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Proteomic Analysis on Symbiotic Differentiation of Mitochondria in Soybean Nodules

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Symbiotic interactions between legume plants and rhizobia induce specific metabolisms and intracellular organelles in nodules. For surveying symbiotic differentiation of a key organelle, mitochondria, protein constituents of soybean nodule and root mitochondria were compared after two-dimensional (2-D) electrophoresis, and the proteins were characterized in combination with matrix-assisted desorption/ionization time-of-flight mass spectrometry, electrospray ionization mass spectrometry and N-terminal amino acid sequencing. Of the proteins that were detected only in nodule mitochondria, phosphoserine aminotransferase, flavanone 3-hydroxylase, coproporphyrinogen III oxidase, one ribonucleoprotein and three unknown proteins were identified. Seven up-regulated, eight down-regulated and two strongly suppressed protein spots in nodule mitochondria were also assigned protein identities. The physiological roles of these differential expressions were discussed in relation to nodule-specific metabolisms in soybean nodules.

Keywords: 2-D electrophoresis — MALDI-TOF mass spectrometry — Nodule mitochondrial proteins — Root mitochondrial proteins — Soybean.

Abbreviations: β-HBDH, β-hydroxybutyrate dehydrogenase; 2-D, two-dimensional; ESI MS, electrospray ionization mass spectrometry; IEF, isoelectric focusing; pI, isoelectric point; MALDI-TOF MS, matrix-assisted desorption/ionization time-of-flight mass spectrometry; MDH, malate dehydrogenase; MnSOD, manganese superoxide dismutase; PMF, peptide mass fingerprint; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Introduction

The symbiotic interaction between legume plants and nitrogen-fixing rhizobia leads to the formation of root nodules, specialized symbiotic organs. During nodule development, the plant cells and the microsymbionts undergo differentiation to develop specific structures and metabolic functions, which are essential for effective and stable nitrogen fixation. In soybean nodules, the central part of the tissue is called the infected zone, and around half of the plant cells are carrying rhizobia as endo-symbionts. In this infected zone, the shape of mitochondria in the plant cells was observed to be large, elongated and cristae-rich by intensive folding of the inner membranes (Werner and Mörschel 1978). These morphological changes of mitochondria are believed to be due to microaerobic conditions in the region. It is believed that O_2 concentration in the infected zone was extremely low (5–50 nM), and the values were much lower than the K_m (O_2) value for terminal oxidase of nodule mitochondria (Tajima and Kouchi 1995, Kuzma et al. 1999). This condition would relate to metabolic modification of mitochondria to generate large amounts of organic acids, mainly in the form of C_4-dicarboxylic acids, to meet the large demand for maintaining bacteroid respiration for nitrogen fixation (Tajima and Kouchi 1995). Although mitochondria isolated from soybean nodules exhibited higher oxidative and phosphorylative enzyme activities than those from the roots (Suganuma and Yamamoto 1987), very little biochemical evidence has been reported on mitochondria differentiation in this symbiotic condition.

The recent development of proteome analysis has provided a powerful tool for surveying the molecular mechanisms in symbiotic interaction. The pioneering work on the interaction between the bacterium *Sinorhizobium meliloti* strain 1021 and the legume *Melilotus alba* (Natera et al. 2000) revealed over 250 proteins induced or up-regulated, which were mainly of bacterial origin, in the nodules as compared with the roots and over 350 proteins were down-regulated in the bacteroids as compared to cultured bacteria. Among them, leghemoglobin, nifH and nifK, as well as proteins involved in bacteroid carbon metabolism were identified as nodule specific proteins. Symbiotic interaction was also studied in terms of proteins involved in the early stages of nodulation like those from a subterranean clover cultivar Woogenellup inoculated with *Rhizobium leguminosarum* bv. *trifolii* strains ANU843 and ANU794 (Morris and Djordjevic 2001). They included a α-fucosidase, a Cu/Zn superoxide dismutase, a chaperonin 21 precursor and several ethylene-induced proteins. Protein profiles of inoculated roots

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Table 1 Purification of mitochondria fraction from soybean nodules and roots

Marker enzyme [a]	Nodule				Root		
	Cytosol	Crude Mt	Purified Mt	Bacteroids	Cytosol	Crude Mt	Purified Mt
Cyt. *c* oxidase [b]	0.21	64.40	198.1	2.18	0.96	109.31	211.48
Alcohol dehydrogenase [b]	21.33	n.d.	n.d.	n.d.	0.77	n.d.	n.d.
Catalase [c]	0.16	71.43	26.68	1.05	0.24	54.82	11.89
Alkaline pyrophosphatase [d]	10.95	6.61	2.48	0.06	19.40	38.04	11.44
β-hydroxybutyrate dehydrogenase [b]	1.86	1.79	n.d.	9.88			

[a] Mitochondria purification was monitored with organelle marker enzymes: cytochrome *c* oxidase (mitochondria), alcohol dehydrogenase (cytosol), catalase (peroxisomes), alkaline pyrophosphatase (plastids) and β-hydroxybutyrate dehydrogenase (bacteroids).

[b] Specific enzyme activity are expressed in nmol per minute per mg of protein (nmol min^{-1} (mg protein)$^{-1}$); [c] μmol min^{-1} (mg protein)$^{-1}$; [d] μmol P_i min^{-1} (mg protein)$^{-1}$ (P_i, inorganic phosphate). Data are means of 3–5 replicates. Mt, mitochondria. Cytosol refers to the supernatant after the first centrifugation to discard cell debris and bacteroids. Crude mitochondria refers to mitochondria fraction obtained before being further purified by Percoll/PVP gradient centrifugation. n.d., not detectable.

from the model plant *Medicago truncatula* were investigated with time-course analysis, which showed 46 newly induced polypeptides, 4 up-regulated and 1 down-regulated ones (Bestel-Corre et al. 2002). Some other proteome analyses of symbiosis have focused on bacteria (Dainese-Hatt et al. 1999) or peribacteroid membrane proteins (Panter et al. 2000). However, there has been no published research on symbiotic differentiation of plant organelles like mitochondria in legume nodules. In addition to symbiotic metabolism, mitochondria might have functions for establishing symbiotic signal exchange because mitochondria are believed to be evolved from ancestral bacteria (Karlin and Campbell 1994) and play a key role to apoptosis (Green and Reed 1998).

For investigating this, we compared protein profiles in soybean nodule and root mitochondria using proteomic approach. In our best knowledge, this is a first report on proteome analysis of symbiotic differentiation of legume mitochondria.

Results

Comparative image analysis and characterization of mitochondria proteins from soybean nodules and roots

Table 1 shows four preparations during mitochondrial isolation. Distribution of marker enzyme activities suggested that purified nodule mitochondria preparation contained no cytosol proteins and no significant contamination of bacteroids because no marker enzyme activity of bacteroids (β-hydroxybutyrate dehydrogenase, β-HBDH) was detected in the preparation. However, observation of nodule mitochondria preparation by light microscopy after Gram-staining always detected trace amounts of bacteroids (data not shown). Small amounts of catalase and alkaline pyrophosphatase activities in mitochondria preparation also suggested slight contamination or adsorption of them to the mitochondria membrane as previously reported (Neuburger et al. 1982, Day et al. 1986).

Two-dimensional protein patterns of nodule and root mitochondria were compared (Fig 1). Data of triplicate gels obtained from separately extracted proteins indicated high reproducibility in protein patterns of mitochondria from both tissues. Image analysis using ImageMaster 2D software (Amersham Pharmacia Biotech) revealed 465 different protein spots in nodule mitochondria fraction and 383 spots in root mitochondria fraction.

The computerized matching of two mitochondrial protein patterns suggested 275 common protein spots in mitochondria of both tissues. They included most of the abundant spots, and the remaining protein spots, 190 in nodules and 108 in roots, were designated as putatively tissue-specific ones. Based on spot quantification analysis, 98 among 275 common protein spots from nodule mitochondrial fraction were found to have clear differences in abundance when compared to those from root mitochondria. Forty-nine of them were defined as putatively up-regulated in nodules, and the other 49 spots were down-regulated.

As the result of matrix-assisted desorption/ionization time-of-flight (MALDI-TOF) peptide mapping, tandem mass spectrometry analysis and Edman degradation analysis, identities of 43 protein spots, representing 34 different proteins, were successfully determined in nodule mitochondria (Table 2, 3, Fig. 1A). In addition to these plant proteins, there were 65 spots identified as *Bradyrhizobium japonicum* proteins (data not shown). The presence of these bacterial proteins in the mitochondria fraction may be caused by the adsorbing of bacteroid proteins to mitochondrial membranes or by contamination of bacteroids which lost β-HBDH activity. Such contamination by bacteroids was frequently reported like in the case of peribacteroid membrane preparation, and more precise isolation technique should be developed (Saalbach et al. 2002).

In root mitochondria, identities of 20 proteins were obtained for 39 spots as the result of mass spectrometry analysis (Table 2, 3, Fig. 1B). Localization prediction by PSORT and MITOPROT programs as well as reported location of the proteins indicated that total of 77 spots representing 41 proteins in both nodule and root tissues were mitochondrial proteins (Table 2, 3). Three of them were outer membrane proteins, nine

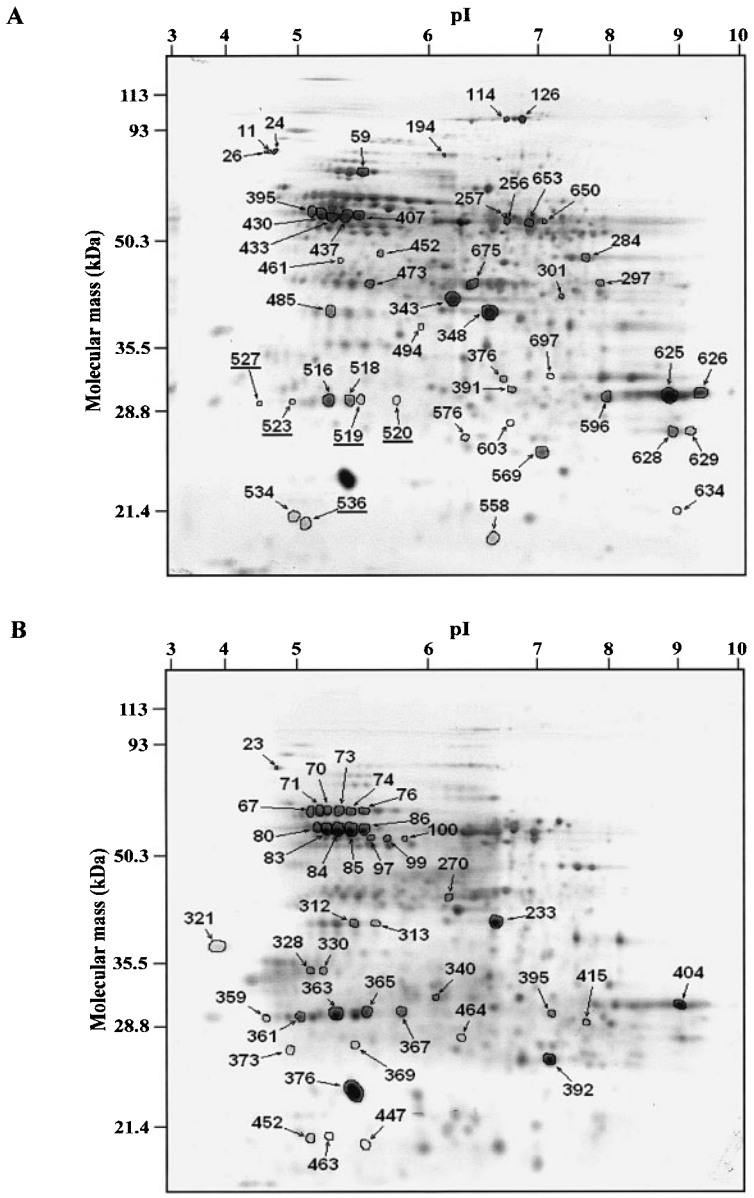

Fig. 1 Comparison of mitochondrial protein patterns from soybean nodules (A) and roots (B). Protein spots were detected by silver staining. The numbers above the gels indicate calibrated isoelectric point (pI) values in pH units. The numbers on the left indicate standard molecular mass in kDa. All detected spots were numbered using ImageMaster 2D software. Identified protein spots were circled and marked with arrows and numbers. Protein spots with numbers underlined (Fig. 1A) were identified using root mitochondrial fraction.

were inner membrane proteins and 29 were matrix proteins. They included three proteins encoded by mitochondrial genes.

Identification of protein spots which were detected only in nodule mitochondria

Among protein spots which could not be detected in root mitochondria, seven spots were assigned plant protein identities (Table 2). They are a phosphoserine aminotransferase (spot 297), flavanone 3-hydroxylase (spot 301), and coproporphyrinogen III oxidase (spot 494). Spot 461 was matched to a putative transformer serine/arginine-rich ribonucleoprotein. An unknown protein from *Arabidopsis* (At2g37930, spot 452) and two hypothetical proteins from rice (OSJNBb0053G03.2, spot 634; P0446B05.4, spot 697) were also identified. These proteins under significant differential expression were identified for the first time, and further characterization of the function is in progress in our laboratory.

Sixty-five protein spots which were not detected in root mitochondria were identified with high score as proteins of *B. japonicum* (data were not shown), and they were excluded from the data of mitochondrial proteins. Although they were faint protein spots, MALDI-TOF peptide mapping analysis showed reproducible annotation data with high score value due to the established genome sequencing data by the Kazusa DNA Research Institute.

Identification of putative up-regulated and down-regulated proteins in nodule mitochondria

All of the putative up-regulated proteins, except one (spot 114), appeared as abundant spots on 2-D gels (Table 2, Fig.

Table 2 Identification of putatively specific, up-regulated, down-regulated and suppressed protein spots from soybean nodule mitochondria as compared to root mitochondria

Spot (N/R)	Protein	Database Accession No. (Organism)	Sequence	Iden. (%)	Calculated Mr/pI	Cov. (%)	Observed Mr/pI	Psort
Specific proteins								
297/	Putative phosphoserine aminotransferase	REF NP_179354(Arath)			47.1/8.3	15	42.1/8.0	Cl.s (0.881)
301/	Flavanone 3-hydroxylase	DBJ BAC58033 (Raphanus)	AREFFALAPR	80	21.4/5.2		40.3/7.4	M.m (0.696)
452/	At2g37930, unknown protein	GB AAO63447 (Arath)	KAEEVLFGVD	80	52.0/8.8		47.4/5.7	M.m (0.462)
461/	Putative transformer serine/ arginine-rich ribonucleoprotein	REF NP_563787 (Arath)			41.4/11.6	10	45.9/5.4	N (0.993)
494/	Coproporphyrinogen III oxidase	SP P35055 (Glycine)	TVSIEKETPE	100	43.3/6.7		36.9/6.0	Cl.s (0.938)
634/	OSJNBb0053G03.2, hypothetical protein	DBJ BAC10753 (Oryza)			31.2/11.8	33	21.3/9.0	M.m (0.475)
697/	P0446B05.4, hypothetical protein	DBJ BAB89557 (Oryza)	SVVRGGVLRR	80	25.1/6.9		31.9/7.3	C (0.450)
Up-regulated proteins								
59 [a]/49	Heat shock protein 70 (Hsc70-5)	GB AAF27638 (Arath)	SKKPAGNDVI	90	73.0/5.6		72.4/5.5	M.m (0.907)
114 [a]/114	Putative glycine dehydrogenase	GB AAC31228 (Arath)			113.7/6.2	15	97.8/6.8	M.m (0.661)
126 [a]/120	Glycine dehydrogenase [decarboxylating]	SP P26969 (Pisum)	ISVEALQPSD	90	114.7/7.2		97.8/6.9	M.m (0.869)
536/452 [b]	ATP synthase D chain	SP P31399 (Rattus)			18.8/6.2	44	20.8/5.1	M.m (0.360)
626 [a]/405	Porin-like protein	GB AAO72587 (Oryza)	MRGPGLFSDI	90	29.5/9.2		30.4/9.4	C (0.450)
628 [a]/408	ATP synthase delta chain (OSCP)	SP P17604 (Pisum)	ANVPGQKETK	70	4/9.7	45	27.1/9.0	ER.m (0.550)
629 [a]/409	$NADH_2$ dehydrogenase 27 kDa chain	PIR PQ0785 (Vicia)	ANVPGQKENK	80	1.1/8.6		27.1/9.2	
Down-regulated proteins								
433 [a]/84 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	28	56.3/5.3	M.m (0.920)
437 [a]/85 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	22	56.7/5.4	M.m (0.920)
519/365 [b]	ATP synthase alpha chain	SP Q01915 (Glycine)			55.3/6.2	22	29.5/5.6	M.m (0.621)
520/367 [b]	ATP synthase alpha chain	SP Q01915 (Glycine)			55.3/6.2	18	29.5/5.8	M.m (0.621)
523/361 [b]	Dihydrolipoamide dehydrogenase	SP P31023 (Pisum)			53.3/6.7	16	29.0/5.1	M.m (0.585)
527/359 [b]	ATP synthase beta chain	SP P29685 (Hevea)			60.3/5.9	23	28.9/4.6	M.m (0.920)
603 [a]/388	Hypothetical protein	GB AAP06826 (Oryza)			21.7/10.1	31	27.7/6.8	N (0.968)
653 [a]/163	ATP synthase alpha chain	SP Q01915 (Glycine)	MEFSVRAAEL	100	55.3/6.2	37	54.5/7.0	M.m (0.621)
Suppressed proteins								
/369 [b]	Pseudo-atpA	EMBL CAA78408(Glycine)			34.8/5.6	31	26.7/ 5.5	M.in (0.566)
/373 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	15	26.4/5.0	M.m (0.920)
/415 [b]	Porin	SP Q9XH72 (Prunus)			29.7/7.1	29	28.5/7.7	C (0.450)
/447 [b]	NADPH:quinone oxidoreductase	SP Q9XFI5 (Arath)			21.5/6.8	40	20.4/5.5	M.m (0.529)
/463 [b]	ATP synthase D chain	SP P31399 (Rattus)			18.8/6.2	37	21.0/5.3	M.m (0.360)
/464 [b]	Pseudo-atpA	EMBL CAA78408(Glycine)			34.8/5.6	34	27.3/6.3	M.in (0.566)

Spot (N/R), the spot number of nodule (N) and root (R) mitochondria as given in Fig. 1A and 1B. Specific spots appeared only in nodule mitochondria (Fig. 1A) and suppressed spots only in root mitochondria (Fig. 1B). [a] Protein spots identified from nodule mitochondria (Fig. 1A) [b] Protein spots identified from root mitochondria (Fig. 1B). Databases: DBJ, Development Bank of Japan; EMBL, European Molecular Biology Laboratory; GB, GenBank; PIR, Protein Information Resource; REF, NCBI Reference Sequence; SP, SwissProt. Accession no., protein entries in the databases. Organism abbreviations: Arath, *Arabidopsis thaliana*; Glycine, *Glycine max*; Hevea, *Hevea brasiliensis*; Oryza, *Oryza sativa* (japonica cultivar-group); Pisum, *Pisum sativum*; Prunus, *Prunus armeniaca*; Raphanus, *Raphanus sativus*; Rattus, *Rattus norvegicus*; Vicia, *Vicia faba*; Zea, *Zea mays*. Sequence, ten-residue N-terminal amino acid sequence obtained from Edman-degradation-based sequencing. Iden. (%), the percentage of identity calculated between the amino acids present in the Edman sequence and the sequence found in the databases. Calculated Mr/pI, the calculated molecular mass and theoretical isoelectric point of the protein match. Cov. (%), Coverage, the percentage of the full-length sequence covered by the matching peptides. Observed Mr/pI, the molecular mass and isoelectric point observed on the 2-D gel. Psort, predicted localization ($P = 0–1$) using PSORT software; C, cytoplasm; Cl.s, chloroplast stroma; ER.m, endoplasmic reticulum (membrane); M.in, mitochondrial inner membrane; M.m, mitochondrial matrix; N, nucleus.

1A). Spot 59 shared 90% identity in N-terminal sequence with a mitochondrial matrix heat shock protein 70 kDa (Hsp70). Two other protein spots, 114 and 126, showed homology to glycine dehydrogenase, which catalyzes the oxidation of glycine to form CO_2, NH_3, NADH and methylenetetrahydrofolate, an important step of photorespiratory pathway (Turner et al. 1992a). Spot 626 was identified as a porin-like protein. Like other constitutively expressing porins (spots 596, 625), this protein may not need N-terminus cleavage import so it was not predicted as a mitochondrial protein by PSORT or MITOPROT programs.

Table 2 shows eight putative down-regulated proteins in nodule mitochondria, which were dihydrolipoamide dehydrogenase (spot 523), a FAD-containing polypeptide catalyzing

Table 3 Identification of constitutively expressed protein spots in soybean nodule mitochondria as compared to root mitochondria

Spot (N/R)	Protein	Database Accession no. (Organism)	Sequence	Iden. (%)	Calculated Mr/pI	Cov. (%)	Observed Mr/pI	Psort
11 [a]/23 [b]	Heat shock protein 70 (Hsc70–7)	GB AAF27639 (Arath)	EKVVGIDLGT	100	77.1/5.1		81.3/4.7	Cl.s (0.874)
24 [a]/24	Heat shock protein 70 (Hsc70–7)	GB AAF27639 (Arath)	EKVVGIDLGT	100	77.1/5.1		81.4/4.8	Cl.s (0.874)
26 [a]/16	Heat shock protein 70 (Hsc70–7)	GB AAF27639 (Arath)	EKVVGIDLGT	100	77.1/5.1		81.1/4.6	Cl.s (0.874)
70/67 [b]	Heat shock 70 kDa protein	SP Q01899 (Phaseolus)			72.5/5.9	20	62.0/5.1	M.m (0.920)
71/71 [b]	Heat shock 70 kDa protein	SP Q01899 (Phaseolus)			72.5/5.9	21	62.1/5.2	M.m (0.920)
72/70 [b]	Heat shock 70 kDa protein	SP Q01899 (Phaseolus)			72.5/5.9	16	62.4/5.3	M.m (0.920)
73/73 [b]	Chaperonin 60	GB AAA33450 (Zea)			61.2/5.7	16	62.1/5.4	M.m (0.637)
74/74 [b]	Chaperonin 60	GB AAA33450 (Zea)			61.2/5.7	16	62.0/5.4	M.m (0.637)
75/76 [b]	Chaperonin 60	GB AAA33450 (Zea)			61.2/5.7	15	62.1/5.5	M.m (0.637)
194 [a]/126	Heat shock protein 70 kDa	SP Q01899 (Phaseolus)			72.5/5.9	15	79.7/6.2	M.m (0.920)
256 [a]/168	SRPK4 (serine/threonine protein kinase)	EMBL CAC03678 (Arath)	STSAAIELDL	80	59.4/6.0		55.1/6.8	C (0.450)
257 [a]/151	SRPK4 (serine/threonine protein kinase)	EMBL CAC03678 (Arath)	STSAAIELDL	80	59.4/6.0		56.8/6.8	C (0.450)
284 [a]/155	Cysteine proteinase isoform B (fragment)	PIR T08844 (Glycine)			34.9/7.6	27	46.3/7.8	C (0.450)
339/270 [b]	Glutamate dehydrogenase 1	SP Q43314 (Arath)			44.5/6.4	24	41.8/6.2	M.m (0.665)
343 [a]/264	Malate dehydrogenase, MDH1	GB AAD56659 (Glycine)	ASEPVPERKV	100	36.1/8.2		40.0/6.3	M.m (0.483)
343 [a]/264	Malate dehydrogenase, MDH2	PIR T06326 (Glycine)			27.5/5.6	25	40.0/6.3	M.m (0.100)
348 [a]/233 [b]	Malate dehydrogenase, MDH1	GB AAD56659 (Glycine)	ASEPVPERKV	100	36.1/8.2	47	38.4/6.6	M.m (0.483)
376 [a]/241	Predicted NADH dehydrogenase 24 kDa subunit	REF NP_567244 (Arath)	STALNYHLDT	90	28.4/ 8.1		31.7/6.7	M.m (0.717)
378/340 [b]	Cytochrome P450 93A1	SP Q42798 (Glyine)			57.8/9.0	20	30.8/6.1	ER.m(0.820)
391 [a]/242	Ferrochelatase (Heme synthetase)	SP O59786 (Schizo)	GELVADHLKA	70	42.7/6.1		30.7/6.8	C (0.450)
395 [a]/80 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	20	57.9/5.2	M.m (0.920)
407 [a]/86 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	20	56.8/5.5	M.m (0.920)
422/97 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	29	53.9/5.6	ER.m(0.550)
424/99 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	28	53.6/5.7	ER.m(0.550)
426/100 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	28	53.5/5.8	ER.m(0.550)
430 [a]/83 [b]	ATP synthase beta chain	SP P19023 (Zea)			59.1/6.0	20	57.3/5.2	M.m (0.920)
473 [a]/294	Succinyl-CoA ligase [GDP-forming] beta-chain (Succinyl-CoA synthase, beta chain)	SP O82662 (Arath)	LNIHEYQGAE	100	45.3/6.3		42.1/5.6	M.m (0.508)
485 [a]/311	Pyruvate dehydrogenase E1 beta subunit isoform 1	GB AAC72192 (Zea)	SSAAQEITVR	80	39.8/5.5		38.6/5.3	V (0.464)
486/312 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	22	38.5/5.5	ER.m(0.550)
487/313 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	34	38.5/5.6	ER.m(0.550)
499/330 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	16	33.5/5.2	ER.m(0.550)
505/328 [b]	F1-ATP synthase, beta subunit	EMBL CAA75477 (Sorgh)			49.1/5.2	16	33.5/5.1	ER.m(0.550)
507/321 [b]	Cytochrome oxidase subunit 6b-1	DBJ BAA76393 (Oryza)			18.9/4.3	22	36.0/3.9	C (0.650)
516 [a]/363 [b]	ATP synthase subunit	PIR S48643 (Glycine)	AKESAPPALK	100	20.3/8.9	32	29.7/5.3	M.m (0.731)
518 [a]/364	ATP synthase subunit	PIR S48643 (Glycine)	AKESAPPALK	100	20.3/8.9		29.7/5.4	M.m (0.731)
533/376 [b]	Elongation factor G	SP P34811 (Glycine)			86.9/5.5	16	23.6/5.5	M.m (0.474)
534 [a]/451	NADH-ubiquinone oxidoreductase 22.5 kDa subunit (fragment)	SP P80266 (Solanum)	AKVKASTGIV	80	4.1/9.4		21.1/5.0	ER.m(0.550)
558 [a]/438	At1g64250.1, hypothetical protein	REF NP_176607 (Arath)	VTPARIEEHG	70	90.1/5.1		19.4/6.6	M.m (0.477)
569 [a]/392 [b]	Manganese-superoxide dismutase	EMBL CAD29434 (Glycine)			15.4/6.1	48	25.4/7.2	P (0.640)
569 [a]/392	Superoxide dismutase [Mn]	SP P27084 (Pisum)	LHVYTLPDLD	80	25.8/7.2		25.4/7.2	M.m (0.867)
576 [a]/382	Probable acetaldehyde dehydrogenase oxidoreductase protein	REF NP_522455 (Ralstonia)			31.7/7.1	34	26.6/6.4	B.c (0.070)
589/395 [b]	Unknown mitochondrial protein	SP Q9SMN1 (Arath)			28.0/8.9	35	29.3/7.2	M.m (0.908)
596 [a]/401	36 kDa porin I	EMBL CAA56601 (Solanum)	VKGPGLYSDI	100	29.36/7.8	16	30.0/8.0	C (0.450)
	36 kDa porin II	EMBL CAA56600 (Solanum)			29.39/7.8			
625 [a]/404 [b]	36 kDa porin I	EMBL CAA56601 (Solanum)	AKGPGLYSDI	90	29.36/7.8		30.1/8.9	C (0.450)
	36 kDa porin II	EMBL CAA56600 (Solanum)			29.39/7.8			
650 [a]/162	Putative 26S proteasome regulatory subunit	REF NP_174210 (Arath)			46.7/6.2	17	54.9/7.2	M.m (0.594)
675 [a]/268	Hypothetical protein 11 (coxI 5′ region)	PIR S37678 (Oryza)	VSFRRTKLLV	70	11.2/6.6		42.0/6.4	M.m (0.360)

Spot (N/R), the spot number of nodule (N) and root (R) mitochondria as given in Fig. 1A and 1B.
[a] Protein spots identified from nodule mitochondria (Fig. 1A).
[b] Protein spots identified from root mitochondria (Fig. 1B).
Databases: DBJ, Development Bank of Japan; EMBL, European Molecular Biology Laboratory; GB, GenBank; PIR, Protein Information Resource; REF, NCBI Reference Sequence; SP, SwissProt. Accession no., protein entries in the databases. Organism abbreviations: Arath, *Arabidopsis thaliana*; Glycine, *Glycine max*; Oryza, *Oryza sativa* (japonica cultivar-group); Phaseolus, *Phaseolus vulgaris*; Pisum, *Pisum sativum*; Ralstonia, *Ralstonia solanaearum*; Rattus, *Rattus norvegicus*; Shizo, *Shizosacharomyces pombe*; Solanum, *Solanum tuberosum*; Sorgh, *Sorghum bicolor*; Zea, *Zea mays*. Iden. (%), the percentage of identity calculated between the amino acids present in the Edman sequence and the sequence found in the databases. Calculated Mr/pI, the calculated molecular mass and theoretical isoelectric point of the protein match. Cov. (%), Coverage, the percentage of the full-length sequence covered by the matching peptides. Observed Mr/pI, the molecular mass and isoelectric point observed on the 2-D gel. Psort, predicted localization ($P = 0–1$) using PSORT software; B.c, bacterial cytoplasm; C, cytoplasm; Cl.s, chloroplast stroma; ER.m, endoplasmic reticulum (membrane); M.m, mitochondrial matrix; P, peroxisome; V, vacuole.

the reduction of lipoamide group and acting as L protein of glycine decarboxylase complex (Turner et al. 1992b), α subunit (spots 519, 520, 653), β subunit (spot 433, 437, 527) of F_1-ATP synthase and a hypothetical protein (spot 603).

Proteins which were suppressed the expression in nodule mitochondria

Six identified protein spots were detected only in root mitochondria (Table 2, Fig. 1B), indicating that these proteins were strongly suppressed in nodule mitochondria. Two spots (369, 464) shared 31–37% sequence coverage with pseudo-atpA, a product of the pseudogenes truncated in the *atpA-orf214* region (*atpA* encodes α subunit of F_1-ATPase and *orf214* is an unidentified reading frame) in soybean mitochondrial DNA (Chanut et al. 1993). Three other spots were matched to proteins involved in electron transport: β subunit which bears catalytic site for conversion of ADP and P_i to ATP of F_1-ATP synthase from maize (373); NADPH:quinone oxidoreductase from *Arabidopsis* (447), a flavoprotein catalyzing two-electron reduction of hydrophilic quinones by NADPH (Chareonthiphakorn et al. 2002); and D chain of F_0-ATP from rat (463). The other protein spot corresponded to a porin of apricot, which forms voltage-dependent channel for transport of small molecules across the mitochondrial outer membrane.

Identification of constitutively expressed proteins

Most of the abundant spots classified as constitutively expressed proteins in mitochondria from both tissues were also subjected to identification in order to understand basic protein constituents in mitochondria. The identity of 27 proteins was obtained with 52 protein spots in nodule and root mitochondria as shown in Table 3. They represented housekeeping functions of mitochondria and can be divided into several function groups. The first group represented major function of mitochondria, electron transport and ATP synthesis, and accounted for about one-third of the total identified spots. The second group was heat shock proteins, Hsp70s and chaperonin 60. Many of them appeared as abundant spots on 2-D gels, indicating their important role in processing and translocating mitochondrial proteins. The third group consisted of proteins participating in pyruvate decarboxylation and tricarboxylic acid cycle. The fourth group was outer membrane proteins (voltage-dependent anion-selective channel protein allowing transport of small hydrophilic molecules). Spot 596 and 625 in nodule mitochondria had 100% and 90% sequence homology, respectively, to both isoforms I, II of 36 kDa porin from potato. There are of two isoforms of porin in *Arabidopsis* mitochondria, one of which has Mr/pI as 29.1/7.98 and the other 29.3/8.7 (Kruft et al. 2001), that are very similar to those two protein spots. The fifth group included proteins involved in various metabolisms including Mn-dependent superoxide dismutase (MnSOD) (spot 569/392), which is involved in the protection of mitochondria against oxidative stress and showed a high specific activity in soybean nodule mitochondria (Iturbe-Ormaetxe et al. 2001).

Discussion

The combination of mass spectra data and N-terminal amino acid sequence has been indicated to be a useful tool for identification of unknown proteins (Natera et al. 2000, Kruft et al. 2001, Bardel et al. 2002). Due to the relatively poor genome DNA sequencing database of soybean plants, we employed the combination analysis of large-scale MALDI-TOF MS, LC-MS/MS and N-terminal amino acid sequencing for identifying proteins in nodule and root mitochondria. As a result, we identified 43 spots from nodule mitochondrial preparations and 39 spots from root mitochondria. On the way to identify symbiosis-related proteins, we add several proteins to the mitochondrial database, which were not yet declared in previous reports on plant mitochondrial proteome of *Arabidopsis*, pea and rice (Millar et al. 2001, Kruft et al. 2001, Bardel et al. 2002, Heazlewood et al. 2003). They were 27 kDa, 22.5 kDa subunits of NADH:ubiquinone reductase, ferrochelatase, hypothetical protein 11 (coxI 5′ region), NADPH:quinone oxidoreductase and pseudo-atpA.

In the results of protein identification, approximately 24% of the identified mitochondrial proteins were present in at least two spots (Table 2, 3), like the β subunit of F_1-ATP synthase. The β subunit of F_1-ATP synthase in *Nicotiana syvestris* mitochondria was reported to have three isoforms encoded by a small multi-gene family of differentially expressed genes (Lalanne et al. 1998). Such multi-spots were also observed in heat shock protein 70 (Hsc70–7) and heat shock 70 kDa protein. A series of experimental approaches need to be established in order to define the nature of those modifications as well as their impact on protein functions.

From image analysis of the 2-D gels, we detected a significant difference in protein spot profiles between nodule and root mitochondria. Although 65 spots were identified to be *B. japonicum* proteins and those proteins were successfully excluded from the data, significant modification of protein constituents was supposed. As a result, we identified 28 protein spots which were differentially expressed (Table 2). Although many of the proteins concerned have not been identified with well-matching data, the data suggests new metabolic profiles.

With respect to nodule-specific metabolism, phosphoserine aminotransferase (spot 297) and glycine dehydrogenase complex (spot 114, 126) were identified as significantly up-regulated proteins. Phosphoserine aminotransferase acts in the production of phosphoserine to yield L-serine, involved in carbon supply for purine synthesis in ureide biogenesis via the production of glycine and FH_4 (Reynolds and Blevins 1986). The role of this enzyme is significant for such tropical legumes as soybean, which transport fixed-nitrogen in the form of ureides. Although this enzyme was predicted to be located in nodule protoplastids by the same activity distribution as serine hydroxymethyltransferase and phosphoglycerate dehydrogenase (Reynolds and Blevins 1986), it was estimated by MITOPROT to have a high probability to target into mitochondria (*P*

= 0.799). Glycine dehydrogenase complex proteins (spot 114 and 126) are well known to be responsible for the decarboxylation step of the photorespiratory pathway. The increase in abundance of these proteins in soybean nodule mitochondria (3- and 69-fold, respectively) suggested they play a role in the maintenance of L-serine pool necessary for purine biosynthesis. These data suggested that amplified L-serine formation can be a regulatory point for the ureide production in soybean nodules. This is a new finding that these enzymes for L-serine production in nodule mitochondria were coordinately up-regulating and this would be a possible regulatory point for ureide accumulation in soybean nodules.

Flavanone 3-hydroxylase (F3H) is involved in formation of dihydroflavonols, various isoflavones and anthocyanins. The enzyme was thought to participate in modifying the pool of flavonoids and isoflavonoids in nodules (Charrier et al. 1995). The physiological role of this enzyme in nodules is not clear yet, but the enhancement of this enzyme suggested the contribution of flavonoids in the late stage of nodule formation.

The microaerobic condition is a most influential factor for plant metabolism in nodules. Various proteins involved in maintenance of cellular conditions and energy metabolism in mitochondria were expected. In our data, coproporphyrinogen III oxidase (spot 494), an enzyme for heme biosynthesis, was observed as a protein which was detected only in nodules (Table 2). The mRNA was already found to be highly expressed in soybean nodules, corresponding to increased demand of symbiotic leghemogobins, which is essential for microaerobic respiration of bacteroids (Madsen et al. 1993). Many down-regulated proteins were α and β subunits of F_1-ATP synthase (spots 373, 433, 437, 519, 520, 527, 653). This would suggest the inhibition of ATP production under microaerobic conditions in nodules, although further characterization would be necessary because ATP synthase subunits were also detected as constitutive proteins and previous reports claimed the decrease of only non-phosphorylating respiratory pathway in nodule mitochondria (Day et al. 1986, Bryce and Day 1990). Among the protein spots corresponding to the D chain of ATP synthase, spot 463 (root) was much less abundant than spots 452 (root) and 536 (nodule), indicating the protein was up-regulated in the nodule. This protein was already found in *Arabidopsis* as a novel mitochondrial protein (Kruft et al. 2001) and the function is still not understood although it was reported to be a tumor-related protein. For example, it was over-expressed in human thyroid papillary carcinoma and thus proposed as a marker for diagnosis of that disease (Srisomsap et al. 2002).

Interestingly, pseudo-atpA that was found in root mitochondria as two distinct spots (369, 464) was suppressed in nodule mitochondria. Pseudo-atpA is a product of the pseudogene truncated in N-terminal coding region and upstream region of *atpA* gene encoding α subunit of F_1-ATPase. The expression at protein level of this pseudogene has not been characterized yet but together with *atpA* gene it was supposed to be involved in the homologous recombination of mitochondrial DNA (Chanut et al. 1993). Therefore the transcription and subsequent translation from this gene under some specific conditions may have an effect on the expression of other genes in mitochondrial DNA. The truncated *atpA* pseudogenes in both male-fertile and cytoplasmic male-sterile (CMS) petunia were investigated and the creation of CMS-specific locus was supposed to occur via several steps of mitochondrial DNA homologous recombination (Yesodi et al. 1997).

Among putative up-regulated protein spots in nodule mitochondria, ribonucleoprotein (spot 461) and a heat shock protein 70 (spot 59, mtHsp70) were suggested to function for protein synthesis in nodules. The ribonucleoprotein might be involved in the RNA editing process, and MtHsp70 contributes to the folding and assembly of imported mitochondrial proteins during or after the import. MtHsp70 was highly expressed during seed maturation and germination together with some other hsp70s, relating to protein metabolism such as segregation of storage proteins, aggregation of unfolded and misunfolded proteins during seed desiccation or translocation of proteins (Sung et al. 2001).

In summary, proteomic analysis was utilized to investigate protein profiles of mitochondria isolated from soybean nodules and roots. The results presented various potentially symbiosis-related proteins, which were reported first in this work. Since the proteomic approach analyzes relatively abundant and existing proteins, major metabolic reactions can be identified, compared to transcriptome work. In the future, other organelles such as plastids and peroxisomes should be analyzed to elucidate metabolic compartments in nodule tissues.

Material and Methods

Preparation of plant materials

For nodule mitochondria preparation, soybean (*Glycine max* L. Merr. cv. Akishirome) seeds were surface-sterilized (Natera et al. 2000), inoculated with *B. japonicum* USDA110, and transferred to water culture after germination on moist vermiculite (Tajima and Yamamoto 1975). At 4–5 weeks after inoculation, when the acetylene reduction activity per gram fresh weight of nodules reached a maximum, nodules were harvested from the roots. For preparing root mitochondria, roots of 7- to 10-day-old uninoculated seedlings were harvested.

Mitochondrial isolation and purification

Mitochondria were isolated and purified from nodules as described by Day et al. (1986). The procedure included two centrifugation steps for purification by Percoll and Percoll/PVP gradients. In the case of mitochondria from roots, the purification was carried out based on the method of Neuburger et al. (1982). The purity of mitochondria was evaluated by light microscopy observation after Gram staining and by measuring marker enzyme activities (Day et al. 1986, Kar and Mishra 1975, Smith and ap Rees 1979). Protein concentration was determined according to the Lowry method, modified by Peterson (1977).

Protein extraction, separation and visualization

Proteins were extracted from purified mitochondria fractions of roots or nodules by freeze-thawing in lysis solution containing 40 mM Tris-HCl (pH 7.5), 50 mM DTT and 2% (w/v) TritonX-100 (Stasyk et al. 2001) and precipitated in 80% (v/v) acetone at –28°C for 2 h.

For preparation of analytical 2-D gels, 150 μg of proteins were solubilized in buffer consisting of 6 M urea, 2 M thiourea, 2% (w/v) Triton X-100, 20 mM DTT, 0.001% (w/v) bromophenol blue, 2 mM tributylphospine and 1% (v/v) pH 3–10 IPG buffer (Amersham Biosciences, Uppsala, Sweden). IEF was performed using 13 cm non-linear pH 3–10 Immobilline DryStrip gels (Amersham Biosciences, CA, U.S.A.) following the manufacturer's instructions. Then the gel strips were equilibrated and subsequently transferred onto 10% SDS-polyacrylamide gels in the second dimension as described by Berkelman and Stenstedt (2001). The 2-D gels were visualized by silver staining based on the method of Heukeshoven and Dernick (1985). In the case of preparative gels, 800 μg proteins was loaded to rehydrate the gel strips and 7 M urea and 3% (w/v) Triton X-100 were used in the rehydration solution for better solubilization and resolution.

Image analysis

The 2-D gel images of nodule and root mitochondrial proteins were digitalized with an Epson ES-2200 scanner using Photoshop 6.0 (Adobe System Inc., U.S.A.). Digitalized images were analyzed by ImageMaster 2D version 3.01 software (Amersham Pharmacia Biotech). Spot detection and density normalization was performed according to Bestel-Corre et al. (2002). Spots were considered as putatively specific when they were detected in the gel of nodule mitochondria but not in that of root mitochondria and vice versa. A protein considered as being up- or down-regulated when its normalized volume was at least a 3-fold increase or decrease, respectively.

Protein identification by MALDI-TOF MS, ESI-MS and N-terminal amino acid sequencing

Sample preparation for MALDI-TOF MS was carried out based on the method reported by Millar et al. (2001). Spots of interest were excised from CBB-stained gels, and the gel pieces were reswelled in 25 mM NH_4HCO_3 containing 12.5 ng μl^{-1} trypsin (Promega, Madison, WI, U.S.A.). Mass spectrometry was performed using Autoflex MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany). Peptide mass values except those derived from blanks or autolysis of trypsin were used in peptide mass fingerprinting to search the NCBInr database with Mascot search engine. Based on the wide range of soybean EST sequences available (286,868 ESTs by February 2003), unsuccessful searches were done again with EST database. The confidence of matches relied on the number of peptide matches (usually more than three) and the overlap of matched peptide masses with the major peaks of the mass spectra as described by Mathesius et al. (2001). Only matches with ESTs considered similar to known proteins in the databases were accepted.

For the ESI-MS assay, peptide mixtures were prepared from CBB staining gels, and the fragments were separated by a C18 column (Magic C18 P/N 902-61260-00, Michrom BioResources, Inc., Auburn, CA, U.S.A.) in linear gradient (5% to 60%) of acetonitrile containing 0.1% formic acid. Separated peptides were eluted directly into a hand-made nanospray ionization apparatus (Kaneko et al. 2002), and analyzed by Magic 2002 LC-MS/MS system (Michrom BioResources, Inc. Auburn, CA, U.S.A.).

For N-terminal amino acid sequencing, proteins on CBB gels were blotted onto PVDF membranes using the Trans-Blot Semidry Transfer Cell from BioRad according to instructions of the manufacturer. Selected spots were excised from dried membranes with sterile scalpels, and were subjected to sequencing on a Procise® protein sequencing system (Applied Biosystems, Foster City, CA, U.S.A.). The N-terminal sequences were used to search using the BLAST tool available from NCBI to identify homology to proteins in databases. Sequences of protein matches were applied for calculations of molecular masses and isoelectric points using Compute pI/Mw available at ExPASy Molecular Biology Server, Geneva (http://us.expasy.org/tools/pi_tool.html).

Subcellular localizations of protein matches from mass spectrometry and N-terminal sequencing analysis were predicted based on known protein sorting signals by the PSORT program (http://psort.ims.u-tokyo.ac.jp/form.html) and on putative mitochondrial targeting sequences by the MITOPROT program (http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter).

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