BMB
reports

Proteome analysis of chloroplast proteins in stage albinism line of winter wheat (*triticum aestivum*) *FA85*

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The "stage albinism line of winter wheat" *FA85* was a specific natural mutant strain on leaf color. This physiological mutation was controlled by cytogene. In order to reveal the genetic and biochemical mechanism of albinism, 2-DE was used to investigate the difference of chloroplast protein expression pattern between *FA85* and its parent wheat *Aibian 1*. From the results of 2-DE gels analysis, approximately 683 spots were detected on each gel, and 57 spots were expressed differently at least two-fold. Using MALDI-TOF/TOF MS, 14 of 57 spots were identified, which could be categorized into four classes: carbon metabolism, energy metabolism, defense/stress response and signal transduction. Compared with the parent wheat, the expression of ATPase-γ and GP1-α was up-regulated in *FA85*, and of other proteins was down-regulated. Together, we concluded that the expression of chloroplast proteins had changed obviously in *FA85*, which might be related to the leaf color mutant. [BMB reports 2009; 42(7): 450-455]

INTRODUCTION

The leaf color mutants have been reported in many high plants such as rice (1), tomato (2) and Arabidopsis thaliana (3). Up to now, most of the mutants were controlled by the nucleus genes (4, 5), however, there are few reports on plant albinism controlled by cytogene. Luckily, one specific "stage albinism line of winter wheat" has been obtained in our laboratory since 1985, called as *FA85*. It is a natural mutant strain from the parent dwarf wheat *Aibian 1*, could show albinism of all aerial part in the green up period in early spring, and would return green with temperature increasing gradually. In the past twenty years, the cell ultrastructure, physiological, biochemical, molecule and genetic characters of *FA85* have been studied well (6-8). It has been proved that the leaf color was controlled by the cytogene, especially related to the chloroplast (6). We persuaded that it was the chloroplast genomic DNA to determine the mutation of *FA85*. The physiological quality of *FA85* has been transferred into other common wheat by hybridization and backcross (6).

Proteome analysis at the level of subcellular structures represents an analytical strategy. One of the key potentials of this strategy is the capability to enhance the understanding of the biochemical machinery for subsequently functional studies. The progress of proteomics and its related technologies over the last decade are based on two major developments- the progress of proteomics and its related technologies. (9-11).

2-DE gel eletrophoretics (2-DE gels) is a classical method for proteomics analysis, which procedures based on isoelectric point (pI) and Mass for separation, in spite 2-DE gels has some shortcoming, for example, it is difficult to analyze the low abundance and limited solubility proteins (12). Biological mass spectrometry (MS) is the key technique used for proteome research. The successful combination of 2-DE and MS/MS analysis allowed the comparison of theoretical pI and molecular mass with the experimentally determined values for each of the pI (13).

Chloroplasts are of particular interest for plant biologists because of their complex biochemical pathways for essential metabolic functions. Information from the chloroplast proteome will provide new insights into pathway compart mentalization and protein sorting (13) in many species, such as the chloroplast proteome of arabidopsis (14), rice (15) and maize (16). And they can identify the differential expression of chloroplast proteins under the adversity conditions between the wild type and the mutant.

The aim of this work is to analyze the chloroplast proteome of the *Aibian 1* and *FA85* and the difference or relation between the investigated species, and to explore the mechanism of leaf color mutation of *FA85*.

RESULTS

Chloroplast isolation and protein seperation

As a special material, *FA85* has been found since 1985. With

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Received 31 October 2008, Accepted 3 March 2009

Keywords: Chloroplast proteins, *FA85* mutant, MALDI-TOF/TOF MS, Proteome, 2-DE gel

the accumulation of low-temperature, its younger leaves will gradually turn albinism in January in the Northwest of China (Fig. 1B, C, D), and will turn into green with the increase of temperature in March. Using the method of Gong (17), the complete wheat chloroplasts were obtained. After the extraction by lysis buffer (18) and ultracentrifugation at 4°C, we got chloroplast proteins. The concentration was 9.31 mg/ml in *FA85* and 11.17 mg/ml in *Aibian 1*, respectively, measured with the Bradford method (19).

The results of 2-DE

Results from 2-DE gels showed differential protein expression patterns between A and F (Fig. 2). Moreover, we analyzed the image gels with the PDQuest8.1.0 (Bio-Rad). Approximately 683 protein spots were reproducibly detected in gels with silver staining. However, 57 spots were expressed differentially more than two-fold between A and F proteins. Among these spots, we found 5 proteins were up-regulated and 45 were down-regulated in F chloroplast proteins compared with A, however, 7 spots are absent in F.

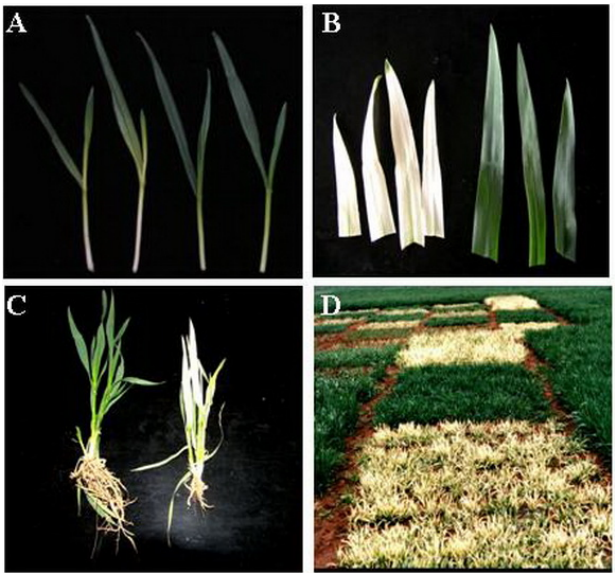

Fig. 1. The material picture of dwarf wheat *Aibian 1* and *FA85* mutant. (A) Young plants (just before albinism), (B) Mature leaves, (C) Plants of *Aibian 1* and *FA85* in field, (D) *Aibian 1* and *FA85* in field.

Identification of proteins

To the chosen 14 spots, the image also showed that 10 spots displayed more than ten-fold expression variation and 4 spots that nearly did not exist in F. All the 14 spots were tested by MALDI-TOF/TOF-MS. Then, the mass spectra fragments data were searched in the Swiss-Prot database. Results showed that 14 spots belong to 11 different proteins, and each one has more than 98% homology to the known related proteins (Table 1).

Among the 14 spots, 2 were up-regulated in F compared with A, and the others were down-regulated (Fig. 3). Most of the down-regulated proteins in F were related to carbohydrate and energy metabolism (Table 1). We could predicate that the metabolism system of the *FA85* have been influenced apparently. The 11 proteins could be classified to four groups related to carbohydrate metabolism, signal transduction, energy metabolism and defense/stress response (Table 1).

Rubisco large and small chain (spot 9, 1001, 2003 and 8003), 2,3-bisphosphoglycerate (2,3-BPG) mutase (spot 3707), Glutamine synthetase (GS) leaf isozyme (spot 403) and cp31BHv (spot 114) are the main enzymes and proteins related to the carbohydrate metabolism. The expression of them was down-regulated in F. Maybe this change would have influence on the structure formation and energy metabolism of chloroplast in the *FA85*.

ATP synthase subunit gamma subunit (ATPase-γ, spot 6315) and cytochrome b6-f complex iron-sulfur subunit (cyt b6/f FeS, spot 5005) belong to energy metabolism proteins. They are the important enzymes in the energy metabolism. Our results showed that expression of the ATPase-γ was significantly increased in the *FA85* compared with *Aibian 1*.

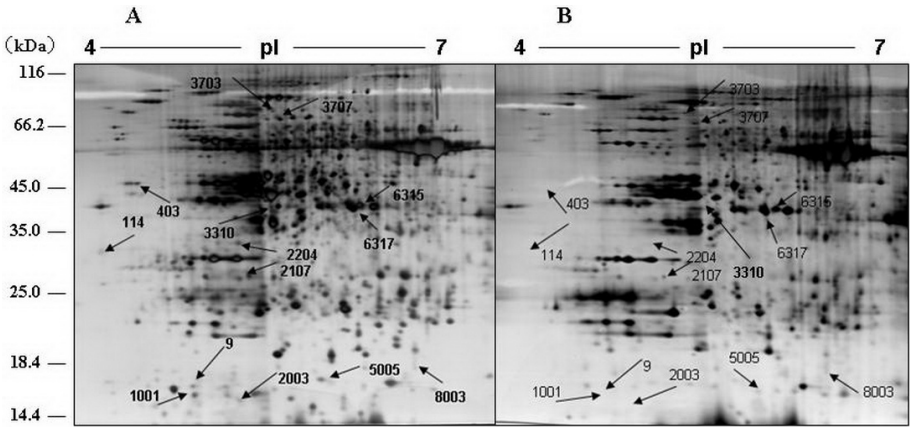

Fig. 2. 2-DE analysis of 14 spots from the 57 spots selected from chloroplast proteins. (A) dwarf wheat *Aibian 1*, (B) *FA85*.

Table 1. Protein identification of spots on 2-DE gels in *Aibian 1* (A) and *FA85* (F)

Spot no.	Protein name	SwissPort no.	PM	SC	Mw/pI	Ratio F/A	Ca
1001	Ribulose bisphosphate carboxylase large chain precursor	P31198	3	P	5.92/4.81	0	A
8003	Ribulose bisphosphate carboxylase large chain	Q9GCW1	7	P	52.6/6.24	0.23	A
9	Ribulose bisphosphate carboxylase large chain	Q8MFQ0	4	P	48.9/6.77	0	A
2003	Ribulose bisphosphate carboxylase small chain	Q40004	11	N	19.4/8.98	0	A
3707	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	P35494	11	N	61.0/5.98	0.4	A
2204	Fructose-bisphosphate aldolase	Q40677	6	N	42.0/6.38	0.15	A
3310	Fructose-bisphosphate aldolase	Q40677	5	N	42.0/6.38	0	A
403	Glutamine synthetase leaf isozyme	P13564	2	N	47.1/5.11	0.12	A
114	cp31BHv	O81988	10	N	30.7/4.76	0.1	A
6315	ATP synthase subunit gamma	P0C1M0	6	N	39.8/8.44	12.66	B
5005	Cytochrome b6-f complex ironsulfur subunit	Q7X9A6	6	P	23.7/8.47	0.2	B
2107	Non-intrinsic ABC protein 7	Q9CAF5	5	N	36.9/6.62	0.46	C
6317	Guanine nucleotide binding protein alpha-1 subunit	P18064	15	N	44.5/5.96	3.17	C
3703	Heat shock 70 kDa protein	Q01899	13	N	72.5/5.95	0.19	D

PM: number of peptides matched; SC: coding site; Ca: category; A: carbohydrate metabolism; B: energy metabolism; C: signal transduction, D: defense/stress response; P: plastid; N: nucleus.

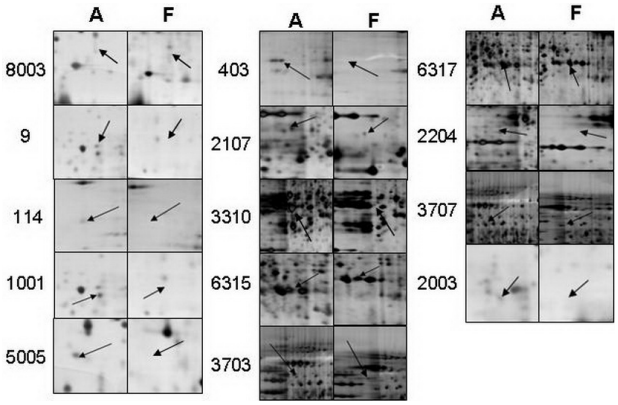

Fig. 3. The expression difference of every protein spots showed in 2-DE gels. (A) dwarf wheat *Aibian 1*, (F) *FA85*.

We also detected the variation of non-intrinsic ATP-binding cassette (ABC, spot 2107) protein 7 and Guanine nucleotide-binding protein alpha-1 subunit (GP1-α, spot 6317), which are the important signal transduction proteins in chloroplast. Expression of GP1-α have been up-regulated in the *FA85*, but non-intrinsic ABC protein 7 was down-regulated.

Hsp70 (spot 3703) is one of defense/stress response proteins, and it was down-regulated in the *FA85*, too.

DISCUSSION

Selected proteins analysis

Proteome analysis of the *FA85* suggested the functions of chloroplast proteins and the biochemical mechanism of the wheat leaf albinism. Among the investigated proteins, only two are encoded by chloroplast DNA (Rubisco-L and cyt b6/f FeS), most of them are encoded by nucleus. Why the identified proteins encoded by chloroplast were few? Indeed, the plastid DNA in higher plants encodes for less than 100 proteins, and the nuclear genome is responsible for the encoding of more than 95% proteins inside the chloroplast proteome (20). Probably, the identified proteins may be related directly or indirectly to *FA85*, the function of every special protein would be verified further.

Molecular masses and pI

Our experimental results suggest that most of the spots are matched closely between the predicted and experimental pI, but the predicted pI of 3 spots (2003, 6315, 5005) is much higher than of the experimental on the gels. This discrepancy might be a consequence of post-translational modification (20). On the other hand, some proteins appeared with more than one spot (Table 1), which suggest these proteins might be isoforms, post-translational modifications of one protein or specific or non-specific protein degradation. The deduced pI and MS of the few spots related to the same protein are variable too. For example, 3 different MS are predicted for the Rubisco-L, the reason of which is possible that the spots matched the fragment of wheat chloroplast from different species in the database. Similar results were reported in other higher plants (21).

Carbon metabolism

Among all the biochemical reaction of the chloroplast, carbo-

hydrate metabolism is generally considered as the major process. In our study, many proteins taking part in this process have been varied obviously in leaf color mutant–*FA85*, such as cp31BHv, GS etc.

2, 3-BPG mutase and Fructose bisphosphate aldolase are the important enzymes of the Carbon Metabolism in chloroplast. They were down-regulated largely in *FA85* (Table 1). Regina *et al*. (22) has reported that FBPase was related to the heat-induced accumulation in the oat chloroplast. Perhaps, to our study, FBPase down-regulated expression is also related to the low-temperature accumulation.

The protein cp31BHv is one of chloroplast RNA-binding proteins. This protein was thought to be associated with the chloroplast ribosomal complex, and correlated with the stage of the leaf development (23). Northern blotting in the barley has showed that the transcript accumulation of cp31BHv was clearly stimulated by light (24). This protein may be relate to the albinism of the mutant by influencing the process of leaf development and photosynthesis. Another, the albinism of *FA85* was sensitive to the low temperature in winter. Whether the cp31BHv of *FA85* was clearly influenced by the lower nature temperature in fields, needs to be proved by further work.

GS is a key enzyme in the nitrogen assimilatory process as it catalyses the first step in the conversion of inorganic nitrogen into an organic form (25). Plant GS is an octameric enzyme that occurs as distinct isoenzymes located in the cytosol (GS_1) and in the plastids (GS_2). GS_2 is regulated by phosphorylation and 14-3-3 interaction (25). In our study, GS_2 expression is down-regulated in the *FA85*. So, the down regulation must have influenced the nitrogen assimilatory and carbon metabolism largely in the chlorolplast of *FA85*.

Energy metabolism proteins

Energy metabolism is the basis of all the life activities. ATP synthesis widely exists in the mitochondria and chloroplast as the main enzyme of the ATP biosynthetic pathway. As membrane-bound enzyme complexes and ion transporters (26), it produces ATP from ADP in the presence of a proton gradient across the membrane (27). In the chloroplast, it mainly takes part in the photophosporylation reaction. In our study, with the accumulation of low temperature, it's expression was significantly up-regulated in the F compared with A.

Cytb6/f FeS mainly take part in the transfer electrons from a liposoluble quinol to a hydrosoluble protein, plastocyanin or cytochrome *c*, and couple the resulting electron free energy drop to setting up a transmembrane proton electrochemical potential (28). In *FA85*, its down-regulation should impact on the electron transfer and oxidative phosphorylation, and lead to the disorder of ATP synthesis.

The two enzymes above-mentioned both attend the energy metabolism process, but locate in different site of the path, and have dissimilar expression patterns in *FA85*. We could not explain the reason now, but we hypothesized that the activity of ATPase simulated obviously in *FA85* could cancel out the damage of the electron transfer by down-regulation of cyt b6/f FeS, which was the active response to the surrounding low temperature in *FA85*.

Defense/stress response

The heat-inducible proteins termed "heat-shock proteins (Hsps)" constitute an important part of the stress-responsive proteins (29). Hsp70 was thought to act as molecular chaperons, repair and aid in the renaturation of stress damaged proteins. In addition to heat stress, plant Hsp are also accumulate in response to a large number of other stress, such as heavy metals, oxidative stress, salt, chilling and anoxic conditions (30). Some reports revealed that the levels of Hsp70 do not change to any extent during the stress treatments in maize (31) and rice (32). In *FA85*, the expression of Hsp70 was down-regulated, which suggested that the stress-response system of *FA85* was abnormal, this may be another important reason of the mutation of leaf color in chill condition.

Signal transduction

ABC protein shares a highly conserved ATPase domain. It has been demonstrated to bind and hydrolyze ATP, thereby providing energy for a large number of fundamental biological processes (33). Møller *et al*. (34) reported that ABC plays a role in phytochrome signal transduction. Plants share a specific set of ABC families with bacteria and most notably cyanobacteria. ABC X proteins are located or predicted to be located in chloroplast (35). They involved in intercompartmental communication of light signaling between plastids and the nucleus. Non-intrinsic ABC protein 7 belong to the ABC X proteins, were down-regulated in *FA85*. It provided that the light signal transduction were influnced in the mutant, the photosystem maybe abnormal and the synthesis of photopigment could be blocked.

G protein is involved as modulators or transducers in various transmembrane signaling systems. It participates in signal transduction by transferring signals from cell surface receptors to intracellular effector molecules (36). G protein is composed of three units-alpha, beta and gamma. The alpha chain contains the guanine nucleotide binding site, is believed to mediate signal transduction between a variety of receptor and effectors. Our results revealed that the expression of the GP1-α was up-regulated in *FA85*, which developed the important clue on the signal transduction path of *FA85* response to the lower temperature.

Through the 2-DE gels analysis of the chloroplast proteins, we know that the expression of many proteins changed between *FA85* and the parent wheat *Aibian 1*. All of them were the important proteins to the chloroplast development and function. Their variation has effected the abnormal metabolize of the mutant, especially on energy metabolism and signal transduction, and were related to the albinism of the *FA85* in chill condition. We hope the biochemical and physiological mechanism of albinism would be soon described clearly by

our further works.

MATERIALS AND METHODS

Plant materials and chloroplast isolation

The dwarf wheat *Aibian 1* (A) and albinism mutant *FA85* (F) were grown under natural field conditions on October. The samples were observed and checked every two days from the January of next year. When the tender leaf just before albinism turn on and temperature at about 0°C (Fig. 1A), 30 g leaf materials were used for the isolation of chloroplast using the method of Gong (17).

Protein extraction and quantification

Chloroplast pellets were suspended in 40 ml resuspension buffer (20 mM Mops, 1 mM phenylmethylsulfonyl fluoride, 50 mM EDTA [pH7.0]) (20) and proteins were precipitated 2 h at -20°C using 10% trichloroacetic acid, then centrifuged for 15 min at 15,000 g. The supernatant was discarded. Etioplast were washed three times with cold acetone. Further, the etioplasts were suspended in an extraction buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS and 65 mM DTT). Proteins were extracted for 1 h at room temperature with vortex. After centrifugation at 40,000 g for 1 h at 4°C, insoluble material was discarded and soluble fraction was used for 2-DE gels. The quantification protein was determined by the Bradford method (19) with bovine serum albumin (BSA) as a standard.

2-DE gels

Extracted proteins were analyzed by 2-DE and repeated three times independently using proteins from each of the two samples A and F. IEF was performed using an IPGphor IEF system (Bio-Rad). To each sample, 200 μg protein extract was mixed with rehydration buffer to 350 ul, and the samples were loaded onto 17 cm IPG dry strips (pH4-7, Bio-Rad) and rehydrated 50 V for 12 h and focused 20 V for 2 h, 500 V for 2 h, 1,000 V for 2 h, 8,000 V for 4 h and finally 8,000 V until the total V hours reached at least 60,000.

Before SDS-PAGE, the IPG strips were equilibrated for 15 min in an equilibration buffer (6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 50 mM Tris-HCl) containing 10 mg/ml DTT followed by 15 min in an equilibration buffer containing 40 mg/ml iodoacetamide. After equilibration, strips were applied to 12% [w/v] SDS-PAGE gels and sealed with agarose sealing solution. After eletrophoresis, the SDS-PAGE gels were silver-strained according to the method of Blum *et al*. (37).

Image and data analyses

Stained gels were scanned using an image scanner (Amersham Biosciences) in transmission mode. Analysis of the gels was accomplished using the PDQuest analysis software including background subtraction, spots detection and the establishment of a reference gel (38). Protein spots were selected based on the more than a two-fold variation of expression between A and F. 500 μg protein of each sample was used for 2-DE. Data analyses was performed using MALDL-TOF/TOF. At last, the 2-DE gels were CBB-stained according to the method of Kim *et al*. (39).

MALDI-TOF/TOF-MS analysis and database search

Selected protein spots were excised manually from the CBB-stained gel and digested according to the method from Shevchenko *et al*. (40). Then, 1 μl of the mixture was spotted onto a target plate. Protein analysis was performed using MALDI-TOF/TOF AB4800 mass spectrometer (Applied Biosystems). The laser energy is MS 5,200 and MSMS 5,700. Peptides were selected in the mass range between 700 and 3,500 Da. Swiss-PROT (20070123, 254609 sequences) was used for database search.

Acknowledgements

This work has finished at the Beijing Proteome Research Center (BPRC). We thank very much to Ph.D. Ying Jiang and technicist Yu-Juan Yan in BPRC for the technical assistance. The work was supported by Grants from the Program of 2008 New Century Excellent Talents of Ministry of Education (02240601) and National R.&D. Project of Transgenic Crops of Ministry of Science and Technology of China (JY03-A-13-01).

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