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# Comprehensive analysis of differently expressed genes and proteins in albino and green plantlets from a wheat anther culture

P. ZHAO[1], K. WANG[1], W. ZHANG[1], H.Y. LIU[1], L.P. DU[1], H.R. HU[2], and X.G. YE[1]*

*Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R. China*[1]
*College of Life Sciences, Northwest A&F University, Yangling, Shanxi, P.R. China*[2]

## Abstract

The anther culture technique, although it has been widely used in wheat breeding programs, is associated with a high albinism rate that limits its application. The mechanism of albinism has not been studied explicitly at the molecular level. In this study, differently expressed genes (DEGs) and proteins (DEPs) of albino and green seedlings from a wheat anther culture were analyzed and compared using transcriptomic sequencing (RNA-Seq), two-dimensional electrophoresis, and mass spectrometry. A total of 1 892 DEGs (1 115 down-regulated and 777 up-regulated) and 43 DEPs (15 down-regulated and 28 up-regulated) that are primarily involved in photosynthetic pathways and porphyrin and chlorophyll metabolism was identified when the albino group library was used as the control. Most DEGs participated in constructing components of thylakoids and the chloroplast envelope as determined *via* gene ontology analysis. The expression patterns of 12 selected genes were evaluated using real-time quantitative PCR, and the results were in agreement with the RNA-Seq analysis. Our results may assist the development of new methods to decrease the albino seedling rates in wheat anther cultures.

*Additional key words*: chloroplast, photosynthesis, transcriptomic sequencing, *Triticum aestivum*.

## Introduction

Anther cultures can be used to induce haploid or doubled haploid plants. This method has been successfully used to obtain haploids for *Datura* and wheat more than 40 years ago (Guha and Maheshwari 1964, Ouyang *et al*. 1973). Since then, the technique has been gradually adopted in breeding of many new wheat cultivars (Hu *et al*. 1986, De Buyser *et al*. 1987, Cistué *et al*. 2003, Ren *et al*. 2006, Tang *et al*. 2009). This breeding strategy has many advantages, including a shortened breeding time, an accelerated homozygote production, and improvements in selection efficiency. However, its applications are limited by some problems, including a high albino seedling frequency, a low callus induction frequency, a strong genotype and environmental dependence, a low chromosome doubling rate, *etc*. Among these problems, the high albino rate is the key limiting factor because the albino seedlings cannot further grow and develop into adult plants. Although the albinism rate was between 30 and 50 % for most wheat cultivars, it has been reported at an average of 88 % in four German spring wheat cultivars (Ziegler *et al*. 1990) and as high as 97 % in cv. Edwall (Zhou and Konzak 1989). The high albinism rate has also been reported in other cereal plants, including rice (Harada *et al*. 1991), barley (Yaronskaya *et al*. 2003), and maize (Schmitz-Linneweber *et al*. 2006).

The genetic variation in both the plastid and nucleic genes of albinism in a cereal anther culture was investigated from the 1980s to the 1990s. Using the Southern blot, it has been determined that a wide variety of chloroplast genomic (ctDNA) molecules are deleted in some albino wheat plants, and a heterogeneous population of ctDNA molecules is present in most albino plants (Day and Ellis 1984). The plastid genome in some albino wheat and barley plants exists in linear form (Day

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*Abbreviations*: 2-DE - two-dimensional electrophoresis; CI - confidence interval; DEGs - differently expressed genes; DEPs - differently expressed proteins; GO - gene ontology; KEGG - Kyoto encyclopedia of genes and genomes; MS - mass spectrometry; PS - photosystem; RPKM - reads per kilo base per million reads.
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* Corresponding author; e-mail: yexingguo@caas.cn

and Ellis 1985). Similar result was also observed in rice. Plastid DNA suffered a large scale deletion in approximately 35 % of albino plants derived from anther cultures for different reduction sizes and locations (Harada *et al*. 1991). Almost all of the albino plants suffer from reduced amounts of ptDNA, especially for the transcripts from the plastid genes of *rbcL*, *psbD-psbC*, from 16S, and from 23S rRNAs. The amounts of ptDNA were either undetectable or significantly reduced compared with the corresponding green plants. Other studies have suggested that the production of green plants in anther cultures are mainly affected by the alteration of nuclear genes (Tuvesson *et al*. 1989, Larsen *et al*. 1991) with reduced transcription of the nuclear genes *rbcS* and *cab* encoding the chloroplast proteins in albino plants (Dunford and Walden 1991).

However, it is not clear whether the occurrence of wheat albino seedlings from anther cultures are primarily caused by nuclear gene variations or plastid DNA deletion. The albino rate varies significantly among wheat cultivars, and the plastid or chloroplast DNA deletion cannot easily explain the phenomenon. Therefore, it is necessary to further investigate the mechanism of the albinism using newly developed technologies that were not previously available. In recent years, two-dimensional gel electrophoresis (2-DE) and transcriptome sequencing (RNA-Seq) have been widely used to identify the differently expressed genes and to screen proteins (Nagalakshmi *et al*. 2008, Wang *et al*. 2009, Lopez-Casado *et al*. 2012, Wang *et al*. 2013, Zhou *et al*. 2013). Both the albino and green plants from the wheat anther culture are precise (rigorous) near-isogenic lines, except for the seedling color trait. In this research, albino and green plants obtained from the anther culture of cv. Shimai15 were analyzed using 2-DE and RNA-Seq to differently screen the expressed genes and proteins. The expression patterns of some key differently expressed genes were evaluated using real-time quantitative PCR (qPCR). Our results could provide additional information to understand the molecular basis for the albinism phenomenon in anther cultures of wheat and other cereal plants.

## Materials and methods

**Plants: C**ommon wheat (*Triticum aestivum* L.) cv. Shimai15, was kindly provided by Prof. Li Hui at the Institute of Cereal and Oil Crops of Hebei Academy of Agriculture and Forestry Sciences in China. The seeds were sowed in the autumn 2011 at the experimental station of the Institute of Crop Science at the Chinese Academy of Agricultural Sciences.

During the booting stage, wheat tillers that contained pollens in the anthers at the mid-uninucleate stage were collected in addition to the top two nodes and the flag leaf. The developmental stage of the anthers was assessed *via* microscopic examination and spike morphology. The samples were wrapped in wet plastic paper and stored in a refrigerator at 5 °C. Three days later, the young spikes were carefully removed after the tillers were wiped on the surface with cotton that contained 70 % ethanol. The anthers were inoculated on W14 basal medium (Ouyang *et al*. 1989) (pH 5.8) with 2.0 mg $dm^{-3}$ of 2,4-dichloro-phenoxyacetic acid, 0.5 mg $dm^{-3}$ of kinetin, 100 g $dm^{-3}$ of sucrose, and 2.4 g $dm^{-3}$ of phytagel, and cultured at a temperature of 28 °C in the dark for 30 - 40 d for callus induction. The embryonic calluses were quickly moved onto a regeneration medium (Murashige and Skoog salts and vitamins) containing 1.0 mg $dm^{-3}$ of kinetin, 0.5 mg $dm^{-3}$ of 1-naphthaleneacetic acid, 20 g $dm^{-3}$ of sucrose, and 2.4 g $dm^{-3}$ of phytagel, pH = 5.8) for shoot induction at 25 °C, a 16-h photoperiod, and a photosynthetic photon flux density of 100 μmol $m^{-2}$ $s^{-1}$. The green and albino plants produced *via* anther culture were collected in May 2012 for analyses (Fig. 1 Suppl.).

**RNA isolation and cDNA library preparation:** Total RNA was extracted from the frozen samples using *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). First strand cDNA was synthesized using a *TaKaRa* (Dalian, China) reverse transcription kit and stored at -20 °C in a refrigerator for further investigation.

Sequencing libraries were performed in parallel using an *IlluminaTruSeqTM* RNA sample preparation kit (*Illumina*, San Diego, USA) according to the manufacturer's instructions. Using poly-T oligo-attached magnetic beads, the extracted mRNA was purified from 3 μg of total RNA. The fragmentation was performed randomly in a proprietary *Illumina* fragmentation buffer. By employing random primers, the first strand cDNA was synthesized. The second strand of cDNA was synthesized using DNA polymerase I and RNase H. *Illumina PE* adapter oligonucleotides were added to the cDNA fragments after adenylation of the 3' ends. To achieve the cDNA fragments in 200 bp-lengths, the fragment libraries were selected using the *AMPure XP* system (*Beckman*, Coulter, USA). The cDNA fragments that had adaptor molecules on both ends were collected *via* a PCR reaction. Finally, the products were quantified using an *Agilent* high sensitivity DNA assay and the *Agilent Bioanalyzer 2100* system. The sequencing of the cDNA library was performed at *Novogene Bioinformatics Technology Company*, Beijing, China.

**Identification of differently expressed genes:** The original data were transferred into sequence data, which were defined as raw reads. The raw reads were filtered according to the *Illumina pipeline* before data analysis. The filtering steps included removing reads with

adaptors, removing low-quality reads, and removing reads that included more than 10 % of unknown bases. Meanwhile, the Q20 content, GC content, and sequence duplication level of the clean data were calculated. Then, clean tags were mapped to the wheat genome database for annotation using *SOAPaligner/soap2* by allowing a 2-bp mismatch (Li *et al*. 2009). The gene expressions were calculated by counting the number of reads that mapped to the reference genes, and they were measured *via* the reads per kilobase per million reads (RPKM) method using the formula developed by Mortazavi *et al*. (2008). To evaluate the genes that had different expression between the two kinds of samples, a rigorous algorithm was used, as described by Wang *et al*. (2010). A threshold with $P \leq 0.05$ and an absolute value for fold changes $\geq 2$ were selected to determine significant differences in gene expression.

**Gene ontology and pathway enrichment analyses:** The DEGs were classified according to the genome gene ontology (*GO*) annotations (Ashburner *et al*. 2000). The *GO* enrichment analysis was used to describe the product characteristics and reaction network of the DEGs. The DEGs were mapped to *GO* terms in the database (http://www.geneontology.org/), and gene numbers for every term were calculated. To find the significantly enriched *GO* terms in the DEGs as compared with the genome background, a hypergeometric test was used:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

where N represents the number of all of the *GO* annotation genes, n is the number of DEGs in N, M is the number of all of the annotated genes for certain *GO* terms, and m is the number of DEGs in M. The *GO* terms with a corrected *P* value of $\leq 0.05$ were considered significantly enriched by the DEGs.

The pathway enrichment analysis was based on the Kyoto encyclopedia of genes and genomes (*KEGG*) database, and significantly enriched metabolic pathways in the DEGs were identified and compared with the entire genome background. The formula was the same as for the *GO* analysis. In the formula for the enrichment pathway analysis, N is the number of all of the *KEGG* annotation genes, n is the number of DEGs in N, M is the number of all of the genes that were annotated to specific pathways, and m is the number of DEGs in M. A pathway (*P* value was corrected as $\leq 0.05$) was defined as a significantly enriched pathway.

**Analyses of proteins:** The proteins in the albino and green wheat seedlings were extracted according to the standard protocol for the *TRIzol* reagent (*Invitrogen*) in three biological replicates from each sample group. The protein content was determined with a *2-D Quant* kit (*Amersham Biosciences*, Piscataway, USA) using bovine serum albumin (2 mg $cm^{-3}$) as the standard. The 600 µg protein samples were loaded onto *Immobiline DryStrips* (18 cm, pH 3 - 10, *GE Healthcare*, Milwaukee, USA) using an *EttanTMIPG-phorIITM* system. The first-dimension strips were equilibrated twice in an equilibration solution for 15 min. The first equilibration buffer contained 50 mM Tris-HCL (pH 8.8), 6 M urea, 30 % (v/v) glycerol, 2 % (m/v) sodiumdodecyl sulphate (SDS) and trace amounts of bromophenol blue that contained 1 % dithiotreitol (DDT). The second equilibration buffer contained 4 % (m/v) iodoacetamide instead of 1 % DTT. After equilibration, the first-dimension strip was loaded on 12 % SDS-PAGE for the second dimension. When the electrophoresis was complete, the 2-DE gel was stained with colloidal Coomassie brilliant blue and analyzed using *ImageMasterTM 2D Platinum* software (*v. 5.0*, *GE Healthcare*). Protein spots that showed statistically significant changes (vol. % > 2-fold and $P \leq 0.05$) were chosen for identification *via MALDI-TOF/TOF* analysis.

The *MALDI-TOF/TOF* analysis was conducted at *Applied Protein Technology Co.*, Shanghai, China, using *GPS* explorer software and *MASCOT*, the MS and the MS/MS spectra were input for search within the non-redundant green plant database of the *NCBI*. The parameters included a maximum missed cleavage of 2, a peptide mass tolerance of ± 0.2 Da, and a fragment tolerance of ± 0.3 Da. Additionally, proteins with a score confidence interval (CI) and a total ion score CI above 95 % were deemed a credible index for the MS/MS assay.

**Real-time quantitative PCR analysis** was performed using hot start fluorescent PCR core reagent kits (*SYBR Green I*) and a *Life Tech 7500* real-time PCR system. A 0.020 $cm^3$ reaction volume was used to perform the PCR reactions, with 100 ng of cDNA as the template. The real-time PCR system was performed under the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, 72 °C for 20 s, and then a disassociation stage. Twelve DEGs were selected and specific primers for them were designed using the *Premier v. 5* (*PREMIER Biosoft*, CA, USA) software. The *TaADP* gene was employed as the internal control gene. The relative expressions of these genes were calculated based on the threshold cycle using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). All of the reactions were performed in biological triplicates, and significant differences were calculated using a paired *t*-test.

## Results

Overall, there were 20 474 903 and 22 940 683 raw reads obtained by the RNA-Seq from the green and albino seedlings, respectively. After filtering the dirty tags (*i.e.*, low quality tags), we obtained 20 286 483 and 2 262 341 clean tags from the two groups of plantlets, respectively (Table 1 Suppl.). The sequencing depth was enough for transcriptome coverage in the wheat. The GC content in the two libraries was approximately 50 % and stable, and it was invariable during the entire sequencing progress. The sequencing error rates of the libraries from the green and albino samples were 0.04 and 0.03 %, respectively. These tests indicated that the RNA-Seq quality was reliable. All of the clean reads were mapped onto the wheat reference genome in the *NCBI* for gene annotation. In total, 44.13 and 40.17 % of the clean reads from green and albino seedlings, respectively, were mapped to the reference genes. Among them, 39.92 and 37.04 % of the clean reads from the two groups, respectively, were uniquely mapped to reference genes, and 4.21 and 3.13 % of each type of read, respectively, was multiply mapped. Because of the incomplete gene annotation of wheat, more than 60 % of the clean reads could not be mapped to the reference genes. The RNA-Seq data were deposited in the Sequence Read Archive of the NCBI. The accession numbers of experiment-SRX1056057 (run-SRR2058178) and experiment-SRX1055042 (run-SRR2058329) were for the dataset of the albino and green seedlings, respectively.

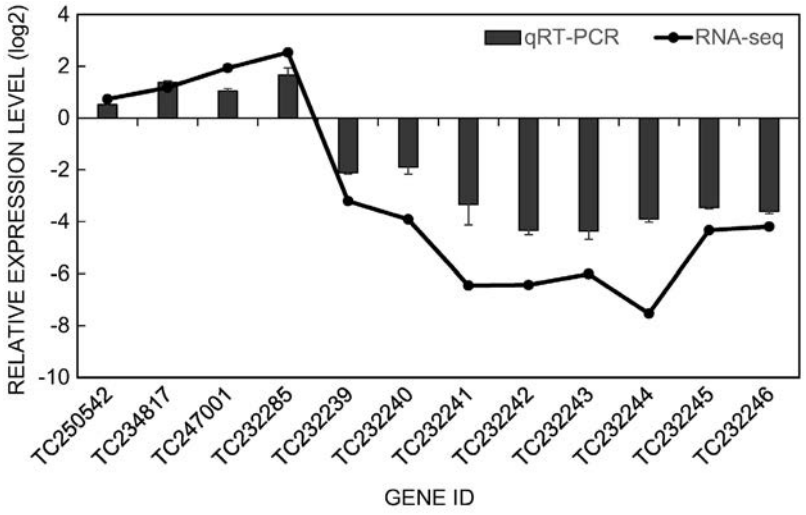


Fig. 1. The real time qPCR validation of the 12 differentially expressed genes that were selected from the albino and green plants. The histogram represents the relative expression determined by real time qPCR (means ± SEs), and the line chart represents the expression determined by the RNA-Seq.

To define the DEGs between the two cDNA libraries that were generated from the green and albino plants, the RPKM value was used to normalize the gene expression, and the combined criteria of at least a two-fold change and a $P \leq 0.05$ were used to determine the significance. The relative expressions were analyzed by using the library of the albino group as the control. There were 1 115 down-regulated and 777 up-regulated DEGs that were obtained after comparing the two libraries of the green and albino samples. Among them, 9 and 1 DEGs were specifically expressed in the libraries of the green and albino plants, respectively. Some significant DEGs were detected and considered to be related to the albinism (Table 2 Suppl.). Among them, the functions and structures of several genes were predicted; however, most of them should be confirmed by further studies.

Twelve DEGs that were identified by the RNA-Seq were chosen to evaluate the validity using real time qPCR, including 4 up-regulated and 8 down-regulated genes in the albino plants. The expressions of these genes were generally in agreement with the results of RNA-Seq, indicating the data from the RNA-Seq were reliable (Fig. 1). However, some fold differences exist between the RNA-Seq and real-time qPCR, which may be due to different experimental methods and different algorithms (Ekman *et al*. 2003).

Using the *GO* enrichment analysis, 1 892 DEGs within the two cDNA libraries were derived from both types of plants, and they were classified into 387 categories. However, as described previously, the *GO* terms with a corrected $P \leq 0.05$ were considered significantly enriched with DEGs. Thus, only 21 categories were enriched *GO* terms (Fig. 2, Table 3 Suppl.). In categories of biological processes, the metabolic process is the most dominant group, followed by translation, the defense response to bacteria, cation

transport, photosynthetic pigments, and isoprenoid biosynthesis. There were 32 down-regulated DEGs in the albino plants that were placed in the metabolic process category. This primarily included protochlorophyllide reductase A, α-glucan phosphorylase, glutamate synthase 1, and ferredoxin-dependent glutamate synthase. Six down-regulated DEGs in the albino plants participated in the light harvesting processes, such as the photosystem (PS) I antenna protein, the light-harvesting complex IIa protein, and the chlorophyll *a*-*b* binding protein. The molecular functions of most of the DEGs were associated with structural constituents of the ribosome, calcium ion binding, and hydrogen antiporter activity. All of the DEGs in the enriched molecular *GO* terms were up-regulated. Thirty up-regulated DEGs in the albino plants functioned as structural constituents of the ribosome, eleven functioned as calcium ion binders, and three exhibited hydrogen antiporter activity. Additionally, the chloroplast, membrane, and ribosome occupied the top three classes of cell components. There were 78 down-regulated DEGs of the albino plants that were placed in the *GO* terms for the chloroplast, and they primarily included protochlorophyllide reductase A, alanine aminotransferase 2, phytoene desaturase, and arginase. These results indicate that albinism in wheat anther culture is a complex process that is associated with dynamic changes in the expression of many genes.

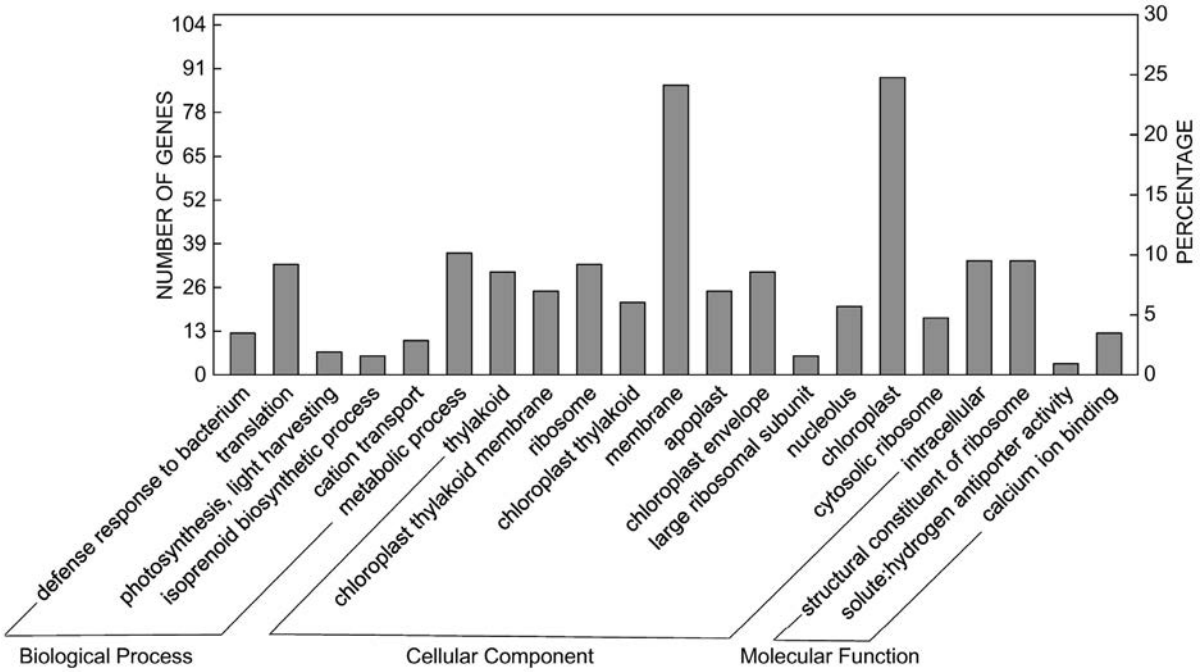


Fig. 2. Histogram of the gene ontology enrichment analysis of the differentially expressed genes (DEGs). The y-axis indicates the number and percentage of DEGs in the cluster. The x-axis indicates the names of the clusters.

To further determine the potential functions of DEGs, a pathway enrichment analysis was performed. Twenty enriched pathways were selected to produce an enrichment scatter diagram (Fig. 2 Suppl.). There was a positive correlation between the enrichment factor and the enrichment degree, and there was a negative correlation between the *P*-value and the enrichment degree. The most significant enrichment pathway should have a higher enrichment factor and a lower *P*-value. Meanwhile, 14 significantly enriched pathways were obtained that involved 306 DEGs (Table 1). Photosynthesis, ribosome metabolism, photosynthesis-antenna protein metabolism, porphyrin metabolism, chlorophyll metabolism, and other metabolic processes were implicated. The DEGs involved in photosynthesis were significantly down-regulated in the albino plants, suggesting that reduced photosynthesis is associated with albinism in wheat anther cultures. All of the DEGs detected in the photosynthesis pathway were exclusively down-regulated in the albino plants, including 6 transcripts of the plastocyanin precursor gene, 2 transcripts of the ATP synthase subunit gene, 5 transcripts of the ferredoxin gene, 4 transcripts of the oxygen-evolving enhancer protein gene, 2 transcripts of the photosystem II 10 kDa polypeptide gene, and some predictable protein genes.

The green leaf color may be primarily determined by porphyrin and chlorophyll metabolism (http://www.kegg.jp/kegg-bin/show_pathway? map00860). Several mutants for leaf color may have a gene defect for the chlorophyll metabolism (Zhang *et al*. 2006). In this pathway, the aminolevulinic acid dehydratase gene, 3 transcripts of the Mg-chelatase subunit genes, the leucine zipper protein gene, 3 transcripts of protochlorophyllide reductase, the

geranyl-geranyl hydrogenase gene, and 2 transcripts of the chlorophyllide *a* oxygenase gene were determined to be down-regulated in the albino plants (Table 2 Suppl.). Protochlorophyllide reductase participates in the biosynthesis of divinyl chlorophyllide *a* and chlorophyllide *a*. Protochlorophyllide reductase catalyzes the rate-limiting steps of these reactions (Fujita *et al*. 1998). Chlorophyllide *a* oxygenase catalyzes the conversion of chlorophyllide *a* into chlorophylide *b*, and chlorophylide *b* can then transform into chlorophyll *b* after one step (Lee *et al*. 2005, Oster *et al*. 2000). Thus, the down-regulated genes of protochlorophyllide reductase and chlorophyllide *a* oxygenase may result in a decrease of chlorophyll content in leaves.

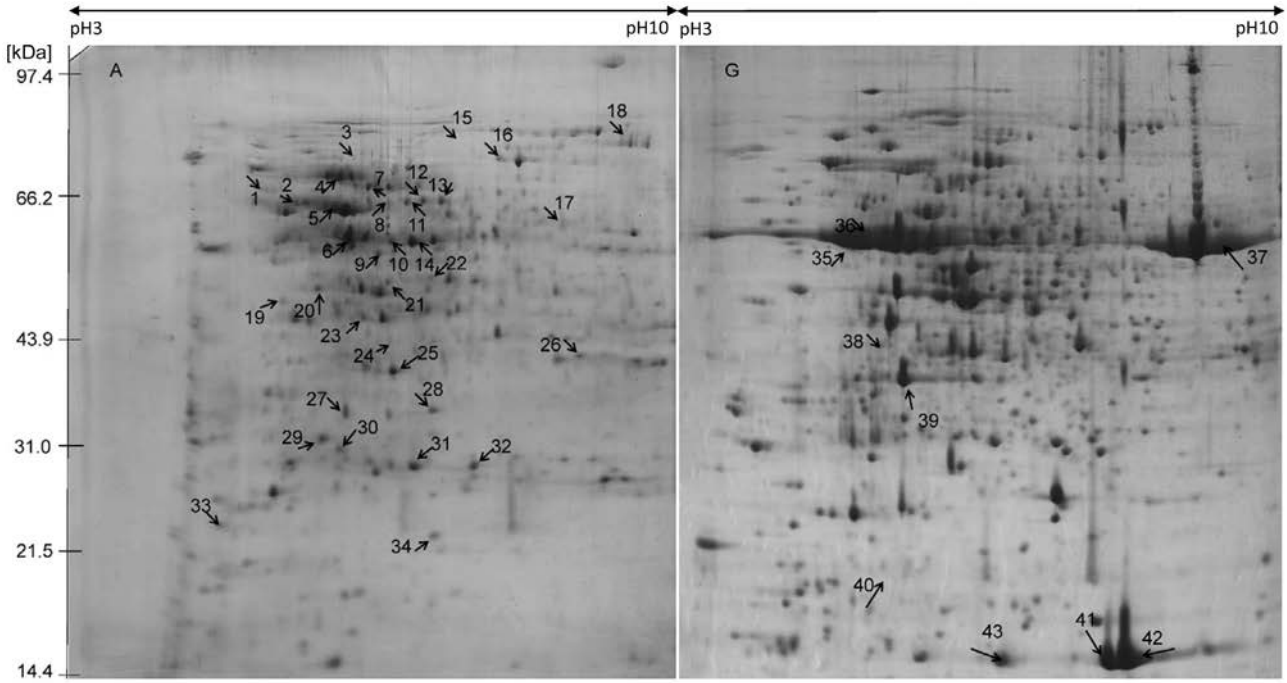


Fig. 3. 2-DE analysis of the total proteins extracted from the albino (*A*) and green (*G*) plantlets. The experiment was repeated three times. In total, 42 differentially expressed proteins exhibited significant volume changes for the albino and green seedlings that were labeled on the 2-DE gels.

Table 1. Pathway enrichment analysis of the differentially expressed genes based on the *KEGG* database. A pathway with $P \leq 0.05$ was defined as a significantly enriched pathway.

| Term | ID | Sample number | Background number | *P*-value |
|---|---|---|---|---|
| Photosynthesis | ath00195 | 37 | 77 | 1.04E-10 |
| Ribosome | ath03010 | 83 | 311 | 2.43E-06 |
| Photosynthesis - antenna proteins | ath00196 | 13 | 22 | 7.28E-06 |
| Carbon fixation in photosynthetic organisms | ath00710 | 26 | 79 | 0.000248 |
| Porphyrin and chlorophyll metabolism | ath00860 | 15 | 47 | 0.006741 |
| Nitrogen metabolism | ath00910 | 14 | 43 | 0.007184 |
| Ascorbate and aldarate metabolism | ath00053 | 11 | 34 | 0.017184 |
| Pentose phosphate pathway | ath00030 | 15 | 53 | 0.021361 |
| Proteasome | ath03050 | 16 | 58 | 0.022619 |
| α-Linolenic acid metabolism | ath00592 | 10 | 31 | 0.023105 |
| Peroxisome | ath04146 | 18 | 69 | 0.028202 |
| Valine, leucine, and isoleucine degradation | ath00280 | 13 | 46 | 0.031272 |
| Protein processing in endoplasmic reticulum | ath04141 | 31 | 137 | 0.037407 |
| Linoleic acid metabolism | ath00591 | 4 | 9 | 0.046417 |

Leaf proteins extracted from the green and albino plants were separated *via* high-resolution 2-DE, and protein spots were analyzed using *ImageMaster 2D Platinum*. The results indicated that most of the proteins distributed in the pH range of 3 - 10 on the gels (Fig. 3). Forty-three protein spots changed by more than two times

in abundance ($P < 0.05$) and were considered to be differently expressed proteins (DEPs). Among them, 15 down-regulated and 28 up-regulated DEPs were produced for the two kinds of plants by using the albino group as the control, and 3 up-regulated and 9 down-regulated DEPs were specifically expressed in the albino and green plants, respectively. Then, the 43 DEPs were analyzed using *MALDI-TOF/TOF-MS*. Based on the database searching result provided in Table 4 Suppl., 35 isoforms were identified with different molecular masses or isoelectric points, and each had two or three protein spots located at different positions on the same gel. These included the ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (spots 35, 36, and 37), the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (spots 41, 42, and 43), ascorbate peroxidase (spots 29 and 30), phosphoglycerate mutase (spots 11 and 13), the RuBisCO large subunit-binding protein (spots 1 and 5), and the heat shock protein 70 (HSP 70) (spots 3 and 4). According to their functions, the identified DEPs were classified into 5 main groups that are involved in antioxidant, binding, transporter, molecular, and catalytic activities, as shown in Fig. 4. The DEPs in these functional groups are shown in Table 5 Suppl. The majority of the DEPs functioned in binding and catalytic activities, and only a small number of DEPs processed antioxidant, transporter, and molecular activities.

There was a linear correlation between the RNA-Seq and 2-DE results. Based on the integrative analysis of the two results, 18 genes were found to express differently in the two experiments (Table 2). The relative trends for the expression of the 16 genes among the 18 selected genes

Table 2. The selected genes and proteins *via* the integrative analysis of the combined RNA-Seq and 2-DE and their predicted subcellular localization. A/G of 2-DE indicates the different expression patterns of proteins. $Log_2$A/G of RNA-Seq indicates the different expression patterns of genes. ER - endoplasmic reticulum.

| Spot No. | Gene ID | A/G of 2-DE | $log_2$A/G of RNA-Seq | Description | Subcellular localization |
|---|---|---|---|---|---|
| Down regulated in albino seedlings | | | | | |
| 35(36,37) | TC264087 | 0 | -3.48 | ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | chloroplast |
| 38 | TC249041 | 0 | -5.23 | 30S ribosomal protein S14p/S29e | chloroplast |
| 39 | TC234416 | 0 | -3.33 | chloroplast oxygen-evolving enhancer protein 1 | chloroplast |
| 40 | TC269752 | 0 | -2.73 | putative peptidyl-prolyl *cis-trans* isomerase | chloroplast |
| 41(42,43) | TC263601 | 0 | -6.489 | RuBisCO small subunit | chloroplast |
| 24 | TC232210 | -7.69 | -6.04 | fructose-bisphosphate aldolase | cytoplasm |
| 20 | TC247042 | -4.16 | -6.01 | plastid glutamine synthetase isoform GS2c | cytoplasm |
| 32 | TC250393 | -2.78 | -4.53 | oxygen-evolving enhancer protein 2 | chloroplast |
| Up-regulated in albino seedlings | | | | | |
| 19 | TC232235 | 2.28 | 1.29 | 40S ribosomal protein S26 | cytoplasm |
| 16 | TC232326 | 2.31 | 0.61 | methionine synthase 2 enzyme | chloroplast |
| 8 | TC234744 | 2.79 | 1.12 | arabinoxylan arabinofuranohydrolase | ER |
| 34 | TC235102 | 2.86 | 1.24 | glycine-rich protein | cytoplasm |
| 14 | TC232333 | 2.89 | 0.61 | enolase 2 | chloroplast |
| 2 | TC250542 | 4.39 | 0.71 | protein disulfide isomerase 3 precursor | chloroplast |
| 3(4) | TC234817 | DIV/0 | 1.18 | HSP70 | chloroplast |
| 17 | TC247001 | DIV/0 | 1.93 | aldehyde dehydrogenase 7b | chloroplast |
| Expressed differentially by two methods | | | | | |
| 18 | TC247435 | DIV/0 | -2.16 | lipoxygenase 2 | cytoplasm |
| 1(5) | TC234574 | 3.08 | -1.75 | RuBisCO large subunit-binding protein | chloroplast |

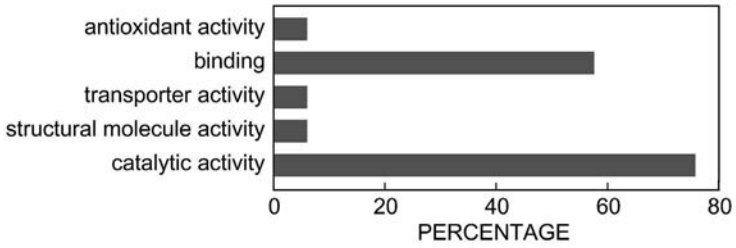


Fig. 4. Bar chart of the distribution of differentially expressed proteins (DEPs) between the albino and green groups according to their functions. The y-axis indicates the names of the functional groups. The x-axis indicates the percentage of DEPs in the functional groups.

were consistent for the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, the chloroplast oxygen-evolving enhancer protein 1, the fructose-bisphosphate aldolase, and the HSP 70. Additionally, the lipoxygenase 2 and RuBisCO large subunit-binding proteins were not completely consistent with each other in 2-DE and RNA-Seq, which were expressed as down-regulated in RNA-Seq but up-regulated in 2-DE. This inconsistency may be the result of post-translational modification of the encoding genes and different metabolism processes of the corresponding proteins.

Using *Plant-PLoc* software, the subcellular location of these proteins were predicted (Chou and Shen 2008). Many DEPs were located in the chloroplast (66.7 %), some DEPs were located in the cytoplasm (27.8 %), and only one protein was located in the endoplasmic reticulum. The prediction indicated that most of the DEPs that are associated with albinism play a role in the chloroplasts.

## Discussion

Understanding the mechanism of crop albinism is important for haploid breeding improvement. In recent years, many efforts have been made to identify genes that are associated with albinism. In this study, genes and proteins that regulate some aspects of albinism were identified. RuBisCO is the most abundant protein, accounting for approximately 12 - 35 % of the total leaf proteins in $C_3$ plants (Kokubun *et al*. 2002). There are two types of subunits in most photosynthetic organisms: the 15 kDa small subunit and the 55 kDa large subunit (Spreitzer 2003). RuBisCO is the key enzyme in photosynthetic carbon assimilation and the main storage protein in plants (Yosef *et al*. 2004). However, the catalytic efficiency of RuBisCO is very low, and carboxylation and oxygenation activities can only be activated by RuBisCO activase (Portis 2003). Co-suppression of an *Arabidopais* RuBisCO small subunit gene (*RBCS3B*) in *Arabidopsis* resulted in albino or pale green phenotypes, and in some transgenic lines, chloroplast numbers in mesophyll cells decreased and stacked thylakoids broke down (Zhan *et al*. 2014). Additionally, at the albino stage of a greenable albino rice mutant, lower content of RuBisCO and RuBisCO activase was found in the leaves. However, during the greening period, the content increased and reached the same level as in the control on the 30$^{th}$ day of greening. In a “stay green” durum wheat mutant, RuBisCO activase was primarily found in green leaves or other photosynthetic tissues, and it first degraded when the chloroplasts began to disassemble during senescence (Rampino *et al*. 2006). Similarly, we found that genes encoding the RuBisCO large and small subunits, and RuBisCO activase were significantly down-regulated in albino plants in our study.

Carotenoids are photosynthetic pigments participating in a wide range of physiological processes (Liu *et al*. 2014a). They are essential components of the photosynthetic apparatus and are coordinately synthesized with chlorophylls in chloroplasts (Andrade-Souza *et al*. 2011). Phytoene synthase is the first dedicated enzyme in the carotenoid biosynthetic pathway (Just *et al*. 2007). Transgenic orchid plants with phytoene synthase-RNAi transformed into a protocorm-like body has phenotype with yellow-greenish leaves and significantly reduced content of chlorophyll *a* and *b*. Additionally, the expression of some chlorophyll biosynthetic-related genes in transgenic plants is dramatically decreased (Liu *et al*. 2014b). However, seed-specific overexpression of an endogenous phytoene synthase gene results in increased content of carotenoids and chlorophylls in *Arabidopsis* (Lindgren *et al*. 2003). The phytoene desaturase enzyme is also an important enzyme in the carotenoid biosynthetic pathway. Disrupting the phytoene desaturase gene resulted in albino phenotypes in *Arabidopsis* and in the inhibition of several other genes that are associated with carotenoid and chlorophyll biosynthetic pathways (Qin *et al*. 2007). Our results suggest the down-regulated genes for phytoene synthase and phytoene desaturase may play important roles in the albino process of wheat anther cultures. Additionally, phytoene synthase gene is expressed consistently with numerous photosynthesis-related genes, and this transcriptional regulation is very important. It plays a significant role in regulating the synthesis of carotenoids and chlorophylls (Meier *et al*. 2011).

Some genes that have functions in ubiquitination and stress resistance were found to respond to albinism in wheat. Ubiquitination is involved in many important biological processes in plants, especially in protein degradation and abiotic and biotic stress responses (Hershko and Ciechanover 1998). Due to the lack of chlorophyll, there is no photosynthesis progress that occurs in albino seedlings. Many proteins involved in photosynthesis may degrade *via* ubiquitination. Meanwhile, the albino seedlings may suffer from biotic and abiotic stresses. Thus, genes that are associated with stress resistance and ubiquitination progress are accordingly up-regulated in albino plants.

Among the identified DEPs, ATP synthase was up-regulated and fructose-bisphosphate aldolase was down-regulated in the wheat albino plantlets. The results were similar to the findings of Hou *et al*. (2009). ATP synthase is the main enzyme in ATP biosynthesis and is present in

the chloroplast (Tucker *et al*. 2001). Fructose-bisphosphate aldolases are important enzymes in carbon metabolism in the chloroplast, and their down-regulated expression may affect the formation of the chloroplast structure in albino plants (Hou *et al*. 2009). Glutamine synthetase and heat shock protein 70 were up-regulated in our 2-DE results, and we observed opposite results from those of Hou *et al*. (2009). The difference may be related to the plant materials used in this study.

Higher plants collect and transfer light energy to their photosynthetic reaction centers *via* light-harvesting chloroplast *a*/*b*-binding proteins (LHCP) (Paulsen *et al*. 2010). Genes encoding LHCPs in plants are divided into 10 members, including light-harvesting proteins of PS I (LHCA) 1-4, which are associated with the PS) I complex, and light harvesting proteins of PS II (LHCB) 1-6 (Jansson 1999, Teramoto *et al*. 2001, Nick *et al*. 2013). Previous studies analyzed the relationship between LHCPs and antenna pigments in two nuclear mutants of *Chlamydomonas* that had low amounts of chlorophyll. The LHCP II in mutant *PA2.1* was soluble and rapidly degraded before it was inserted into the thylakoid, and mutant GE2.27 was defective for thylakoid insertion of the LHCP II. In our libraries, the *Lhcb1* (TC263155) and *Lhcb4* (TC263456) genes, which encoded LHCP II and CP29, respectively, were found to be down-regulated significantly in the albino group. The down-regulated *Lhcb1* gene may lead to decreased LHCP II content in the albino plants. There may be no sufficient amount of LHCP II to bind chlorophyll molecules, especially chlorophyll *b*. Free LHCP IIs may not insert into the chloroplast and are degraded in vacuoles or are returned to the cytoplasm. Meanwhile, free chlorophyll molecules act as the feedback signals to repress the synthesis of chlorophyll. The accumulation of chlorophyll is influenced by the LHCP II content (Plumley and Schmidt 1995). Thus, the down-regulated *Lhcb* gene may cause plant albinism *via* feedback regulation.

By comparing differently expressed genes and proteins in green and albino plants, several genes that are associated with the photosynthetic pathway were obtained. Because there is a strong relationship between the photosynthetic capacity, albinism, and grain yield in crops, genes involved in photosynthesis are worthy of study. Sedoheptulose-1,7-bisphosphatase (SBPase) plays a key role in controlling the inflow and regeneration of carbon in the Calvin cycle (Geiger and Servaites 1994). Previous studies have indicated that overexpressing the SBPase gene in *Arabidopsis* would result in more and greener plant leaves, larger plants, a shorter vegetative phase, and a higher photosynthetic rate. The activity of SBPase in transgenic tobacco increased *via* the overexpression of *Arabidopsis* SBPase sense cDNA, and the photosynthetic capacity per unit area and grain yield increased synchronously to the photosynthetic rate, leaf area, and biomass (Lefebvre *et al*. 2005). Fructose-1,6-bisphosphatase (FBPase) catalyzes the conversion of triose-phosphates to sucrose during photosynthesis (Daie 1993). The rice pale green *OscFBP1* mutant exhibits a significantly decreased photosynthetic rate and chlorophyll content (Lee *et al*. 2008). In our results, *SBPase* and *FBPase* genes were all significantly down-regulated in the albino plants (Table 2 Suppl.), which may be major reason for the occurrence of albinism in anther cultures.

## Conclusion

Although albino mutants are important materials for chlorophyll biosynthesis research and for the cloning genes that are associated with these processes in plants (Peng *et al*. 2012), albinism is still a crucial obstacle for cereal improvement programs that use the double haploid strategy. We identified 1 892 DEGs and 43 DEPs in the albino and green seedlings produced from a wheat anther culture. The DEGs were mainly involved in photo-synthesis, porphyrin, and chlorophyll metabolism pathways. The DEPs were classified into 5 main groups involving antioxidant, binding, transporter, structural molecule, and catalytic activities. The results of the RNA-seq and 2-DE were analyzed, and 16 genes were detected and uniformly regulated during both experiments. The expression patterns of 12 key genes were evaluated using real-time qPCR, and the results are in agreement with the RNA-Seq analysis patterns. Our results provide additional insights into the molecular basis of the albinism phenomenon in wheat, and may facilitate development in methodology to decrease the predominance of albino seedlings in the anther culture of this crop.

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