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Identification of Differentially Expressed Proteins Associated with Chlorophyll-Deficient Mutant Rice

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Abstract: In plant, chlorophyll-deficient mutants have been employed to study the mechanism of chlorophyll and chloroplast biogenesis. Here, we found a new chlorophyll-deficient mutant rice line (W02S) whose leaves became etiolated at two-leaf stage and turned into green at three-leaf stage different from that of its isogenic rice line (Peiai64S). Compared with Peiai64S, 44 differentially-expressed proteins were selected in the two-leaf stage of W02S by the means of 2-D gel electrophoresis and MALDI-TOF MS analysis. Among the identified ten categories, four highly expressed protein spots involved in the EMP-TCA pathway and protein expressions were selected for real-time quantitative PCR analysis. The mRNA levels of three genes encoding an enolase, a chloroplast 29 kDa ribonucleoprotein and a proteasome alpha subunit were significantly increased in the two-leaf-stage W02S, indicating that a substantial proportion of protein changes is the consequence of altered mRNA levels during the seedling stages of the mutant rice. These new findings lead us to better understand regulatory mechanisms of chlorophyll-deficient phenotype in rice and other plants.

Key words: *Oryza sativa* L., 2-D gel, Real time PCR, Chlorophyll-deficient mutant

INTRODUCTION

With the completion of draft genome sequences of *Oryza sativa* ssp. *indica* (Yu *et al*., 2002) and *japonica* (Goff *et al*., 2002) and the completion of map-based sequences of chromosome 1 for *Oryza sativa* sp. *japonica* cultivar *Nipponbare*, it became feasible to perform large-scale proteomics studies. Many proteomics studies with different cells, tissues and subcellular organelles have been reported in rice (Komatsu *et al*., 2003; Komatsu and Tanaka, 2005; Komatsu, 2005). However, identification of all or most of the expressed proteins in cells remained the greatest challenge due to the huge diversity and dynamic range of the proteins being expressed (Wu and Han, 2006). Recently, functional genomics has been employed to determine the global protein expression profile in biological systems. For example, many studies have utilized genome-wide cDNA or oligonucleotide microarray to measure the mRNA levels in rice (Yazaki *et al*., 2000; Rabbani *et al*., 2003). Although mRNA quantification provides important information with regard to the early stages of transition from genome to cellular machinery, it is not always consistent with the abundance of corresponding proteins (Gygi *et al*., 1999; Ideker *et al*., 2001). In addition, due to various alternative splicing, mRNA processing, protein proteolysis and post transcriptional modifications, many different protein species can be produced from a single gene (Pandey and Mann, 2000; Service, 2001). More importantly, subcellular localization of proteins cannot be accurately predicted based on mRNA abundance. Therefore, gene expression at protein level will ultimately be needed to explain the complex biological phenomenon.

Chlorophyll is a green pigment identified in most plants, bacteria and algae. It plays an indispensable role in the light harvesting in antenna systems and energy transfer in the photosynthesis reaction centers. Chlorophyll biosynthesis is very important to all bacteria, algae and plants. Genetic mutants deficient in chlorophyll biosynthesis and chloroplast biogenesis are important resorts for functional analysis of genes that are involved in the pathways of chlorophyll synthesis. In addition, it will elucidate the relevant biological metabolisms with a consecutive series of intermediates in various organisms by both biochemical and genetic approaches (Alberte *et al*., 1974; Markwell and Osterman, 1992; Markwell *et al*., 1986; Galova *et al*., 2000; Sugimoto *et al*., 2004; Pasini *et al*., 2005). Until now, more

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than 70 chlorophyll-deficient mutants have been identified in rice (Kurata *et al*., 2005). However, the genes that are responsible for the phenotypes of these mutants have not been identified yet (Liu *et al*., 2007). In 2004, we reported a novel TGMS (Thermal-sensitive Genic Male Sterile line) rice mutant (W02S) with chlorophyll-deficient leaf at seedling stages. This albino morphological character has been utilized in purity identification of two-line hybrid rice seed production (Deng *et al*., 2004). While, W02S developed etiolated leaves at the two-leaf stage, its leaves at the three-leaf stage of development were normal. Apart from this phenomenon, W02S and its wild type rice line (Peiai64S) showed identical agronomic characters including the sterility character. However, a comprehensive proteomics analysis for the chlorophyll-deficient rice mutant seedlings has not been performed until now. In this study, we carried out a comprehensive analysis of chlorophyll-deficient rice mutant via proteomics profiling in order to unravel the underlying mechanisms of etiolating phenomenon during the course of chlorophyll metabolism, biogenesis and biochemical processes of the chloroplasts in rice seedlings.

MATERIALS AND METHODS

Plant materials: The research was undertaken from 2004 to 2008 in the Huazhong Agricultural University. A chlorophyll-deficient Thermo-photoperiod sensitive Genic Male Sterile (TGMS) mutant rice line (W02S) and its isogenic rice line (Peiai64S) with normal chlorophyll content were used in this study. The leaves of W02S became etiolated in the seedling stage, while in the mature stages the leaves of W02S were green and normal. W02S was obtained by transferring a mutant character, which was discovered in pollen culture into a new TGMS line (Peiai64S) (Bao *et al*., 2005). The seeds of W02S and Peiai64S were soaked in water for 2 days and then germinated in a thermostatic container for another 2 days before being transferred to the pot culture in late April. In parallel, Peiai64S plants grown in the same conditions were included in the experiment as a control. As the third leaves were fully expanded, the second leaves were harvested from randomly selected seedlings, immediately frozen in liquid nitrogen and stored at -80°C for protein extraction. The third leaves were collected after all the leaves turned into green.

Transmission electron microscopy: The tissues from fresh leaves were collected randomly from the two stages of W02S (W02SW, W02SG) and Peiai64S (P2 and P3). The leaf tissues were cut into pieces of approximately 1 mm in length and pre-fixed in a solution containing 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4). Prefixed samples were fixed in 2% OsO4 in phosphate buffer (0.1 M, pH 7.4) and then dehydrated and embedded in epoxy resin according to Luft (1961) and SPI-812, respectively. Ultrathin sections (70 nm) obtained with a Leica UC6 ultramicrotome were stained with uranyl acetate (Gibbons and Grimstone, 1960) and subsequently with lead citrate (Reynolds, 1963). Images were obtained with a HITACHI H-7650 transmission electron microscope at 80 KV and a Gatan 832 CCD camera.

Protein extraction: Protein extraction by TCA/acetone precipitation was performed according to Damerval *et al*. (1986) but with some minor modifications. Frozen tissues of rice leaves from the two stages of W02S and Peiai64S (W02SW, W02SG, P2 and P3, respectively) were grounded in a mortar with liquid nitrogen and the rice proteins were precipitated with cold acetone containing 10% trichloroacetic acid and 0.07% β-mercaptoethanol. After incubation at -20°C overnight, the mixture was centrifuged at 12000 rpm at 4°C for 1 h. The pellets were washed several times with acetone containing 0.07% β-mercaptoethanol until the pigment was removed. The pellets were lyophilized by SpeedVac Concentrator. The resultant powder was resuspended in 1 mL lysis buffer containing 8 M of urea, 2 M of thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-lyte (pH 4-7). The protein solution was stored at room temperature for 30 min and then centrifuged at 12000 rpm at 10°C for 1 h. RC DC Protein Assay Kit determined the protein concentration.

4. 2-D gel electrophoresis: The protein samples collected from W02SW, W02SG, P2 and P3 were separated by 2-D gel electrophoresis. Isoelectric focusing (IEF) was carried out according to the Bio-Rad manufacturer's methods and product manual. The extracted proteins (2 mg gel^{-1}) were mixed with rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-lyte (pH 4-7), 0.002% bromophenol blue and rehydrated with IPG strips (17 cm with a linear pH gradient from 3 to 10) at 20°C for 14 h. Electrofocusing was performed by using PROTEAN IEF Cell (Bio-Rad, Hercules, CA) at 20°C following the manufacturer's instruction. Prior to the second dimension, the IPG strips were equilibrated with equilibration buffer I (6 M urea, 20% v/v glycerol, 2% SDS, 2% DTT and 0.375 M Tris-HCl pH 8.8) for 15 min and equilibration buffer II (6 M urea, 20% v/v glycerol, 2% SDS, 2.5% iodoacetamide and 0.375 M Tris-HCl pH 8.8) for 15 min. Equilibrated gel strips were placed on top of the SDS-polyacrylamide gel (12% polyacrylamide) prepared according to Laemmli (Laemmli, 1970) and sealed with ReadyPrep overlay agarose (Bio-Rad). Electrophoresis

Table 1: Primers used for the real time quantitative PCR

Gene	Accession No.	Forward primers	Reverse primers
AAC49173	NP001051733	GAGTGAGGGAGTTGATTGT	GGAAACCGAGATGAGATT
NP910055	NP001064223	GGTGATAGCCTGAAGAATG	CAAACCAACAGCCAAATC
XP483663	ABR26075	GCCTCGGGATGATTTCGC	TGATATGGCATTGTTGACTTCT
ABR26075	NP001062437	CCCTCTACCAGACCGACCCA	GCAAATAGCCATATCCACTTCA

was carried out using a PROTEAN II XL Multi-Cell apparatus (Bio-Rad) with a programmable power control at 50 V/gel for 1 h and then 200 V/gel until the dye front reached the bottom of the gel. The separated proteins were visualized by Coomassie Brilliant Blue G-250 staining (Candiano *et al*., 2004). Proteins samples extracted from two independent experiments were analyzed in triplicates for each stage of wild-type and mutant rice lines.

Image analysis and protein identification: Images of the stained gels were acquired with Gel Explorer (Ultra-Lum, Claremont, CA). Detection of the spots and comparison of the images for different gels were performed by using GELLAB II+ (Ultra-Lum). The protein spots that were present only in the samples from W02SW stage were excised from the stained gels. Each small piece of gels with proteins of interest was washed with distilled water for approximately 10 min at the room temperature and destained with 50% acetonitrile in 50 mM ammonium bicarbonate for 1 h at 37°C. Proteins in the gel pieces were reduced with 10 mM DTT in 25 mM of ammonium bicarbonate for 1 h at 56°C and alkylated with 50 mM iodacetic amide in 25 mM ammonium bicarbonate for 45 min at room temperature in a dark environment. Finally, the gel pieces were successively washed by 25 mM ammonium bicarbonate, 25 mM ammonium bicarbonate in water/ACN (50/50) and before being completely dried by SpeedVac Concentrator. The gel pieces were incubated in 2-3 μL modified trypsin solution (10-20 ng μL^{-1} in 25 mM ammonium bicarbonate) for 30 min and digested with 10-15 mL ammonium bicarbonate (25 mM) at 37°C overnight before addition of 0.1% TFA to stop the digestion reaction. All these steps were performed precisely as described by operation manual.After digestion, the protein peptides were loaded onto a target well of the AnchorChip plate. After drying, 0.1 μL CHCA (4 mg mL^{-1} in 70% ACN, 0.1% TFA) matrix solution was added onto the target well, followed by air drying. Subsequently, the samples were washed twice with 0.1% TFA for desalting. Tryptic peptide masses were determined with Ultraflex II MALDI-TOF/TOF. The PMFs obtained from MALDI-TOF MS were searched against rice data in the NCBInr database using the MASCOT software (Matrix Science, London, UK). The proteins were identified according to their MOWSE score, sequence coverage and functions as documented in the NCBI database.

Real-Time quantitative PCR analysis: Total RNA was isolated from W02SW and W02SG by using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. In order to synthesize single-stranded cDNA, reverse transcription reactions were performed using Reverse Transcription System (Promega, Madison, WI). Real-time PCR was performed using a SYBR Green PCR MasterMix on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). PCR reaction (20 μL) included 1 μL template cDNA, 10 μL SYBR Green PCR Master Mix, 0.5 μL ROX reference dye (Invitrogen, Carlsbad, CA), 0.5 μM forward primer and 0.5 μM reverse primer. The reactions were incubated in a 96-well plate at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were run in triplicates. Amplification of *Actin* gene was included as an internal reference. The threshold cycle (Ct) is defined as the fractional cycle number at which the florescence passes the fixed threshold. The primers for each gene (Table 1) were designed by Primer premier 5.0.

RESULTS AND DISCUSSION

Phenotypic observations of chlorophyll-deficient mutant W02S and wild type Peiai64S seedlings: Our earlier report showed that the W02S white leaf phenotype is recessive (Deng *et al*., 2004) and it is controlled by a pair of recessive genes. In this study, we further determined the physiological and biochemical alteration in this mutant. The seedlings of W02S showed an apparent white color until the three-leaf stage (Fig. 1a). The 1st to 3rd leaves turned into green after the 4th leaves were fully expanded (Fig. 1c). Peiai64S was slightly bigger and showed a stable chlorophyll content and green color throughout the whole seedling stages (Fig. 1b, d, p2 and p3, respectively).

Transmission Electron Microscopic (TEM) analysis was performed for leaves collected from the two-leaf and three-leaf stages of W02S and Peiai64S. At the two-leaf stage, the W02S seedlings were observed to be pale in color and contain under-developed protoplasts (Fig. 2a-d), but produced normal chloroplasts at the three-leaf stage. In contrast, the color of Peiai64S seedlings was stable and the chloroplasts developed normally during the seedling stage. These results indicated that the chloroplast biogenesis and chlorophyll biosynthesis in W02S was retarded to some degree at the two-leaf stage.

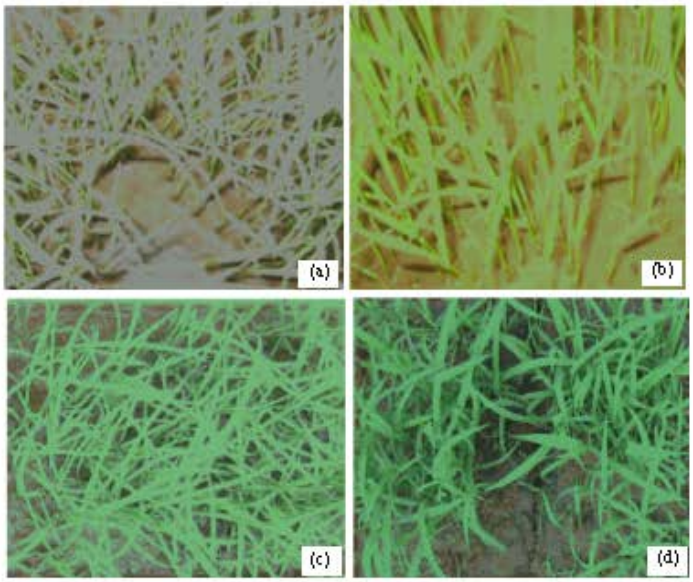

Fig. 1: Phenotypic observations of chlorophyll-deficiency mutant rice line W02S and its isogenic line Peiai64S. (a) The leaves of W02S at two-leaf stage (W02SW) were etiolated and (c) then became green gradually with the development of leaves at four-leaf stage (W02SWG). In contrast, the leaves of Peiai64S were normal and green in both (b) two-leaf (P2) and (d) four-leaf (P3) stages

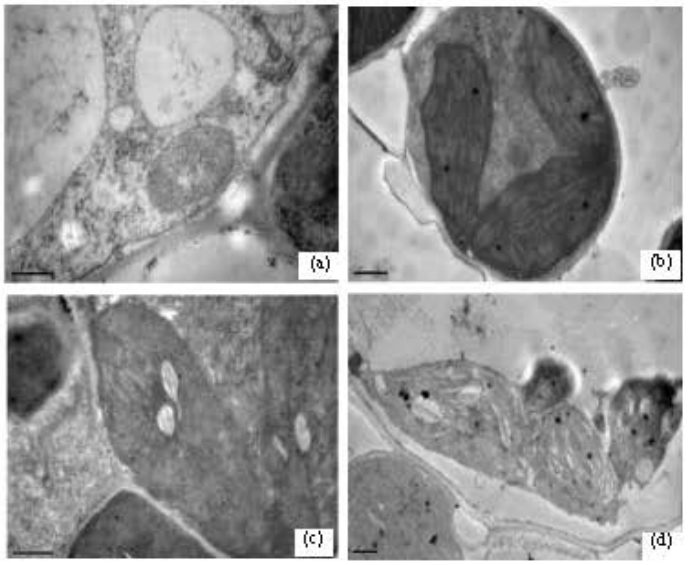

Fig. 2: Transmission Electron Microscopic (TEM) images of W02S and Peiai64S at the two-leaf and three-leaf stages. (a) TEM image for W02S at two-leaf stage of (W02SW), (b) TEM image for W02S at four-leaf stage (W02SG), (c) TEM images for Peiai64S at two-leaf stage (P2) and (d) TEM images for Peiai64S at four-leaf stage (P3)

Proteomic analysis of chlorophyll-deficient mutant W02S and wild type Peiai64S seedlings: To identify the differences of global protein expression between the seedlings of W02S and Peiai64S before the four-leaf stage, we collected leaf samples of both W02S and Peiai64S at two-leaf (W02SW, P2) and three-leaf (W02SG, P3) stages. Global protein expression for all these four samples was performed by 2-D gel electrophoresis followed by Coomassie brilliant blue staining. By using GELLAB II^{+} software, the protein spots in different gels could be easily cross-matched to each other, demonstrating the high reproducibility and low background of this approach.

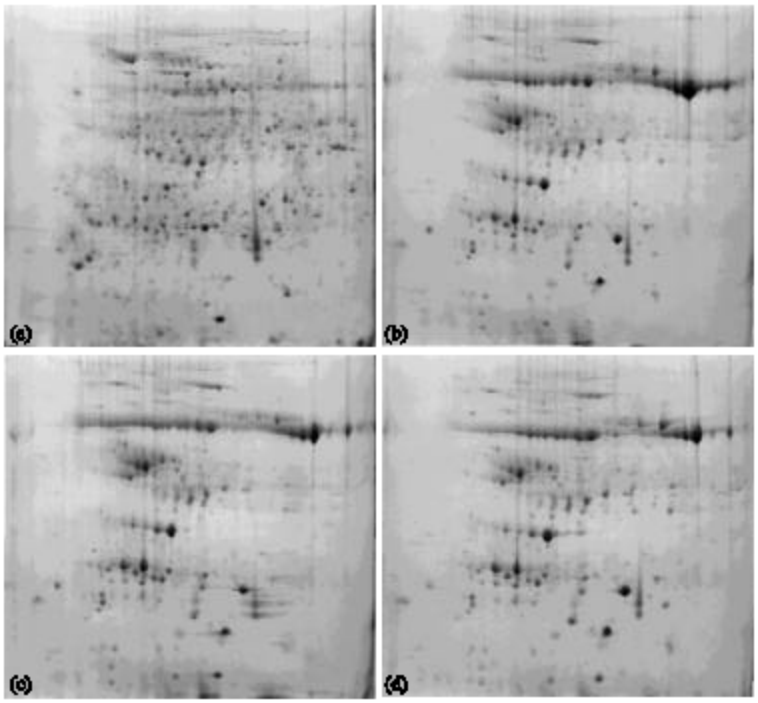

Fig. 3: A total of 44 differential expressed protein spots were identified exclusively in the 2-D gel electrophoresis image of seedling from the (a, b) chlorophyll-deficient mutant rice (W02SW, W02SG) and (c, d) normal variety Peiai64S (P2 and P3). The linear range of pH for 2-D gel electrophoresis was from 4 to 7

All the proteins spots on the 2-D gels were compared for W02SW, W02SG, P2 and P3. Finally, a total of 44 protein spots were identified to be highly expressed only in the leaves of W02SW (Fig. 3a-d).

Putative functions of different categories of highly expressed genes in W02SW: Based on the putative functions, these 44 proteins were classified into 10 categories (Table 2 and 3). The results showed that 9.1% of the highly expressed proteins are involved in the two-component signal transduction system (Mizuno *et al*., 1998) and the regulation of plant response to the light and osmotic stress (Stock *et al*., 2000; Hwang *et al*., 2002; Lohrmann and Harter, 2002; Du *et al*., 2007; Mira-Rodado *et al*., 2007). The second type of proteins plays an important role in the regulation of the EMP-TCA-ETS pathway involved in the basic metabolism of rice seedlings. Surprisingly, only few genes were found to be directly related with the biogenesis of chloroplast and chlorophyll, suggesting that the etiolating process is a regulatory consequence of multiple signal and metabolism pathways.

Real-time quantitative PCR analysis of W02S in the etiolating and green seedlings: To further confirm that the mRNA level is increased for the proteins that were highly expressed in the samples collected from W02S at two-leaf stage, we selected four highly expressed genes (No. 233, No. 491, No. 492 and No. 617) for real-time quantitative PCR analysis. All these four proteins were expressed in the 2-leaf stage of W02S seedlings at a higher level than in any other stages of both rice lines (Table 4). These four genes encode one unknown protein (Os03g0822200), an enolase (Os10g0167300), a chloroplast 29 kDa ribonucleoprotein (gi|149392545) and a proteasome alpha subunit (Os08g0548900). The W02S relative mRNA level of the gene encoding the unknown protein (Os03g0822200) was not significantly changed from two-leaf stage to the three-leaf stage (Table 4). The mRNA levels for the genes encoding the remaining three proteins were significantly increased in the samples of W02S collected at two-leaf stage compared with those collected from three-leaf stage (Table 4). These results indicate that the majority of proteins that were highly expressed in the two-leaf stage of W02S resulted from their high RNA transcription levels.

W02S is a TGMS line with etiolated leaves before 4th leaf stage. This albino characteristic has been used to identify the purity of hybrid rice seeds. Thus, it was necessary to determine the underlying physiological and molecular mechanisms of the albino phenomena. Here, W02S and its isogeneic rice line (Peiai64S) and applied in

Table 2: 44 highly expressed proteins identified in W02SW on 2-D gel

Spot No.	Description	Accession No.	MASCOT score	Sequence coverage (%)	Theoretica-l Mr/pI	Gel Mr/pI
5	Hypothetical protein OsJ_015253	EAZ31770	65	22	94.1/5.5	96.1/6.2
7	Hypothetical protein OsJ_008986	EAZ25503	107	40	11.6/6.4	93.1/6.1
9	aconitate hydratase	NP_001048898	92	25	106.9/6.5	91.6/6.1
23	hypothetical protein OsI_037917	EAY83958	70	39	86.9/6.2	80.8/6.3
24	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	ABG22093	68	33	79.2/7.2	79.9/6.1
25	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	NP_001067313	87	38	84.9/5.9	79.9/6.2
32	NADH-ubiquinone oxidoreductase 75 kDa	NP_001051072	115	50	82.1/5.8	79.5/5.7
37	subunit Arabidopsis thaliana 130.7kDa hypothetical protein family protein.	NP_001049504	92	26	79.8/6.7	76.2/6.5
46	Glycosyl hydrolases family 38 protein	ABG22500	91	23	114.4/5.9	70.4/5.9
47	endosperm lumenal binding protein	NP_001045675	107	52	73.6/5.0	69.3/5.2
50	Hypothetical protein OsJ_017202	EAZ33719	104	49	73.7/5.1	65.8/4.9
54	P0028E10.3	BAB39900	73	30	61.2/5.4	66.2/4.9
55	Hypothetical protein OsJ_017202	EAZ33719	112	50	73.7/5.1	65.2/5.0
57	Heat shock cognate protein 70.	NP_001051724	70	30	71.7/5.1	61.5/5.5
82	Phosphoglyceromutase	NP_001044625	86	38	61.0/5.4	61.5/5.7
83	Peptidylprolyl isomerase	NP_001062292	88	46	64.4/5.2	55.3/5.5
117	Hypothetical protein OsI_017641	EAY96408	92	45	53.5/6.1	52.1/5.5
118	Acetohydroxy-acid reductoisomerase	NP_001056384	113	44	62.7/6.0	55.4/5.6
153	Wheat adenosylhomocysteinase-like protein	AAO72664	82	44	53.9/5.6	52.1/5.5
208	retrotransposon protein, putative	ABB47489	72	32	48.8/5.2	45.5/5.3
233	Enolase	NP_001064223	86	63	48.2/5.5	44.0/6.0
261	Glutamine synthetase	NP_001048045	63	47	39.4/5.5	42.0/6.1
317	Glutamine synthetase	NP_001048045	95	60	39.4/5.5	39.1/5.5
318	Hypothetical protein OsJ_002934	EAZ13109	75	43	43.7/6.6	38.8/5.7
319	Uroporphyrinogen decarboxylase	NP_001050049	82	45	43.0/6.2	39.0/6.0
321	Peroxidase	NP_001042800	76	61	38.4/6.5	38.9/6.7
322	Adenosine kinase-like protein	AAO72629	76	45	40.6/5.6	39.0/5.0
323	Reversibly glycosylated polypeptide	CAA77235	80	37	41.9/5.8	39.0/5.6
380	beta-1,3-glucanase	BAA77785	71	61	35.7/7.0	35.5/6.4
381	hydroxyproline-rich glycoprotein	NP_00104999	58	46	39.2/6.3	35.5/6.5
420	ADP ribosylation GTPase-like protein	AAO72580	57	25	33.5/5.5	33.1/5.7
451	Cysteine synthase	NP_001067319	76	45	33.9/5.4	30.9/5.4
469	Fructose-bisphosphate aldolase	NP_001055494	83	54	36.7/6.6	30.0/6.3
473	Putative GTP-binding protein	BAD23597	79	44	24.1/5.7	29.8/4.9
475	H0323C08.1	CAJ86211	65	44	29.9/9.2	29.8/6.8
491	Unknown protein	NP_001051733	85	55	28.0/6.3	29.1/6.3
492	20S proteasome subunit alpha-4	NP_001062437	72	30	27.4/6.9	29.1/6.4
518	hydroxyacyl glutathione hydrolase	NP_001050016	78	79	29.0/5.4	28.1/5.6
529	hypothetical protein OsI_035415	EAY81456	64	29	27.4/4.9	27.6/4.5
552	Glutathione S-transferase	NP_001048884	87	54	25.8/6.7	26.5/6.8
643	Hypothetical protein OsI_005908	EAY84675	73	31	23.9/6.4	23.4/6.7
656	Unknown protein	BAD25069	48	34	23.9/5.3	22.7/4.4
685	Temperature stress-induced lipocalin	NP_001061901	68	51	20.7/6.1	21.4/6.6
617	Chloroplast 29 kDa ribonucleoprotein	ABR26075	122	68	22.2/4.5	24.2/4.5

Table 3: Categories and functions of highly expressed proteins in W02SW

Category	No. of differential expresses genes	Percent	Putative metabolism pathways
I	15	34.1	Hypothetical proteins
II	6	13.6	EMP-TCA-ETS
III	3	6.8	Met biosynthesis
IV	3	6.8	Glutamine biosynthesis
V	5	11.4	Antioxidation process
VI	2	4.5	Carbohydrate anabolism
VII	2	4.5	Protein anabolism
VIII	1	2.3	Nucleotide anabolism
IX	4	9.1	Signal transduction
X	3	6.8	Chloroplast biogenesis

Table 4: The protein and RNA expression levels of four representative genes

	Protein expression levels			RNA expression level		
Gene	W02SW	W02SG	P2	P3	W02SW	W02SG
AAC49173	19.94	5.49	7.97	9.05	0.10	0.04
NP910055	30.76	3.85	2.20	9.37	1.56	0.41
XP483663	50.62	10.26	1.72	1.66	2.01	0.46
ABR26075	40.35	1.65	4.60	2.35	3.31	0.58

the performed proteomics analysis at their seedling stages. From the above analysis, four representative highly expressed genes encoding the proteins (No. 233, No. 491, No. 492 and No. 617) were further subjected to real-time quantitative PCR in the two-leaf stage of W02S for function analysis.

No.491 was an unknown protein (RF: NP_910055.1) and No.617 was a chloroplast 29 kDa ribonucleoprotein, CP29A (ABR26075) involved in stabilizing the stored

chloroplast mRNAs and recruiting site-specific factors mediating RNA metabolism (Wang *et al*., 2006). cpRNPs are abundant in the stroma of chloroplasts and form complexes with ribosome-free mRNAs to protect them from degradation by RNases (Nakamura *et al*., 2001). In *Arabidopsis*, a nuclear RNA-binding protein was reported to be involved in the stamen and carpel development (Li *et al*., 2001). Here we showed that chloroplast RNA-binding protein, CP29A, was highly expressed in the etiolated leaves in the seedling stage of W02SW (Table 4), suggesting that, it is involved in the initial chloroplast development in the etiolated leaves of the chlorophyll-deficient mutant rice line W02S.

No. 492 was a proteasome alpha subunit (RF: XP_483663.1). Proteasome is a multicatalytic proteinase complex that is involved in the ATP/ubiquitin-dependent proteolytic pathway. There are two types of proteasomes 20S in eukaryotes. 26S proteasome consists of a 19S/22S regulatory complex, the 20S proteasome is a proteolytic complex involved in the degradation of misfolded or truncated proteins (Coux *et al*., 1996; Genschik *et al*., 1998; Woffenden *et al*., 1998; Girod *et al*., 1999) and involved in the oxidative stresses (Chondrogianni and Gonos, 2007; Chondrogianni *et al*., 2005). And interestingly, proteasome alpha subunit was highly expressed in the etiolated mutant rice, endogenous physiological disequilibrium induced the oxidative stress related gene expression, perhaps prepared for turning green after four leaf stage.

The enolase (No.233, GB:AAC49173.1), also known as phosphopyruvate dehydratase, is a metalloenzyme that catalyzes 2-phosphoglycerate (2-PG) to (PEP) phosphoenolpyruvate in glycolysis pathway. This gene transcription was induced and responded to salt, low and high temperature and anaerobic stresses. Enolase also interacted with and induced the tRNA conformational changes in yeast (Entelis *et al*., 2006). In this report, that the elevated levels of protein and mRNA of enolase gene in etiolated mutant at seedling stage (Fig. 3, Table 4) implied its novel function in the mitochondrial respiration to provides cytosolic ATP for activating the outward-rectifying K^+ channels in the chlorophyll-deficient mutant rice (Goh *et al*., 2004).

In conclusion, 44 proteins were identified to be highly expressed in the chlorophyll-deficient rice at the two-leaf stage. Among them, 4 genes were increased at mRNA level and interestingly, these four genes are involved in the EMP-TCA pathway and gene expression in the course of the etiolating of W02S, These new findings leads toward the mechanisms of chlorophyll-deficient phenotype in rice and other plants.

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