Glycoproteomic Analysis of Chloroplasts Isolated from Rice Mature Leaves

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Summary

We performed a glycoproteomic analysis of rice chloroplast. Intact chloroplasts were isolated from mature leaves of rice using discontinuous Percoll density gradient centrifugation. The isolated chloroplast proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by lectinoblotting with peroxidase-conjugated Concanavalin A. Glycoproteins detected with Concanavalin A-peroxidase were analyzed by a mass spectrometry, indicating that phosphoglycerate kinase-like protein which has signal peptide and N-linked oligosaccharide chains occurs in chloroplasts of rice.

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Key words : chloroplast, glycoproteomics, lectin staining, *Oryza sativa* L., phosphoglycerate kinase

The plastid is a cell organelle which is peculiar for the plant, and chloroplast with the photosynthetic capacity and the amyloplast that specialized for the starch integration are included for this. These organelles presumably evolved from photosynthetic bacteria and have original DNA. However, over 90% of the gene which encodes the chloroplast protein exists in the cell nucleus genome. Chloroplast protein gene in the nucleus genome is translated in free ribosome which exists for the cytoplasm, and the precursor with the transit peptide (the plastid localization signal) is formed. This precursor protein is transported to the interior of plastid through the chloroplastic envelope via the membrane permeation machinery, so-called Toc (translocon at the outer envelope of chloroplast)-Tic (translocon at the inner envelop of chloroplast) (Schnell and Hebert, 2003; Soll and Schleiff, 2004; Kessler and Schnell, 2006). From recent proteomic analysis, however, it is suggested that protein without the typical transit peptide exists in the plastids. Furthermore, it is being calculated that the precursor form of protein with the signal sequence for translocating the membrane of endoplasmic reticulum occupies about 8% of all proteins in the isolated chloroplast (Jarvis, 2008). It was discovered that rice α -amylase isoform AmyI-1 known as a secretion enzyme with the typical N- linked sugar chain (Hayashi et al., 1990; Terashima et al., 1994) was concerned in the amylolysis in living cells such as green leaf, and this secretory glycoprotein was transported and localized into the amyloplast and the chloroplast from the secretory pathway (Asatsuma et al., 2005). More recently, convincing evidence for the traffic route from ER-Golgi system to plastid was presented (Villarejo et al., 2005; Nanjo et al. 2006). Both *Arabidopsis* carbonic anhydrase 1 (CAH1) and rice nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) are glycoproteins conjugated with N-linked oligosaccharide chain. These glycoproteins were actually transported and localized to the chloroplasts, but the chloroplast targeting was effectively prevented by a drug Brefeldin A that inhibits the ER-to-Golgi traffic. This indicates that the ER-to-Golgi traffic is essential to the chloroplast targeting of glycoproteins in both monocot and dicot plants. In this study, glycoproteomic analysis of rice chloroplast was attempted to identify the other plastid glycoprotein in rice leaves.

MATERIALS AND METHODS

Plant material

Rice seeds (Oryza sativa L. Nipponbare) were sterilized in a 1% NaOCl solution for 15 min, rinsed several times in sterile water. The seeds were geminated in dark for 7 days at 28℃ on a paper filter which was made to moisten in the sterile water, and further grown on molding over 1 month under a light condition (16h, light; 8h, dark). The harvested mature leaves were used for the chloroplast extraction.

Isolation of intact chloroplasts, micorosome and Golgi membranes

Isolation of intact chloroplasts was carried out according to the procedure described by Tanaka et al. (2004). Rice mature leaves were crushed thoroughly by chopping with a razor blade in two volume of chloroplast extraction (CE) buffer consisting of 50 mM HEPES-KOH (pH 7.5), 330 mM

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Sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA. The extract was filtrated through Miracloth to remove cell debris, and 6 ml of the filtrate was layered on a discontinuous Percoll density gradient solution (upper layer, 3 ml of 40%; bottom layer, 1 ml of 80%) containing CE buffer, and then centrifuged at 8,000 x *g* for 10 min at 4℃. The interface fraction between 40 and 80% Percoll was collected and diluted with 5 volumes of CE buffer, and re-centrifuged at 4,000 x *g* for 10 min at 4℃. The membrane pellet was suspended with 5 μl of 20 mM HEPES-KOH (pH 7.5), 2.5 mM EDTA, 5 mM $MgCl_2$ and 0.2% (w/v) polyvinylpyrrolidone by sonic, and used as intact chloroplast preparation. Preparations of micorosome and Golgi membranes were carried out as described previously (Asakura et al., 2006).

Assays

Assay of alkaline pyrophosphatase was essentially identical to the methods described previously (Nanjo et al., 2006). The enzyme reaction was carried out at 37 ℃ for 10 min in a substrate solution consisting of 50mM Tris-HCl pH8.0, 5mM MgCl2, 1.5mM Na4P2O7. The liberated phosphate was determined by Phosphor C-Test (Wako). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of product per min. Protein contents were measured by Bio-Rad protein assay dye reagent using bovine plasma albumin as a standard.

SDS-PAGE and lectinoblotting

An aliquot of the solubilized membrane proteins was applied to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with 12% separation gel according to Laemmli (1970) and stained with Coommasie brilliant blue (CBB) G-250. The molecular weight marker (Sigma) contained Albumin, bovine (66,000Da), Albumin, egg (45,000Da), Glyceraldehyde-3-phosphate Dehydrogenase, rabbit muscle (36,000Da), Carbonic Anhydrase, bovine (29,000Da), Trypsinogen, bovine pancreas (24,000Da), Trypsin Inhibitor, soybean (20,100Da).

Protein bands excised from CBB-stained gels were re-applied to the second SDS-PAGE. After SDS-PAGE, proteins in the gels were transferred to a nitrocellulose sheet (Advantec, A045A224D) in 25 mM Tris-192 mM glycine- 20 % methanol at 1 mA/cm^2 for 2 h using an electroblotter (Atto, AE-6677). The blotted sheet was washed 3 times with TBST (20mM Tris-HCl (pH7.4), NaCl 150mM, 0.05% Tween20) for 10 min. Lectin staining was performed with peroxidase-conjugated Concanavalin A (Con A) (Mitsui et al., 1990).

MALDI-TOF/TOF MS

Protein bands were excised from the CBB-stained gels, incubated with 100 μl of destaining solution consisting of 25 mM NH_4HCO_3 and 50% acetonitrile at 30 ℃ for 30 min, and further shaken vigorously for 15 min at room temperature. After destained well, the gels were dehydrated with 100% acetonitrile, then dried in Speed-Vac. The dried gels were swollen and incubated in 100 μl of 10 mM dithiothreitol, 25 mM NH_4HCO_3 at 56 ℃ for 45 min. The swollen gels were washed with destaining solution 3 times, and incubated with 100 μl of 1% (w/v) iodoacetamide and 25 mM NH_4HCO_3 at room temperature for 30 min in the dark. The alkilated gels were rinsed with destaining solution, dehydrated with 100% acetonitrile, and dried again in Speed-Vac. The gels were re-swollen and incubated with 20 μl of 100 ng/μl trypsin (Promega) and 20 mM NH_4HCO_3 for 1 h on ice, and further incubated with 25 mM NH_4HCO_3 at 37 ℃ overnight. After removing 25 mM NH_4HCO_3, the gels were treated by sonic in 50 μl of extracting solution consisting of 50% acetonitrile and 5% trifluoroacetic acid at 35-45 ℃ for 30 min, and further incubated at 37 ℃ for 30 min. The extraction process was performed twice, and the combined solution was concentrated to approximately 50 μl in Speed-Vac.

The obtained peptides were subjected to matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS, BRUKER DALTOMICS autoflex III) according to the manufacturer's protocol. The matrix solution was prepared by mixing a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 100% acetonitrile (1:1, v/v). The obtained MS and MS/MS peaks were analyzed by Mascot search system (Matrix Science, http://www.matrixscience.com) under the following conditions: Database ; NCBInr, Taxonomy ; Oryza sativa(rice), Enzyme ; Trypsin, Fixed modifications ; Carbamidomethyl(C), Vatiable modifications ; Oxidation(M), Peptide Mass Tol. ; 0.3Da, Monoisotopic or Average ; Monoisotopic, Data format ; Mascot generic, Instrument ; MALDI-TOF-TOF.

BRAST(http://blast.ncbi.nlm.nih.gov/Blast.cgi)
NetN Glyc 1.0 Server(http://www.cbs.dtu.dk/services/NetNGlyc/)
Signal P 3.0 Server(http://www.cbs.dtu.dk/services/SignalP/)
PSORT Prediction(http://psort.ims.u-tokyo.ac.jp/form.html)

RESULTS AND DISCUSSION

When the extract prepared from rice mature leaves were subjected to discontinuous Percoll density gradient centrifugation, a green membranous substance was constantly distributed at the interface between 40 and 80% Percoll in the gradient (Fig. 1A). Chloroplast marker enzymes, alkaline pyrophosphatase and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), were entirely associated with the green membrane fraction (Fig. 1B,C), indicating that the membrane fraction is chloroplast-enriched, seems to be highly purified from the other organelles and cytosol.

The proteins prepared from the crude extract, microsome, Golgi and chloroplast preparations were applied to SDS-PAGE, followed by lectinoblotting with Con A-peroxidase. Con A specifically binds to mannose and glucose residues of asparagine-linked oligosaccharide side chain conjugated to polypeptide. As shown in Figure 2, Con A-recognized glycoproteins were existed in rice chloroplasts, and the separation profile of chloroplastic glycoproteins in

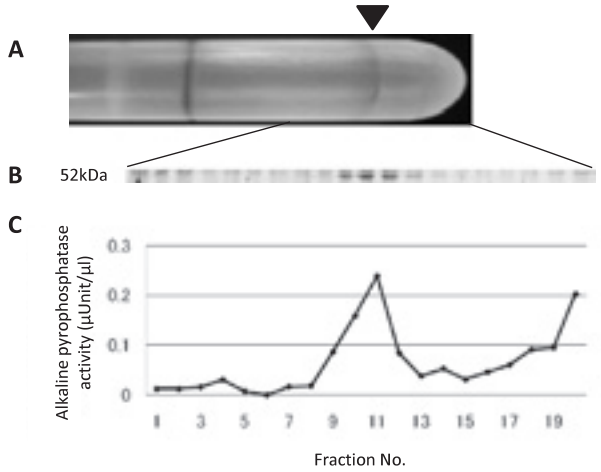

Fig.1 Isolation of intact chloroplast by discontinuous percoll density gradient centrifugation.
(A) Separation pattern of chloroplasts after discontinuous percoll density gradient centrifugation. Arrowhead shows intact chloroplast layer.
(B) Distribution of 52 kDa Rubisco large subunit in the gradient. An aliquot of sample in each fraction was subjected to SDS-PAGE, followed by CBB staining.
(C) Separation profile of alkaline pyrophosphatase activity in the gradient. An aliquot of sample in each fraction was subjected to the enzyme assays.

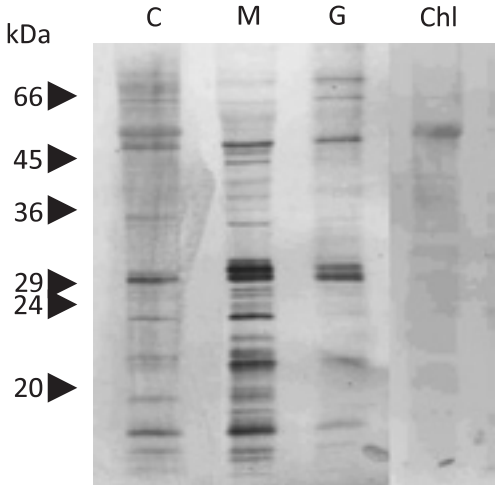

Fig.2 ConA-recognized glycoproteins in different organelles.
C, crude; M, microsome; G, Golgi; Chl, chloroplast.

SDS-gel was apparently distinguishable from those of other organelles.

To further characterize the glycoproteome of chloroplast, chloroplastic Con A-recognized glycoproteins were purified by second SDS-PAGE (Fig. 3) and subjected to MALDI-TOF/TOF MS analysis (Table 1). One of 6 Con A-recognized glycoproteins was identified to be a phosphoglycerate kinase-like protein (Accession no. EAY76155 , EAZ76155). The phosphoglycerate kinase is the enzyme of Calvin cycle which

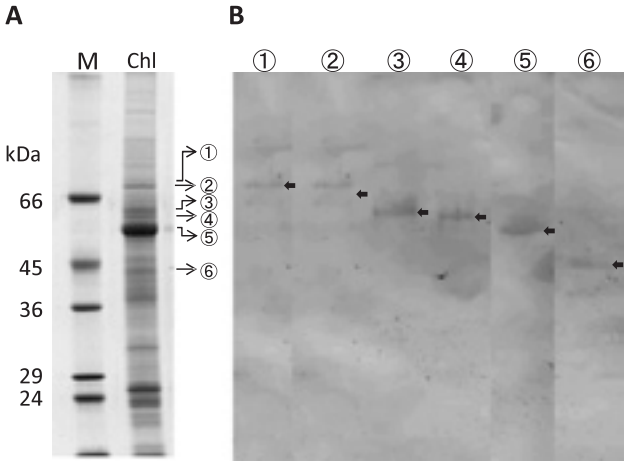

Fig.3 Separation and purification of chloroplast glycoproteins by SDS-PAGE.
(A) Intact chloroplast proteins were separated by 1st SDS-PAGE. The protein bands were visualized by CBB staining. M, molecular weight marker ; Chl, intact chloroplast proteins.
(B) Six chloroplast glycoproteins were purified by 2nd SDS-PAGE. The glycoproteins were detected by lectinoblotting with Con A-peroxidase.

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  1 MARPAARGIVAGAAASTVPLPRAGVASPCPTARSLGFAARGTDPRLAIHV
 51 SSRRRAASASAGSRLARAVATMAKKSVGDLAAADLEGKRVLLRADLNVPL
101 DASQNITDDTRVIAAIPTIKHLIGNGAKVILCSHLGRPKGITPKFSLAPL
151 VPRLSELLGIQVQKADDVIGPEVEKSVSVLPNGSVLLLENVRFYKEEEKN
201 DPEFAKKLASLADLYELDYLVGAVSNPKRPFAAIVGGSKVSSKIGVIESL
251 LEKCDILLLGGGMIFTFYKAQGFPVGASLVEDDKLELATSLLAKAKEKGV
301 SLMLPTDVIVADKFAPEANCQVVSAYAIPDGWMGLDIGPDSZAAFSSALE
351 TTQTVIWNGPMGVFEFEKFAVGTEAIAKKLAELSGKGVTTIIGGKDSVAA
401 VEKVGVANVMSHISTGGGASLELLEGKELPGVVALDEA
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Fig.4 Structural organization of phosphoglycerate kinase like proteins.
The predicted cleavage sites of locations of the N-terminal signal sequence (arrowhead), N-glycosylation sites (underlined).

is the metabolic pathway to fix CO2 in the sugar in the stroma of the chloroplast. It has been reported that the precursor form of some phosphoglycerate kinase has the N-terminal transit peptide (Bertsch et al., 1993). However, the deduced amino acid sequence of phosphoglycerate kinase-like protein contained the signal sequence for translocating the ER membrane and the N-glycosylation sites (Fig. 4), supporting that this protein was synthesized in the ER and gone in the secretory pathway.

The phosphoglycerate kinase-like protein identified in the present study is the fourth example of glycoproteins that are targeted to the plastid through the secretory pathway, next to rice AmyI-1 (Asatsuma et al., 2005) and NPP1 (Nanjo et al., 2006) and *Arabidopsis* CAH1 (Villarejo et al., 2005). The transport of proteins from the secretory pathway to the

Table1. Chloroplast glycoproteins of rice leaves identified by MS/MS.

No.	ACCESSION	DEFINITION	SCORE MS	SCORE MS/MS	Queries matched	Peptide (Observed m/z)	BLAST	NetN Glyc	SignalP	PSORT
①	EAZ33719	hypotherical protein		93	2	K.DIDEVILVGGSTR.I (1373.810) K.AVITVPAYFNDSQR.T (1580.897)	heat shock protein molecular chaperone	Yes	No	chl stroma mit matrix chl thyl mem chl thyl space
②	BAD67886	putative transketolase 1		43	1	K.NPYWFNR.D (996.522)		Yes	No	nucleus microbody
②	EAY99638	hypotherical protein		43	1	K.NPYWFNR.D (996.522)	transketolase	Yes	No	microbody nucleus
③	NP_039380	ATP synthase CF1 alpha subunit		81	3	R.LIESPAPGIISR.R (1252.8385) R.IAQIPVSEAYLGR.V (1416.9145) R.EAYPGDVFYLHSR.L (1552.8656)		No	No	cytoplasm microbody
③	AAM08597	putative ATPase alpha subunit from chromosome 10 chloroplast		81	3	R.LIESPAPGIISR.R (1252.8385) R.IAQIPVSEAYLGR.V (1416.9145) R.EAYPGDVFYLHSR.L (1552.8656)		No	No	cytoplasm microbody
③	AAM12499	ATPase CF1 alpha subunit		81	3	R.LIESPAPGIISR.R (1252.8385) R.IAQIPVSEAYLGR.V (1416.9145) R.EAYPGDVFYLHSR.L (1552.8656)		No	No	cytoplasm microbody
③	AAS46052	ATP synthase CF1 alpha chain; atpA		81	3	R.LIESPAPGIISR.R (1252.8385) R.IAQIPVSEAYLGR.V (1416.9145) R.EAYPGDVFYLHSR.L (1552.8656)		No	No	cytoplasm microbody
④	AAS46052	ATP synthase CF1 alpha chain; atpA	73		7	K.EAIQEQLER.F (1115.6758) R.LIESPAPGIISR.R (1252.8293) R.VINALAKPIDGR.G (1266.8518) MNFYFPLEFR.H (1363.8467) R.IAQIPVSEAYLGR.V (1416.9013) R.EAYPGDVFYLHSR.L (1553.8676) K.QAQAYRQMSLLLR.R (1577.9437)		No	No	cytoplasm microbody
⑤	CAG34174	ribulose biphosphate carboxylase large chain	100		10	K.NHGMHFR.V Oxidation(M) (914.4909) R.ACYECLR.G (971.4929) K.DTDILAAFR.V (1021.609) R.VALEACVQAR.N (1116.6586) R.QKNHGMHFR.V Oxidation (M) (1170.7179) R.DNGLLLHIHR.A (1187.7437) K.NHGMHFRVLAK.A (1309.7631) K.DDENVNSQPFMR.W (1451.7598) K.TFQGPPHGIQVER.D (1465.8462) K.YGRPLLGCTIKPK.L (1502.9673)		Yes	No	microbody cytoplasm
⑤	NP_039391	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	100		10	K.NHGMHFR.V Oxidation(M) (914.4909) R.ACYECLR.G (971.4929) K.DTDILAAFR.V (1021.609) R.VALEACVQAR.N (1116.6586) R.QKNHGMHFR.V Oxidation (M) (1170.7179) R.DNGLLLHIHR.A (1187.7437) K.NHGMHFRVLAK.A (1309.7631) K.DDENVNSQPFMR.W (1451.7598) K.TFQGPPHGIQVER.D (1465.8462) K.YGRPLLGCTIKPK.L (1502.9673)		Yes	No	microbody cytoplasm
⑥	EAY76155	hypotherical protein		45	1	K.FSLAPLVPR.L (999.6366)	phosphoglycerate kinase	Yes	Yes	ER mem
⑥	EAZ13844	hypotherical protein		45	1	K.FSLAPLVPR.L (999.6366)	phosphoglycerate kinase	Yes	Yes	ER mem

No. corresponds to the numbers shown in Fig.3.

organelles of endosymbiotic origin is probably not an exceptionally rare event in higher plant cells.

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イネ葉緑体のグライコプロテオーム解析

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要　約

イネ葉緑体タンパク質のグライコプロテオーム解析を行った。イネ成熟葉から不連続パーコール密度勾配遠心分離法を用いて無傷の葉緑体を単離した。葉緑体タンパク質を調製し、SDS-ポリアクリルアミドゲル電気泳動の後、ペルオキシダーゼ標識 Concanavalin A を用いたレクチンブロッティングを行った。ペルオキシダーゼ標識 Concanavalin A により検出されたいくつかのタンパクバンドを質量分析装置を用いて解析した結果、ER シグナルペプチドと N 結合型糖鎖結合部位を持つホスホグリセリン酸キナーゼ様タンパク質がイネ葉緑体に局在していることが示唆された。

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