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Identification of membrane proteins in maize leaves, altered in expression under drought stress through polyethylene glycol treatment

F. J. Tai [1], Z. L. Yuan [2], X. L. Wu, P. F. Zhao, X. L. Hu, W. Wang *

College of Life Science, Key Laboratory of Physiological Ecology and Genetic Improvement of Food Crops in Henan Province, Henan Agricultural University, Zhengzhou, China

[1&2]These authors contributed equally to this work.

***Correspondence: wangwei@henau.edu.cn**

Abstract

Membrane proteins are involved in many functions due to their cellular locations. Although the effect of drought on plants has been extensively studied, little is known about the changes of membrane proteome in plants under drought conditions. We used gel-based proteomics to study the effect of drought on membrane protein expression in maize (*Zea mays* L.) plants. Moderate drought stress was applied by 16% (w/v) polyethylene glycol (PEG) treatment of maize seedlings for 8 h. Membrane proteins were extracted from membrane fraction of leaf tissues and separated by two-dimensional electrophoresis followed by matrix-assisted laser-desorption ionization time of flight mass spectrometry. Seventeen protein spots were up-regulated and 18 were down-regulated in response to PEG stress. Hydropathicity (gravy) analysis indicated that 8 spots were hydrophobic and 26 spots were hydrophilic. The identification of proteins indicated that these PEG responsive proteins played diverse functions. Furthermore, the prediction of subcellular localization suggested that 25 spots located in chloroplast, 2 in cytoplasm, while others had other location. Therefore, PEG-responsive membrane proteins are mainly from chloroplasts, which are very sensitive in response to drought stress. Besides, the suitability of the present extraction method for membrane proteins was discussed in the text.

Key Words: drought stress; maize leaves; *Zea mays*; membrane proteins; proteomic analysis.
Abbreviations: 2DE - two-dimensional electrophoresis; CBB-Coomassie brilliant blue; IEF - isoelectric focusing; IPG - immobilized pH gradient; MALDI-TOF - matrix-assisted laser desorption/ionization-time of flight; MS - mass spectrometry; PEG - polyethylene glycol; pI - isoelectric point; TCA - trichloroacetic acid.

Introduction

Drought is a major environmental stress factor that affects the growth and development of plants and causes yields loss in cultivated crops worldwide (Waraich et al., 2011). The physiological and molecular basis for plant responses to drought has been the subject of intensive researches (Bartels and Sunkar 2005). To counter the effects of drought, plants undergo a process of stress acclimation. This process may require changes in gene (e.g. Gorantla et al. 2007, Guo et al. 2009, Harb et al. 2010) and protein expression profiles (e.g. Plomion et al. 2006, Bhushan et al. 2007, Aranjuelo et al. 2010). Membrane proteins in plants play a key role in various important cellular mechanisms, such as metabolite, ion transport and responses to biotic and abiotic stresses, etc, according to their location at the interface between cells or between cell compartments (Marmagne et al. 2004). Therefore, the analysis of membrane proteins is highly relevant to our understanding various phenomenon of life, including the mechanisms of stress tolerance. However, despite decades of extensive research, the large-scale analysis of membrane proteins remains a difficult task. This is due to the fact that membrane proteins require a carefully balanced hydrophilic and lipophilic environment, while most protein chemistry methods work mainly, if not only, in water-based media (Rabilloud et al., 2009). Although these limitations, a set of data is now available for membrane proteins using other tools, such as SDS-PAGE, 2D blue native/SDS-PAGE and chromatography etc. (Bell et al., 2001; Zhang et al., 2006; Klepsch et al., 2008). However, SDS-PAGE and 2D blue native/SDS-PAGE can only analyze a limited number of proteins compared with classical 2-DE. Conventional 2-DE remains a method of choice for analysis membrane proteins, at least, those more hydrophilic peripheral membrane proteins. Maize (*Zea mays*) is one of the most important food crops in the world. During the growing season, maize plants often encounter drought and high temperature stresses. In recently years, the physiological and molecular basis for plant responses to dehydration tolerance has been the subject of intense research (Flexas et al., 2002; 2009; Bhushan et al., 2007). At present, we focused to study the response to drought stress of maize (Hu et al., 2010; 2011). Although the effect of drought stress on protein expression in plants has been extensively studied, little is known about the changes of membrane proteome in plants under drought conditions. In plants, membrane proteomics was focused on plasma, chloroplast, mitochondria, etc. in Arabidopsis and rice (Komatsu 2008). The objectives of this study were to determine membrane protein changes of maize plants under drought stress and to provide new insights into maize drought response and resistance. Therefore, we carried out a differential proteome analysis to investigate the alterations of membrane protein expression in maize leaves in response to drought.

Results

In the present study, membrane proteins were extracted from crude membrane fraction of PEG-treated and control maize leaves. After pulverization in liquid nitrogen and homogenization, membrane systems from leaf cells will form micelles in the aqueous environment. Soluble proteins in crude extract were removed by repeated low speed centrifugation and membrane fraction was precipitated at high speed centrifugation. After delipidation with TCA/acetone wash, membrane proteins were extracted from the membrane fraction using the 2DE rehydration solution. The milder nonionic detergents, thiourea and urea allow the solubilization of membrane proteins and membrane protein complexes. 2-DE combined with MALDI-TOF analysis was used as a profiling technique to detect differentially expressed membrane proteins under drought stress. There existed apparent differences in the protein profiles between the PEG-treated and control leaves (Fig. 1). Approximately 170 (±5) distinct protein spots were detected by digital image analysis, with the most noticeable differences being in the range of 14-75 kDa and isoelectric points (pI) 5-7. PDQuest analysis revealed that more than 1/4 spots with significantly different expression levels (t-test, $P<0.05$). 17 protein spots (especially spots 5-7, 11, 18, 20-22, 30) were up-regulated and 18 spots (especially spots 2, 3, 4, 8, 9, 24, 31-35) down-regulated, with their abundance changes higher than 2 times. Spots 34 and 35 were present in abundance in the control leaves, but undetected in PEG-treated leaves. Therefore, PEG-induced alterations of membrane proteins in maize leaves included both quantitative (increased or decreased) and qualitative (newly induced) changes in protein spots. The 35 differentially-expressed protein spots were identified by MALTI-TOF analysis and database search, and 31 spots were matched to known maize proteins, while 3 spots were not matched, and 1 spot (spot 20) was no signal because of some errors (Table 1). Each identified protein was listed by its accession number, molecular weight (kDa) and pI, GRAVY value, expression level, cellular compartment and possible function. Moreover, 5 proteins were identified in more than one spot, e.g. ATP synthase α subunit was identified from three isoforms (spots 32-34), β-D-glucosidase precursor existed in 4 isoforms (spots 27-29, 35). Isoforms are generally considered to diversify the function of a protein (Lockhart and Winzeler 2000, Wang et al. 2004). The presence of isoforms may result from sequence-related proteins encoded by distinct genes and/or polypeptide variants encoded by the same gene (splice variants and/or post-translational modifications). Many proteins identified in the present study, such as ATP synthase, OEE, GAPDH and etc, were found to be involved in diverse stress-responsive processes. Furthermore, our results reveal a wider scope of PEG (drought) responsive proteins, such as hageman factor inhibitor, digalactosyldiacylglycerol synthase 1 and other unknown proteins. Twelve proteins (spots 2-5, 7, 12, 13, 15, 21, 24, 25, 31) have signal peptides, which guide proteins in cells of transport and involved in cellular localization. Twenty-five proteins identified were located at chloroplast (Table 1), indicating that chloroplasts are one of the organelles mostly influenced by drought stress. The PEG responsive proteins identified herein were involved in diverse biological processes (table 2), covering photosynthesis (spots 6, 11, 13, 15, 16, 23, 30-34), carbohydrate metabolism (spots 9, 10, 16, 18, 21, 24-29, 35), protein biosynthesis, hydrolysis and modification (spots 1-5, 17), lipid synthesis (spot 22), cytochrome P450 like protein (19), harpin binding protein (spot 7). Three proteins with unknown functions (spots 8, 12, 14) were also identified as drought response proteins. Our results demonstrated that membrane proteins with minor hydrophobicity could be efficiently identified by 2DE. For example, 8 spots were hydrophobic proteins because of their positive GRAVY index values and 26 spots were hydrophilic proteins (Table 1). All of them are peripheral proteins containing no membrane-spanning domains. For example, ATP synthase α, β, γ subunit (spots 23, 30-34) and leaf ferredoxin-NADP reductase (spots 13 and 15) belong to peripheral proteins and locate at chloroplast thylakoid membranes. Digalactosyldiacylglycerol synthase 1 (spot 22) locates at chloroplast outer membrane. Possibly, β-D-glucosidase precursor (spots 24-29, 35) were cross-contaminative soluble proteins.

Discussion

Membrane proteins extration and separation

Because of their high hydrophobicity，the analysis and separation of membrane proteins is more difficult than soluble proteins. The degree of association of proteins with a cellular membrane varies. Some proteins, such as integral membrane proteins, comprise one or several membrane-spanning regions. Others are more peripheral and associated by reversible interactions with either lipids or other membrane proteins, which do not contain membrane-spanning domains (Marmagne et al., 2004). Thus, peripheral membrane proteins are obviously more hydrophilic than integral membrane proteins. Conventional 2-DE is suitable for soluble proteins but not efficient in membrane proteins separation. At present, there is no a very powerful method for hydrophobic integral membrane proteins. Therefore, we attempted to separate membrane proteins using conventional 2-DE. In the present study, leaf membrane proteins were extracted from membrane fraction, and 34 differentially expressed proteins in maize leaves exposed to PEG stress were identified by 2DE-based proteomics, in which 8 proteins have positive gravy index and high hydrophobicity. However, all the identified proteins were found not containing membrane-spanning domains, perhaps due to the inherent limits of 2DE in resolving membrane proteins. At least, a large number of weakly hydrophobic membrane-associated proteins were extracted and separated by the present method. In addition, these identified membrane proteins are responsive proteins under PEG stress. Therefore, alternative strategies based on two-dimensional blue native/SDS-PAGE (Zhang et al., 2006) must be used in order to separate those integral membrane proteins.

The changes of membrane proteins of maize leaves under PEG stress

Our results here showed that most differentially expressed proteins have a chloroplast location. Six α, β and γ chains of ATP synthase CF1 (spots 23, 30-34) and 4 up-regulated proteins served as probable components of electron transport chain, *i.e.*, oxygen-evolving enhancer protein 1 (spot 11), chlorophyll a/b binding protein 2 precursor (spot 6) and ferredoxin--NADP reductase (spots13, 15) were found. All of them locate at thylakoid membrane and participate in photosynthetic light reaction. The abundance regulated of

Table 1. The differentially expressed membrane-associated proteins in maize leaves under PEG stress.

Spots & Protein name	Accession	Mr	pI	GRAVY	Subcellular location	Gene Ontology
Decreased abundance under PEG stress (18 spots)						
2 Hageman factor inhibitor	gi: 75994175	16.16	7.49	0.162	Extracellular region	Defense-related or PR proteins, serine protease inhibitor
3 Hageman factor inhibitor	gi: 75994175	16.16	7.49	0.167	Extracellular region	Defense-related or PR proteins, serine protease inhibitor
4 50S ribosomal protein L28	gi: 26493229	15.22	11.58	-0.238	Chloroplast	Structural constituent of ribosome
9 Triosephosphate isomerase 1	gi: 168647	27.02	5.52	0.042	Plastid & Cytoplasm	Carbohydrate biosynthesis & degradation, isomerase
24 Granule-bound starch synthase 1	P04713	65.97	6.59	-0.110	Chloroplast	Starch biosynthesis, glucosyltransferase activity
25 Granule-bound starch synthase 1	P04713	65.97	6.59	-0.110	Chloroplast	Starch biosynthesis, glucosyltransferase activity
26 Granule-bound starch synthase 1	P04713	65.97	6.59	-0.110	Chloroplast	Starch biosynthesis, glucosyltransferase activity
27 β-D-glucosidase precursor	gi: 1206013	64.11	6.72	-0.514	Chloroplast	Carbohydrate metabolism, hydrolyzing O-glycosyl compounds
28 β-D-glucosidase precursor	gi: 1206013	64.11	6.72	-0.514	Chloroplast	Carbohydrate metabolism, hydrolyzing O-glycosyl compounds
35 β-D-glucosidase precursor	gi: 1206013	64.11	6.72	-0.514	Chloroplast	Carbohydrate metabolism, hydrolyzing O-glycosyl compounds
23 ATP synthase CF1 β subunit	gi: 11467199	54.04	5.31	-0.078	Chloroplast thylakoid membrane	ATP synthesis, ATP binding
31 ATP synthase γ chain precursor	gi: 26533016	39.79	8.44	-0.143	Chloroplast thylakoid membrane	ATP synthesis, proton-transporting
32 ATP synthase CF1 α subunit	gi: 11467189	55.71	5.87	-0.096	Chloroplast thylakoid membrane	ATP synthesis, proton-transporting
33 ATP synthase CF1 α subunit	gi: 11467189	55.71	5.87	-0.096	Chloroplast thylakoid membrane	ATP synthesis, proton-transporting
34 ATP synthase CF1 α subunit	gi: 11467189	55.71	5.87	-0.096	Chloroplast thylakoid membrane	ATP synthesis, proton-transporting
1 Translation initiation factor 5A-1/2	B4FDE1	13.14	6.58	-0.330	Intracellular	Protein biosynthesis, initiation factor, ribosome binding
8 Expressed protein	C0PNV1	38.24	8.45	-0.211	Intracellular	Zinc ion binding
14 Expressed protein	gi: 26507242	38.39	6.30	-0.620	Intracellular	unknown
Increased abundance under PEG stress (16 spots)						
5 Nucleic acid-binding protein	gi: 168526	33.12	4.60	-0.342	(Nuclear-encoded) Chloroplast	Ribonucleoprotein complex, nucleic acid binding
6 Chlorophyll a-b binding protein	gi: 226502080	27.81	5.45	0.038	Chloroplast thylakoid membrane	Photosynthesis, light-harvesting complex (LHC)
7 Harpin binding protein 1	gi: 38679339	29.26	9.57	-0.23	Chloroplast, plastid	Biotic & abiotic stress responses (probable)
10 β-glucosidase aggregating factor	gi: 162461751	31.78	6.08	0.159	Membrane	Jasmonic acid-induced defense-related protein
11 Oxygen-evolving enhancer protein 1	gi: 195619530	34.52	5.59	-0.221	Extrinsic to thylakoid membrane	Photosystem II stabilization, Ca^{2+} binding
13 Leaf ferredoxin--NADP reductase	gi: 226497434	40.66	8.53	-0.386	Chloroplast thylakoid membrane	Oxidation reduction, NADP or NADPH binding
15 Leaf ferredoxin--NADP reductase	gi: 226497434	40.66	8.53	-0.386	Chloroplast thylakoid membrane	Oxidation reduction, NADP or NADPH binding
16 RuBisCO activase	gi: 162458161	47.94	6.29	-0.401	Chloroplast stroma	Activation of RuBisCO, ATP binding
18 Glyceraldehyde-3-phosphate dehydrogenase	B4F8L7	47.18	5.95	0.054	Chloroplast	Glucose metabolic process, oxidoreductase
21 Phosphoglycerate kinase	gi: 226530482	49.87	6.07	0.197	Chloroplast stroma	Calvin cycle, phosphoglycerate kinase activity
22 Digalactosyldiacylglycerol synthase 1	gi: 226495503	89.12	7.03	-0.516	Chloroplast outer membrane	Biosynthetic process, glycosyltransferase
29 β-D-glucosidase precursor	gi: 1206013	64.11	6.72	-0.514	Chloroplast	Carbohydrate metabolism, β-glucosidase activity
30 ATP synthase CF1 β subunit	gi: 11467199	54.04	5.31	-0.078	Chloroplast thylakoid membrane	ATP synthesis, ATP binding
12 Expressed protein	gi: 257663413	55.87	6.39	-0.239	Chloroplast	Lipid metabolic process, triglyceride lipase activity
17 Methylthioribose-1-phosphate isomerase	B6TZD1	17.90	5.20	0.135	Cytoplasm, nucleus	Amino acid biosynthesis, isomerase
19 Cytochrome P450 like protein	C4J5P6	57.61	6.69	-0.220	Membrane	Oxidation reduction, oxidoreductase
20						

All proteins listed were from *Zea mays*. Accession number with 'gi' was found in NCBI database, others in Swiss-prot database. Spot 20 (up-regulated) failed to be identified. GRAVY is in the range of 2 to -2. Positive GRAVY indicates hydropathicity, and negative GRAVITY indicates hydrophilicity. Theoretical Mr and pI of identified proteins were predicted by sequence entry at http://www.expasy.ch/tools/pi_tool.html. Cellular location of proteins was predicted by TargetP (http:// www.cbs.dtu.dk/services/TargetP).

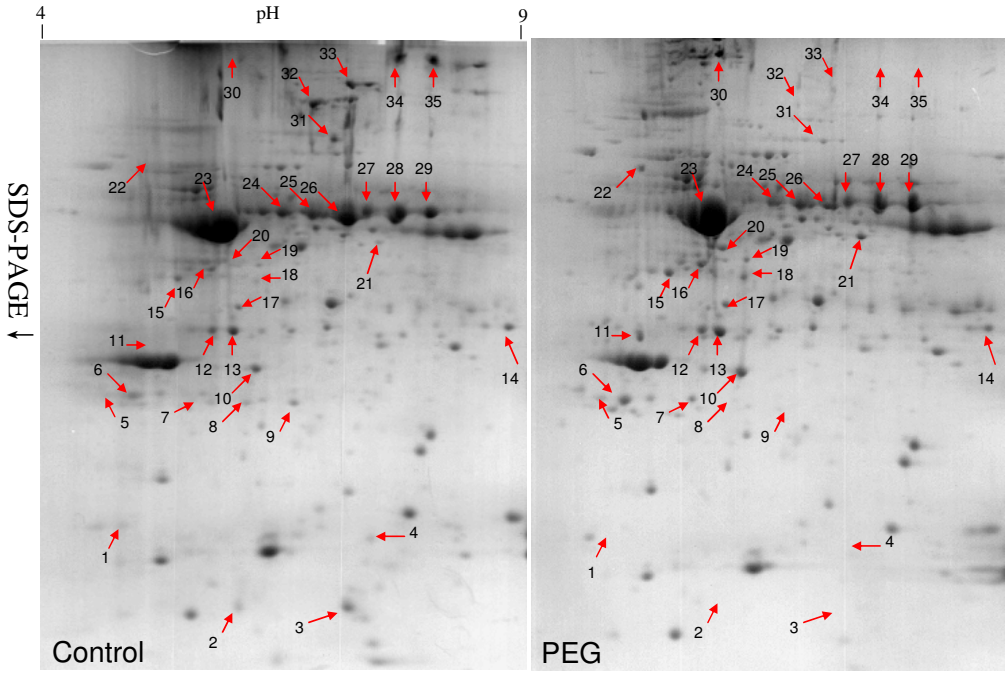

Fig. 1. Comparison of membrane protein profiles between PEG-treated and control maize leaves.

them maybe affect the content of ATP and NADPH, which enter the chloroplast stroma and participate in Calvin cycle. Rubisco, phosphoglycerate kinase (PGK, spot 21), and triosephosphate isomerase (TPI, spot 9) are major enzymes involved in the Calvin cycle. The Rubisco activase precursor (RAC) content up-regulated under drought stress conditions revealed that the higher activation state could also have actively affected the Rubisco activity (Reddy et al., 2004). So the regulation of RAC and PGK may contribute to carbohydrates formation and PGK may be implicated with stress-tolerance in plants (Carpentier et al., 2007). In addition, GAPDH can catalyze the formation of 3-phosphoglycerate, which is glucose and pinitol precursor. Glucose and pinitol are major carbohydrates acting as osmolytes under drought stress conditions (Reddy et al., 2004). Thus, the up-regulation of GAPDH implies more osmolytes synthesis in response to drought stress. However, TPI, the metabolic turning point and participation in many other reactions, is controlled by multiple mechanisms. Drought stress may constantly destroy the cell membrane system and induce the reactive oxygen species (ROS) generation (Chaves and Oliveira 2004; Moreno et al., 2008). The obviously increased abundance of digalactosyldiacylglycerol synthase 1 (DGD1, spot 22) may play a key role in defense the damage of the photosynthetic membranes (Froehlich et al., 2001). In order to protect PSII against increased production of ROS, water-stressed plants improved the mechanism of excess electron removal by up-regulation of well-known proteins involved in detoxification of ROS, such as, glyceraldehyde 3-phosphate dehydrogenase (GAPDH, spot 18), which is involved in ROS scavenging and also known involved in stress tolerance (Hancock et al., 2005). Other redox proteins, such as ferredoxin-NADP reductase (spots 13, 15), involved in the ROS pathway are also slightly increased. In addition, 6 proteins which involved in protein biosynthesis and hydrolysis were found. The abundance of 50S ribosomal protein L28 (spot 4) and translation initiation factor 5A-1/2 (spot 1) decreased remarkably after PEG treatment, suggesting that short-term drought stress represses protein synthesis in vivo (Table 1). Spots 2 and 3 are associated with serine-type endopeptidase inhibitor activity. The sharp down-regulated expression may promote the efficiency of some proteins hydrolysis. These results demonstrated that plant may be through suppression protein biosynthesis and promotion protein hydrolysis in response to drought stress. Nucleic acid-binding protein (spot 5), involved in post-transcriptional gene expression processes, also known involved in various stress tolerance (Kim et al., 2007).

The higher level nucleic acid-binding protein may contribute to regulate gene expressions to response stress. More strategies regulate proteins in plants such as post-translational modifications, which can change the Mr and/or pI of proteins. Alternatively, proteins that were present in multiple spots could result from being translated from alternatively spliced mRNAs. In the study, the observed Mr values of spot 35 and pI values of spots 27, 28 and 29 were differential from the theoretical Mr/pI values, suggesting that possible protein isoforms for which posttranslational modifications may have occurred or they may be isoforms that arise from a same family. Moreover, spots 27, 28 and 35 decreased in abundance under dehydration stress, while spot 29 increased in abundance. The result also highl ights the

Table 2. Functional distribution of protein spots responsive to drought stress

Category	Up-regulation	Down-regulation
Photosynthesis	6	5
Carbohydrate metabolism	5	7
Protein metabolism	2	4
Lipid biosynthesis	1	0
Harpin binding protein	1	0
Cytochrome P450 like protein	1	0
Unknown/ Unclear classification	1	2

Functional categorization of identified proteins was performed using annotation in NCBI database and Swiss-prot database.

spatial complexity of the mechanism of water tolerance and may also suggest differences in function between isoforms. Previous studies showed that β-glucosidases in maize specifically interact with β-glucosidase aggregating factor (BGAF) and also have been proved to play a key role in defense responses (Ogasawara et al., 2009). The higher BGAF precursor (spot 10) level may contribute to defense drought stress. Granule-bound starch synthase (GBSS) is an important enzyme in the synthesis of amylose. In this study, three GBSS1 (spots 24-26) were all decreased under PEG condition, suggesting that the amylose synthesis may be suppressed by PEG stress. β-D-glucosidases can hydrolyze the glucoside and release monolignols for lignin synthesis. As for β-D-glucosidase precursors (spots 27-29, 35), their roles was also thought to be relevant to lignification of cell wall in plant stress response. However, the specific roles of these enzymes in response to the progressively drought stress remain to be established. In addition, three proteins with unknown function (spots 8, 12, 14) were also identified as drought-response proteins in this study. The functions of these novel proteins remain to be investigated. Our result of prediction (Table 1)shows that as much as 73.5% of the expressions changed proteins are located in the chloroplasts. Chloroplast is a sensitive organelle response to drought in plant cell. The limitation of plant growth imposed by drought environment is mainly due to reductions of photosynthetic efficiency, which is dependent on chloroplast (Flexas et al., 2002; 2009). Our results also implies that chloroplasts are one of the mostly influenced organelles inside cells by drought stress and chloroplast membrane proteome is virtually subjective to drought stress. To conclude, the proteomic investigation of maize leaf membrane proteins reveals a complex cellular network affected by PEG stress. PEG responsive proteins are involved in diverse biological processes and located in various cellular compartments.

Mterials and methods

Plant materials

Maize (*Zea mays*) cv. Zhengdan 958, one of the widely grown high-yield maize hybrids in China, was used as the experimental material. Seeds of uniform size were surfaced-sterilized with 0.1% sodium hypochlorite and rinsed with distilled water. The sterilized seeds were soaked in distilled water in darkness (28 ºC, 24 h). Maize seedlings were grown in Hogland's nutrient solution at 400 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation, a 14/10 h day/night cycle, a temperature of 28/22 ºC (day/night) and a relative humidity 75% in a chamber. When the second leaves were fully expanded, seedlings were subjected to 16% PEG 6000 in Hoagland's solution for 8 h. Then, the second leaves from PEG-treated and control plants were collected for protein extraction.

Membrane proteins extraction

Fractionation of cell homogenate was used to isolate membrane proteins of maize leaves. Leaves (ca. 6g) were pulverized in liquid nitrogen. Twenty ml of the extraction buffer (50 mM Tris-HCl, pH 8.8, 5 mM DTT, 5 mM KCl and 1 mM PMSF) were added to the powder and homogenized thoroughly for 10 min. Then, the crude extract was filtrated with coarse gauze and subjected to differential centrifugation: first at 3000 g for 10 min twice and then at 15,000 g for 10 min (4º C). The resultant pellet was designed as membrane fraction which was highly enriched with thylakoids, as its green color indicated. The membrane fraction was washed with 10% TCA/acetone twice and cold acetone twice to removal pigments and other non-protein compounds. The air-dried pellet was extracted using the rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, and 1% IPG buffer) for 30 min at room temperature. After centrifugation at 15000 g for 20 min, protein extract were used for Bradford assays and 2DE analysis.

2-D electrophoresis

Isoelectric focusing (IEF) was performed with the Ettan III system (GE Healthcare, USA). Proteins (400 μg in 250 μl rehydration buffer) were applied into an immobilized pH gradient (IPG) strip (11 cm, pH 3-10 linear) by overnight rehydration. IEF was achieved at 50 V for 12 h, 1,000 V for 1 h, and 6,000 V for 7.5 h (20℃). Focused IEF strips were resolved in 12.5% SDS-polyacrylamide gels. Following SDS-PAGE, gels were stained with 0.1% Coomassie blue R 350, destained in 10% acetic acid until a clear background. Scanning was carried out at 8-bit pixel depth using an image scanner and the protein spots were analyzed with PDQUEST software (Bio-Rad). The changes in abundance of proteins were 2 times and high were subjected to protein identification.

Protein identification by Mass spectrometry

Differentially expressed protein spots were manually excised from 2DE gels, and reduced with 10 mM DTT, and alkylated with 50 mM iodoacetamide. Enzymatic digestion was performed with trypsin (10 μg/μl) in 50 mM ammonium bicarbonate for 16 h at 37 ℃. The supernatants were vacuum-dried and dissolved in 10 μl 0.1 % trifluoroacetic acid and

0.5µl added onto a matrix consisting of 0.5 µl of 5 mg/ml 2, 5-dihydroxybenzoic acid in water: acetonitrile (2:1). The digested fragments were analyzed on Ettan MALDI-TOF Pro mass spectrometer (GE Healthcare, USA), as described previously (Wu et al. 2011). Identification was performed using protein sequences databases downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and maize EST databases from Plant GDB (http://www.plantgdb.org). Gravy value was obtained at the Protparam sever (http://us.expasy.org/tools/protparam.html). Cellular locations of identified proteins were predicted by TargetP (http://www.cbs.dtu.dk/services/TargetP) and PSORT (http://psort.hgc.jp/form.html).

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