Mitochondrial comparative proteomic analysis of sterile line and its maintain line of purple cytoplasmic rice (*Oryza sativa*)

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ABSTRACT

CMS/Rf systems in rice (*Oryza sativa*) have long been exploited for hybrid breeding to enhance productivity. Ying xiang CMS/Rf system is a new type. In this study, a mitochondrial comparative proteomic analysis of Ying xiang Sterile Line and its Maintain Line was started for a comprehensive investigation of the mitochondrial proteins' functions in rice cytoplasmic male sterility. Mitochondria were prepared from rice shoots grown in the dark. Proteins were analyzed by two-dimensional electrophoresis and MALDI-TOF/ MS. Using Mascot, it was found that 7 proteins were not described previously for plant mitochondria, indicating novel mitochondrial functions. 3 of them were characterized.

1. INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited phenotype characterized by the inability of a plant to produce functional pollen [1]. In rice (*Oryza sativa*), several CMS/Rf systems defined by the different CMS cytoplasm with distinct genetic features have been identified [2-4]. These systems have been widely used for hybrid rice breeding in China and other Asian countries as hybrid rice crops often produce higher yields than inbred varieties [5,6].

Cytoplasmic male sterility in plants is always associated with mitochondrial dysfunction. Molecular studies on CMS have revealed the existence of modifications in mitochondrial DNA and a correlation between the presence of chimaeric mitochondrial genes and the synthesis of new proteins [7-10], and some mitochondrial genes responsible for CMS are chimeric in structure [9,11]. Some of these genes encode a cytotoxic protein [12]. Proteomic analysis is a newly developed technique that has been demonstrated as a powerful approach in plant research [13-16].

Genetical resources play a key role in meeting global challenges in the fields of food security. For example, cytoplasmic diversity is very important for resistance to plant diseases and insect pests in agriculture. We constructed a new type of cytoplasmic male sterile (CMS) line ZidaoA. It can help us to reveal the mechanism of rice cytoplasmic male sterility because its microspores abort completely and earlier than the others, which is significant in hybrid breeding in rice [17,18]. Furthermore, it will enrich the diversity of the cytoplasmic genetic resource of rice, which can avoid the reduction of output caused by rice diseases and insect pests because of the genetic frangibility in rice. And Ying xiang CMS line Ying xiang A is a nucleus displacement product of ZidaoA of Purple rice CMS line and it has the identical cytoplasm and the same properties as the Purple-leaf rice CMS line, so research on Ying xiang CMS line and its maintenance can help us to elucidate the mechanism of purple-leaf rice cytoplasmic male sterility and be advantageous in hybrid breeding of rice. Our early study showed that RNA editing may play a role in the CMS. The translation of *atp*9 transcript might interfere with the construction of F0F1-ATPase, resulting in the decrease of ATPase activity and the abortion of pollen in Ying xiang A. We also studied the total proteins of leaves and young panicle by two-dimensional electrophoresis [19,20]. However, little is known about the defects of mitochondrial proteome in the Ying xiang CMS line so far. In the present study, we demonstrate and characterize the mitochondrial proteomics difference of CMS line and its maintain line using 2D-PAGE and MALDI-TOF/MS (matrix-assisted laser desorption/ionisation-time of flight mass spectrometry).

2. MATERIALS AND METHODS

2.1. Materials

Cytoplasmic male sterile (CMS) line and its maintain

line of Zidao (Yunnan purple-leaf rice) type rice (*Oryza sativa*): Ying xiang A and Ying xiang B.

2.2. Rice Mitochondrial Isolation

The methods of rice mitochondrial isolation followed Mignouna *et al.* [21].

2.3. Two-Dimensional Gel Electrophoresis

Mitochondria protein samples (100 μg) were acetone extracted by addition of acetone to a final concentration of 80% (v/v) at –20℃. 1mg dried powder was used for two-dimensional electrophoresis. Proteins were visualized by Coomassie blue R350 staining for 10 min at 100℃ and destained overnight with 10% (v/v) acetic acid. Mw standards were used to identify apparent molecular masses on second dimension separation.

## 2.4. Mass Spectrometry Analysis and Protein Identification

Protein spots were manually excised from stained gels and characterized after in-gel trypsin digestion by Voyager-DE STR (Applied Biosystems, CA) according to Zhang *et al.* [22]. Then the peptide mixture was loaded onto a MALDI plate for MS analysis using a Reflex III type Maldi-Tof-MS (BRUKER) analyses. For protein identification purposes, mass spectrometric data were searched using MASCOT software (http://www.matrix science.com). The search parameters used with this software were as follows: one missed cleavage; 0.1 Da mass accuracy; six peptides allowed. For hits that did not fit with the tolerated mass accuracy, namely the occur-

rence of six peptides per protein and a coverage greater than 20%, cross-matching was carried out by comparing the molecular weight and pI of the predicted protein with the observed molecular mass and pI of the excision site on the two-dimensional gel. Database searches of MALDI- TOF/MS were performed using the NCBI database (*O. sativa* specified databases and the reversed databases as a control), and positive identifications were made when the scores were above the significance threshold value (P < 0.05). The proteomics tools at http://www.expasy.org/ tools/ were used in computing the pI and Mw and functional analysis.

3. RESULTS AND ANALYSIS

3.1. Identification of Proteins via 2DE, Mass Spectrometry and Bioinformatics

In order to get the differentially accumulated mitochondrial proteins in sterile Ying xiang A and maintain lines Ying xiang B, the proteome maps visualized by Coomassie blue R350 of the mitochondia were compared, and protein spots with distinctly difference (**Figure 1**) were analysed using adobe Photoshop software. Only 13 protein spots were chosen (**Figure 1**). The 13 protein spots were excised manually and digested in-gel with trypsin, and analyzed using MALDI-TOF/MS and 12 protein spots were produced peptide mass fingerprint (PMF) data. The 13 spots were numbered, as indicated in **Figure 1** and **Table 1**. Database searches using these PMF data at http://www.matrixscience.com/cgi/ using MASCOT revealed the identities of 12 of the protein
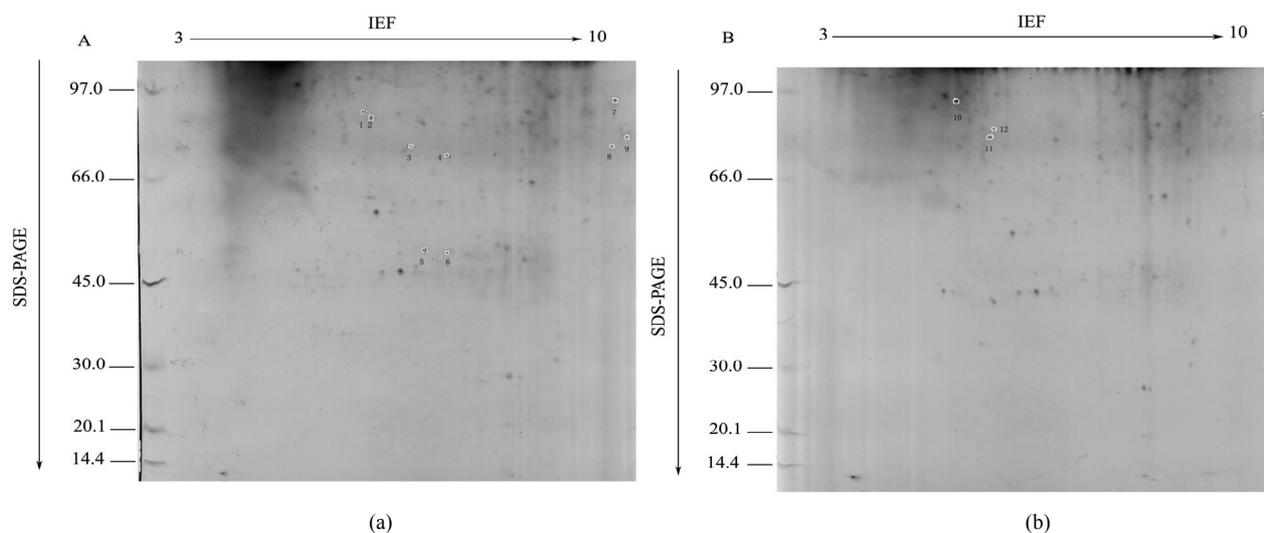
Figure 1. Representative 2-DE maps of the total mitochondrial protein from CMS rice Ying xiang A (a) and its maintainer Ying xiang B (b). IEF in the first dimension was carried out on linear pH 3-10, 18 cm IPG strips loaded with 1 mg of protein. In the second dimension a 13% SDS-PAGE gel was used and proteins were visualised by Coomassie Brilliant Blue R-350 staining. The spots analysed by MALDI-TOF/MS are circled and the arbitrary identifiers correspond to those listed in **Table 1**.

spots and most of the 12 identified protein spots contained only one protein, while 4 spots (spots 5, 6, 12 and 13) contained two or more proteins with the sequences highly conserved to the best matched proteins. But only 3 spots (spots 3, 4 and 10) were significant after being compared with the observed molecular mass and pI of the excision site on the two-dimensional gel.

3.2. Functional Analysis of Identified Proteins

All 3 identified proteins were analyzed using ClustalX at home and BLAST at http://www.ncbi.nlm.nih.gov/. Furthermore, to get the function and other characters of the proteins proteomics, tools at http://www.expasy.org/tools/ were also used. To our knowledge, spot 3 is identifiable as r40c1 protein, whose function is not characterized and that belongs to family PD683597. Spot 4 is identifiable as hypothetical protein OsJ_002947, which has a domain of ATP synthase F1, beta subunit (**Figure 2**). Spots 10 is identifiable as OSJNBb0017I01.1 protein (GenBank: CAE05721.1), which function seem to be Phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (**Table 2**).

As for spot 4, this predicted molecular mass is slightly larger than the apparent molecular mass of the mature peptide as estimated by 2D-PAGE. We think it might be a defective protein. Marilyn and Robert [23] proposed that because the ATPase subunit 2 polypeptide was synthesized in the cytoplasm, it likely was synthesized as a larger precursor with a pre-sequence for mitochondrial targeting and localization. But it is not the case here. Because the protein spot is almost not detectable in the corresponding fertile line's proteomic map, if it is true, the fertile lines can not survive.

4. DISCUSSION

CMS was defined as maternally inherited male sterility resulting from a specific (mitochondrial) gene whose expression impairs the production of viable pollen without otherwise affecting the plant. That means that cytoplasmic male sterility in plants is associated with mitochondrial dysfunction. Up to now, 12 mitochondrion DNA regions associated with CMS have been identified, and most of them are involved in the genes encoding F0F1-ATPase subunits [11]. The N-terminal region of *atp*9 in petunia is present in the CMS-associated *pcf* gene. *atp*6 provides the 5' regulatory sequences for the CMS associated *urf*13-*T* gene in maize [24]. The CMS-associated gene *orf*522 found in sunflowers, co-transcribes with *atpA* and a tissue-specific increase in the level of polyadenylated *atpA-orf*522 transcripts correlates with the tissue-specific instability of *atpA-orf*522 mRNA in male florets of the restored hybrid plants [25]. Rice (*Oryza sativa* L.) CMS-Boro II is associated with an abnormal copy of the mitochondrial gene *atp*6 [12,26] that transcribes aberrant mRNAs containing an additional orf named *orf*79 [27]. In Honglian (HL) rice *orfH*79 is a mitochondria chimeric gene being responsible for the CMS trait [28,29]. Furthermore, RNA editing plays a role in the CMS [10].

Mitochondrion is the site of both the tricarboxylic acid cycle and oxidative phosphorylation pathway and plays a crucial role in energy and carbon metabolism in eukaryotic cells. The oxidative phosphorylation pathway consists of the electron transfer chain (ETC), including complex I–IV, and F0F1-ATPase (complex V). The F1-ATP synthase beta subunit (β-subunit) is a highly con-
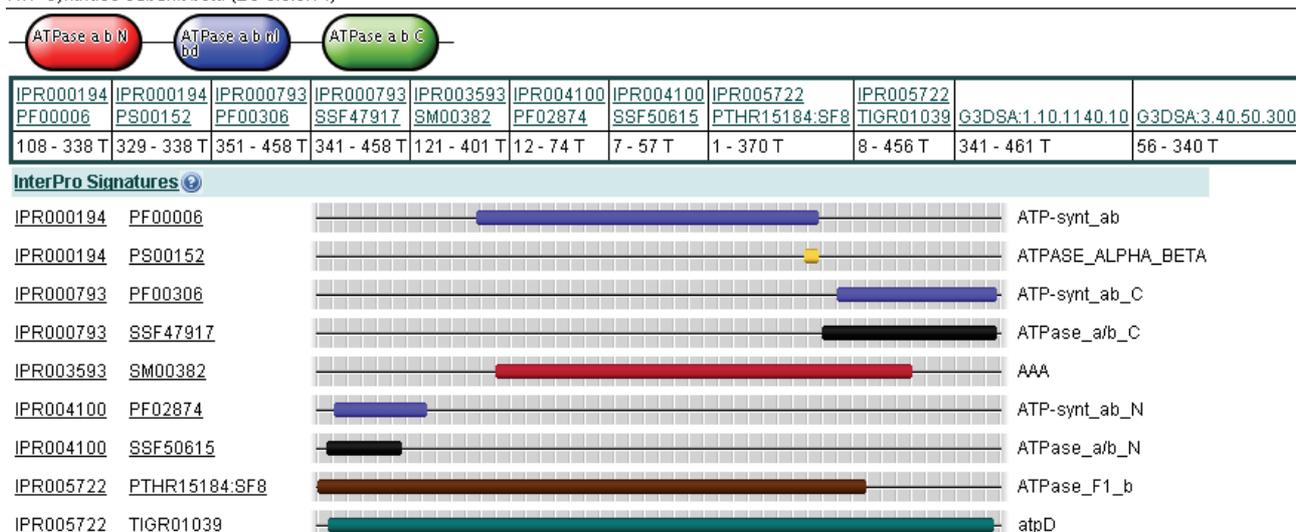
Figure 2. Graphical view of domain structure at http://www.ebi.ac.uk/interpro/ of spot 4.

Table 1. Differentially accumulated proteins identified by mass spectrometry analysis and database searching.

SpotNo	Protein identity and Organism	Accession number	Score [b] (P < 0.05)	Match-ing peptides	Coverage (%)	M_w (KDa)/pI [c]	
						Theoretical	Experimental
1	D-galactose-bind-ing periplasmic protein precursor *Salmonella enterica*	gi|56412897	58	6	24	35.689/6.24	90.0/5.78
2	O-acetylserine lyase [Strepto-coccus suis]	gi|146318090	50	6	27	34.168/ 4.97	87.7/5.85
3	r40c1 protein, *Oryza sativa*	gi|7489571	118	11	47	39.254/6.30	38.70/6.27
	Putative r40c1 protein-rice, *Oryza sativa*	Q8H7M3_OR YSA	115	11	44	42.237/6.25	
	expressed pro-tein, *Oryza sativa*	gi|108707932	96	9	44	31.136/ 6.36	
4	hypothetical protein OsJ_002947, *Oryza sativa*	gi|125571607	95	11	33	49.912/5.05	41.20/6.66
5	Uncharacterized conserved pro-tein, *Streptococcus suis*	gi|146319136	50	5	64	14.271/5.28	52.0/6.42
	Hypothetical protein, *Vibrio cholerae*	gi|9656868	51	6	41	22.527/5.48	
6	hypothetical protein SSU05_1482, *Streptococcus suis*	gi|146319136	53	5	62	14.271/5.28	51.4/6.66
	Uncharacterized conserved pro-tein, *Streptococcus suis*	gi|146321342	48	5	48	18.289/5.19	
7	hypothetical protein At2g22340 [imported], *Arabidopsis thaliana*	gi|25371335	79	6	28	42.644/7.48	94.1/9.58
8	hypothetical protein DDBDRAFT_0 189008, *Dic-tyostelium dis-coideum*	gi|66803747	84	8	11	140.692/ 8.95	78.3/9.16
9	∞	∞	∞	∞	∞	∞	81.4/9.83
10	OSJNBb0017I0 1.1 protein (GenBank: CAE05721.1), *Oryza sativa*	Q7XKF3_OR YSA	81	8	18	41.386 /9.62	41.20/5.37
	H0801D08.7 protein (H0402C08.17 protein), *Oryza sativa*	Q259D2_ORY SA	81	8	18	41.358/9.62	

11	Phage infection protein, Strep-tococcus pyo-genes	Q5XBT6_ST RP6	81	10	31	69.031/8.78	81.0/5.69
	Phage infection protein, Strep-tococcus pyo-genes	gi|50914338	81	10	31	69.031/8.78	
	Monodehydroas-corbate reduc-tase, fruit isozyme (MDAR fruit)	gi|50400840	71	8	15	108205/8.64	
	SalB, Strepto-coccus suis	gi|146320815	48	5	25	25215/7.57	
12	ATP-dependent helicase, *Vibrio cholerae*	gi|9656531	59	7	14	72.324/5.74	83.6/5.73
13	DNA poly-merase, Fowl-pox virus	gi|325379	66	15	?	116.584 /8.34	88.7/9.78
	metallopro-teinase-disintegri n-like protein, Agkistrodon contort*rix* un-known protein, Silkworm	gi|2231613	56	11	35	69.466/6.14	
	acetyl-CoA acetyltransferase , Yersinia pestis	ip|Bmb042428	81	8	78	7.377/4.48	
	acetyl-CoA acetyltransferase , Yersinia pestis	gi|22124378	54	9	29	44.702/6.52	
		gi|16123903	57	9	32	41.239/6.67	

Protein spots (1-13) from **Figure 1** were excised, digested with trypsin, and analysed by MS and database searching. All the proteins were analysed by MALDI-TOF/MS. The GenBank accession number is shown. The Accession numbers correspond to possible proteins matching with the same peptides or part of them. The significance of matches is supported by the number of matching peptides, the combined MOWSE score ($P < 0.05$) of the matching peptides (Score) and the percentage of the predicted protein covered by the matched peptides (Coverage %).

Table 2. Functional analysis of identified proteins by ClustalX, BLAST and proteomics tools.

Spot No	Proteins	Functional analysis	M_w (KDa)/pI		
			Theoreti-cal	Computed	Experi-mental
3	r40c1 protein	Family PD683597 This family was built using psi-blast, with Q8S756_ORYSA as query	39.254/6.30	38.798/6.30	38.70/6.27
4	hypothetical protein OsJ_002947	ATP synthase F1, beta subunit The sequences of ATP synthase F1 alpha and beta subunits are related and both contain a nucleotide-binding site for ATP and ADP. They have a common amino terminal domain but vary at the C-terminus. The beta chain has catalytic activity, while the alpha chain is a regulatory subunit. Proton translocating ATP synthase, F1 beta subunit is homologous to proton translocating ATP synthase archaeal/vacuolar(V1), A subunit.	49.912/5.05	49.798/5.05	41.20/6.66
10	OSJNBb0017I01.1 protein	Phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (By similarity).	41.386 /9.62	41.157/9.62	41.20/5.37

Protein spots (3, 4 and 10) from **Table 1** were analysed and its function were characterised. The Mw (KDa)/pI were computed using Compute pI/Mw tool.

served single copy gene encoding a 480 amino acid protein which is a catalytic site for the ATPase reaction [30]. It plays an important role in the ATPase activity with the alpha subunit. If the β-subunit is defective, it will cause

the dysfunction of F0F1-ATPase, which may impact the energy output of mitochondria, resulting in abnormal anther development with non-functional pollens. It has been reported that there are very highly energy demands

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during pollen development in higher plants [31]. The dysfunction of F0F1-ATPase in CMS line Ying xiang A may impact the energy output of mitochondria, resulting in abnormal anther development with non-functional pollens.

A widely accepted hypothesis on the mechanism of CMS is that the increased demand for respiratory function and cellular energy in the form of ATP during anther development may be compromised by expression of the aberrant mitochondria genes. The mitochondrial enzyme F0F1-ATP synthase synthesizes adenosine triphosphate (ATP), the universal currency of chemical energy in the cell. The ATPase is installed in the inner membrane of the mitochondrion, with the F1-sector protruding into the matrix compartment and the F0-sector embedded in the inner membrane where it forms a proton-translocating channel. Using the pH-gradient between the cytosolic site and the matrix, the membrane embedded F0-part drives the synthesis of ATP in the F1-sector. The latter contains the three nucleotide binding pockets of the enzyme, which are formed mainly by the residues of the three β-subunits, which is thought to be the most conserved in its amino acid sequence [32].

In mitochondria, the majority of ATP is synthesized by F0F1-ATPase and driven by the electron transport on the inner membrane. If there are defects in the F0F1-ATPase, a further disturbance of the electron transport will magnify the disruptions of ATP synthesis, and consequently, the metabolism process in cells will be interfered with. For testing the link between F0F1-ATPase defects and CMS in HL (Hong-lian) rice, Zhang *et al.* [33] used inhibitor of ETC to interfere the respiration of seedlings and observed their growth. The results revealed that seedling growth delayed in the sterile line due to the disruption of phosphorylation, suggesting that F0F1-ATPase activity was suppressed in the CMS plants. Peng *et al.* [29] found that the content of reactive oxygen species (ROS) in the transformants that expressed ORFH79 was increased by 31%, and ATP was decreased by 41% compared with the control. Higher ROS content caused a more swift decrease of F0F1-ATP activity and ATP contents in YtA than those in YtB [34]. Sun *et al.* [35] deduced that reduction of the proteins associated with energy production and lesser ATP equivalents detected in CMS anther indicated that the low level of energy production played an important role in inducing CMS-HL.

In our earlier study, we reported the RNA editing of transcripts of the mitochondrial *atp*9 gene from Ying xiang A and Ying xiang B of the purple rice cytoplasm and discussed the different editing in them [36]. The *atp*9 transcript of Ying xiang A was shown to have no editing sites and the transcript of Ying xiang B was shown to have 2 editing sites with changes affecting the amino acid sequence of the protein product. The editing of the *atp*9 transcript from Ying xiang B was found to change an arginine codon into a stop codon, shortening the protein of Ying xiang B to the "standard" size. And the Ying xiang A transcript, which has no stop codon, cannot be translated to a normal protein. The results demonstrate the important role of RNA editing in the production of the functional ATP9 subunit and RNA editing is associated with cytoplasmic male sterility. In the present study, protein spot 4 was identified to be beta subunit of ATP synthase F1 sector and it is deduced to be a defective protein. Its incorporation into the ATP synthase complex may lead to the dysfunction of F0F1-ATPase. An abnormal ATP synthase may impact the energy output of mitochondria, resulting in abnormal anther development with non-functional pollens in Ying xiang A. Our earlier study also proved that the mitochondrial activity of fertile lines is much higher than that of the sterile lines [17].

In conclusion, CMS of Ying xiang A in rice may have multiple causes. The abnormal protein complex of ATP synthase may cause the dysfunction of mitochondrion. We can propose that the cooperation of abnormal beta subunit and ATP9 protein may cause the cytoplamic male strility of Ying xiang A. Further research should be carried out to characterise the function of other different proteins.

5. ACKNOWLEDGEMENTS

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