA as a standard. Samples were frozen in liquid nitrogen and stored at -80C until use.

3.3. Protein digestion

Protein digestion was performed as described (18). After adjusting the pH to 8.5 with 1 M ammonium bicarbonate (AmBic), the extracted protein was chemically reduced for 45 min at 55C by adding DTT to 10 mM, and carboxyamidomethylated in 55 mM iodoacetamide for 30 min at room temperature in the dark. Then, $CaCl_2$ was added to 20 mM, and endoprotease Lys-C (Roche) was added to a final substrate: enzyme ratio of 100:1 (w/w), and the reaction was incubated at 37C for 12 h. The Lys-C digest was diluted to 1 M urea with 100 mM ammonium bicarbonate, and modified trypsin (Roche) was added to a final substrate:enzyme ratio of 50:1 (w/w). The trypsin digest was incubated at 37C for 12 h. After digestion, the peptide mixture was acidified by 10 µl formic acid for further MS analysis. Samples not immediately analyzed were stored at –80C.

3.4. Analysis by nano-UPLC-MS^E tandem MS

Nanoscale LC separation of Lys-C and tryptic peptides was performed using a nanoACQUITY system (Waters) equipped with a Symmetry C_{18} 5 µm, 180 µm x 20 mm precolumn and a BEH C_{18} 1.7 µm, 75 µm x 250 mm, analytical reversed phase column (Waters). The

samples were initially transferred with an aqueous 0.1% formic acid solution to the precolumn with a flow rate of 7 µL/min for 3 min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a linear gradient of 3 – 40% mobile phase B over 90 min at a flow rate of 200 nL/min followed by a 10 min rinse with 90% mobile phase B. The column was re-equilibrated to the initial conditions for 20 min, and the column temperature was maintained at 35C. The lock mass was delivered from the auxiliary pump of the nanoACQUITY pump with a constant flow rate of 300 nL/min at a concentration of 100 fmol/µL of [Glu1] fibrinopeptide B. All samples were analyzed in triplicate.

Analysis of tryptic peptides was performed using a SYNAPT HD mass spectrometer (Waters). The mass spectrometer was operated in the v-mode with a typical resolving power of at least 10,000 full-width half-maximum for all measurements. The time-of-flight analyzer of the mass spectrometer was calibrated with the MS/MS fragment ions of [Glu1] fibrinopeptide B from m/z 50 to 1600. The reference sprayer was sampled with a frequency of 30 s. Accurate mass LC-MS data were collected in high-definition MSE mode (low collision energy 4 eV, high collision energy ramping from 15 eV to 45 eV, switching every 1.0 seconds, interscan time 0.02 s) (19, 20). The mass range was from m/z 300 to 1990. In order to confirm optimal column loading, all proteins present were quantified by comparison to 100 fmol of rabbit glycogen phosphorylase digest spiked into the sample using the Hi3 quantification method (21).

3.5. Data processing and protein identification

Continuum LC-MS data were processed and searched using ProteinLynx GlobalServer version 2.3 (PLGS 2.3) (Waters). Raw datasets were processed including ion detection, deisotoping, deconvolution, and peak lists generated based on the assignment of precursor ions and fragments based on similar retention times. Components are typically clustered together with a <10-ppm mass precision and a <0.25-min time tolerance. Alignment of elevated energy ions with low-energy precursor peptide ions was conducted with an approximate precision of ±0.05 min.

An NCBI maize database (released in July, 2008; 11653 sequences; 3295141 residues) download from http://www.ncbi.nlm.nih.gov/sites/ was used to search each triplicate run with the following parameters. Peptide tolerance and fragment tolerance: automatic (usually 10 ppm for peptide tolerance, and 20 ppm for fragment tolerance); trypsin missed cleavages: 1; fixed modification: carbamidomethylation of cysteine; variable modifications: N-terminal acetylation, deamidation of asparagine and glutamine, and oxidation of methionine. Rabbit glycogen phosphorylase was appended to the database as an internal standard. The protein identifications were based on the detection of at least three fragment ions per peptide, with more than two peptides identified per protein. A maximum false positive rate of 4% was allowed.

3.6. Quantitative analysis

Measurement of protein abundance, which is based on measuring peptide ion peak intensities observed in low collision energy mode in triplicate, was performed using Waters Expression^{E}. For protein quantification analysis, datasets were normalized using the PLGS "auto-normalization" function. Rabbit glycogen phosphorylase was appended to the database as an internal standard. Identical peptides from each triplicate sample set were clustered based on mass precision of 5 ppm and a retention time tolerance of < 0.25 min using the clustering software included in PLGS 2.3. All proteins were manually assessed by confirming the matched peptide and replication level across samples.

4. RESULTS AND DISCUSSION

4.1. Proteins identified on maize chloroplast thylakoid membranes

In the present study we analysed thylakoid membrane proteins from chloroplasts in young maize leaves by using a shotgun approach based on LC-MS^{E}. Each portion of the study was performed three times to confirm our results, and only proteins that were similarly identified in each replicate were regarded as true positives. A total of 34 non-redundant thylakoid membrane proteins were successfully identified and quantified (Table 1), which varied widely in molecular weight (MW) and isoelectric point (p*I*). For example, the maximum amount of protein was detected for the ATP synthase CF1 beta subunit (P00827) at 10.3728 ng per 0.2 g leaf, and the minimum amount was detected for the NAD(P)H-quinone oxidoreductase subunit K (P06670) at 0.0942 ng per 0.2 g leaf.

According to the annotations in Swiss-Prot database (http://www.expasy.org/), the identified proteins were classified into four functional groups (Table 1 and Figure 1), including chlorophyll biosynthesis, light-reaction (including subunits of PSI and PSII), enzymes involved in carbon assimilation (Calvin cycle and C4 pathway), and proteins which have no function or no direct function on photosynthesis (miscellaneous). As described in Expasy Proteomics Server (http://www.expasy.org/), 16 of the 34 identified thylakoid membrane proteins were identified only at transcript level; 11 proteins were inferred from its homology and 3 proteins were predicted (Table 1).

4.2. Proteins involved in chlorophyll synthesis

We identified three enzymes involved in chlorophyll biosynthesis, and included coproporphyrinogen III oxidase, protoporphyrinogen IX oxidase (PPO), and NADPH-protochlorophyllide oxidoreductase (POR). Coproporphyrinogen III oxidase catalyzes the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, the precursor for both chlorophyll and heme synthesis (22). PPO is a common enzyme of chlorophyll biosynthesis, and it catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX (23). POR is a peripheral, rather than integral, membrane protein (24), and catalyzes the light-driven reduction of protochlorophyllide to chlorophyllide. NADPH is the proximal electron donor

Table 1. Proteins found on thylakoid membrane of *Zea mays* L. with shotgun proteomics method

Accession No.	Protein name	Mw (kDa)	p*I*	Score	Number of Peptides Matched	Coverage (%)	Amount (ngrams)
1 Chlorophyll Synthesis							
Q2F7H8*	Coproporphyrinogen III oxidase	44.61	7.68	277.91	11	34	0.3054
Q9M629*	Protoporphyrinogen IX oxidase	56.84	8.71	389.62	10	20	0.2981
Q70L71*	NADPH-protochlorophyllide oxidoreductase	39.78	9.19	882.02	22	58	0.4986
2 Lightreaction							
2.1 Photosystem II							
P48183#	D1	39.01	5.21	226.26	3	12	0.8214
P48184#	D2	39.60	5.60	261.87	5	12	1.8769
Q41748*	CP24	26.59	7.90	216.15	7	38	0.3860
Q41746*	CP26	29.87	5.49	334.83	7	52	0.8011
O24561*	CP29	31.38	5.33	197.32	8	54	0.5694
P48187#	CP43	51.90	6.54	247.10	6	27	1.1467
P05641#	CP47	56.11	6.06	490.69	8	29	1.4487
Q4178@	LHCP	27.91	5.29	475.78	5	61	0.4833
P12329#	Lhcb1(LHCP)	27.82	5.14	544.20	6	55	1.4524
Q6WF*	PsbS1	27.72	9.07	308.61	5	39	0.8010
Q41048*	OEE3	23.13	9.77	528.29	7	52	1.3078
Q41806#	OEE3	22.84	9.30	382.95	7	62	1.2899
A5JVZ6@	high chlorophyll fluorescence(hcf)	43.09	8.71	385.58	15	60	0.3887
2.2 Photosystem I							
CS485501@	PSI type III chlorophyll a/b-binding protein	30.56	9.01	635.00	8	54	0.9134
O65101*	PSI-H	14.93	10.10	142.43	3	51	0.5412
2.3 Photosynthetic Electron Transfer							
P46617#	Apocytochrome f	35.51	9.07	426.76	8	52	1.0198
P05642*	Cytochrome B6	26.18	9.06	71.92	3	29	0.1123
Q9SLP5*	Ferredoxin--NADP reductase	40.86	7.55	520.14	9	54	1.1924
P27787	Ferredoxin1	15.84	4.62	84.31	7	27	0.3944
Q41864*	Thioredoxin M	18.07	8.69	358.66	7	37	0.4613
P25709#	NADH dehydrogenase subunit 7	45.69	5.62	281.79	5	31	0.1603
P46722#	NAH subunit I	21.16	6.42	140.38	5	17	0.1442
P06670*	NAD(P)H-quinone oxidoreductase subunit K	27.92	8.98	94.54	3	44	0.0942
2.4 ATP Synthase							
P05022#	ATP synthase F1 sector alpha subunit	55.71	5.87	1862.17	15	46	7.9738
P0C1M0	ATP synthase gamma subunit	39.79	8.44	244.72	12	56	0.4476
P00827#	ATP synthase CF1 beta subunit	54.04	5.31	2737.21	25	79	10.3728
3 Carbon Dioxide Fixation							
3.1 Calvin Cyle							
Q7SIC9	Transketolase	72.99	5.47	1057.01	15	49	2.1969
3.2 C4 Cycle							
Q41729*	Carbonic anhydrase	71.34	8.93	485.52	19	46	2.1401
4 Miscellaneous							
Q5XTZ*	Isopentenyl-diphosphate delta isomerase 2	38.56	5.7	245.45	6	30	0.4186
P29036*	Ferritin	31.45	6.13	171.87	3	27	0.6504
P29036	Ferritin 1	28.02	5.56	164.93	4	48	0.4363

@ predicted proteins, * proteins identified only at transcript level, # proteins inferred from its homology

in the POR-catalyzed photoreduction of protochlorophyllide, and requires the formation of a ternary complex containing NADPH and protochlorophyllide as a prerequisite for stable association with the thylakoid membranes, where they are protected against degradation by endogenous stromal proteases (25).

4.3. Proteins involved in the light-reaction of photosynthesis

Light-dependent reactions, which occur in the thylakoid membrane, are the first stage of photosynthesis. In this process light energy is converted into chemical energy in the form of the energy carriers ATP and NADPH. There are four major protein complexes in the thylakoid membrane involved in this process: Photosystem I (PSI), Photosystem II (PSII), Cytochrome b6f and ATP synthase. We identified 26 proteins belonging to these four complexes, accounting for 76% of the identified proteins, suggesting that the thylakoid membrane plays a major role in light-reaction functions.

PSII is the most intricate of the four complexes in that light energy is captured by the peripheral antenna and is transferred to the core complexes, where it is trapped. PSII core complexes consist of CP47, CP43, D1, and D2. It assembles as a dimer with the minor light-harvesting antenna CP24, CP26, and CP29 for each monomer (26). The seven key proteins mentioned above were all identified in our research. In addition, PsbS, which plays an essential role in the dissipation of excessive energy during photosynthesis, was identified and quantified. However, only two PSI subunits, PSI type III chlorophyll a/b-binding protein and PSI-H (Photosystem I reaction center subunit VI) were identified, suggesting it is possible that other subunits of PSI likewise accumulated at much lower concentrations. We identified that three subunits of the ATP synthase were presented in different amounts. For example, the amount of ATP synthase F1 sector alpha subunit and ATP synthase CF1 beta subunit were 7.9738 ng and 10.3728 ng, respectively. However, ATP synthase gamma subunit was present at only 0.4476 ng, indicating

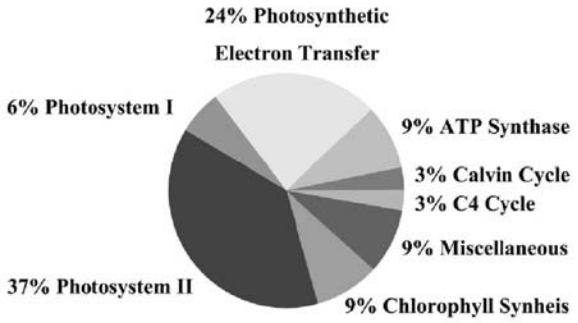

Figure 1. Functional distribution of identified proteins on thylakoid membrane of *Zea mays* L.

that each subunit could play other roles in the chloroplast in addition to assembling into the ATP synthase complex.

4.4. Proteins involved in carbon assimilation and other functions

This study also identified proteins involved in carbon assimilation, including Calvin cycle and C4 cycle. For example, we identified carbonic anhydrase (CA) (Q41729), which catalyzes the first step of C4 photosynthesis, or the reversible hydration reaction that converts CO_2 to HCO_3^-; and transketolase (Q7SIC9), a protein involved in the Calvin cycle that catalyzes the reversible transfer of a two-carbon ketol group from fructose-6-phosphate or sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate to yield xylulose-5-phosphate and erythrose-4-phosphate or ribose-5-phosphate, respectively.

Ferritin is the primary intracellular iron storage protein. It stores iron in a soluble and nontoxic state and releases it into the cell in a controlled fashion. Isopentenyl-diphosphate isomerase plays a regulatory role in terpenoid biosynthesis, which catalyzes the isomerization of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), the latter compound is the precursor of (mero)terpenoid such as certain coumarins, cytokinins, isoflavonoids and anthraquinones. These results indicate that thylakoid membrane proteins exercise other functions apart from photosynthesis.

5. PERSPECTIVE

In the present study, we initiated a proteomic investigation of the maize thylakoid membrane by using a shotgun proteomic approach based on LC-MSE. A total of 34 maize thylakoid membrane proteins were identified, the majority of which are primarily involved in photosynthesis, including the light-reaction and carbon assimilation. These findings will be important cornstones for further study the functions of these proteins and mechanisms of maize leaves.

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