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RESEARCH ARTICLE

Proteomic analysis of differentially expressed proteins between Xiangyou 15 variety and the mutant M15

Zhen-Qian ZHANG[1,2], Gang XIAO[1,2], Rui-Yang LIU[1], Tai-Long TAN[1,2], Chun-Yun GUAN (✉)[1,2], Guo-Huai WANG[1,2], She-Yuan CHEN[1], Xian-Meng WU[1], Mei GUAN[1], Qin LI[2]

[1] *Hunan Branch of National Oilseed Crops Improvement Centre, Hunan Agricultural University, Changsha, Hunan 410128, China*
[2] *Hunan Provincial Key Laboratory of Crop Germplasm Innovation and Utilization,Changsha, Hunan 410128, China*

Abstract **A high oleic acid rapeseed material M15 (derived from Xiangyou 15 variety) has been received more attention for its significant effect for human health. And it has almost the same physiological characteristic with Xiangyou 15 variety. To find out the difference between high oleic acid rapeseed material and Xiangyou 15 seedling, a comparative proteomic approach based on 2-DE and mass spectrometry was adopted. A total of 277 protein spots showed a significant change in intensity by more than 2.0-fold from M15 compared with Xiangyou 15 variety. Among them, 48 spots that changed at least 3.0-fold were excised from gels and successfully identified by MALDI-TOF/TOF MS. The identified proteins involved in metabolism of carbohydrate and energy (75%), stress and defense (8.3%), photosynthesis (6.3%), protein metabolism (2.1%) and other functions (8.3%). Then real-time quantitative PCR (qPCR) analysis was used to verify the expression levels of differentially expressed proteins, but the results did well agree with the proteomic results. In this work, most of the proteins involved in metabolism of carbohydrate and energy have higher expression in M15, which may reveal M15 has higher metabolism ability. These results provided much information to understand the differences between high oleic acid rapeseed material and Xiangyou 15 variety, which will be useful to screen high oleic rapeseed materials in seedling period.**

Keywords proteomic analysis, high oleic acid, 2-D electrophoresis, real-time quantitative PCR

Introduction

High oleic acid oil is one of the interestingly functional food, it has more monounsaturated fatty acids (MUFAs), which is helpful to reduce cholesterol in blood plasma, and lower low density lipoprotein (Robert et al., 2004), thus reduce the risk of coronary heart disease (Iwona et al., 2006), and diets rich in MUFAs would have favorable outcomes on blood lipids and factor VIIc which can be useful to prevent heart disease (Allman et al., 2005; Iwona et al., 2006). In addition, it has lower saturated fat and more heat-stable than commodity-grade oil (Fuller et al., 1967; Mark et al., 2001), which is desirable for frying purposes, refining and storage.

As the largest oilseed crop in China, high oleic acid rapeseed will increase considerably in the near future (Zhang et al., 2011). With Xiangyou 15 variety as raw material treated by ^{60}Co (Guan et al., 2006), more than 100 high oleic acid rapeseed materials (oleic acid content more than 75%) were obtained after 6 years directional selection. The mutants have higher oleic acid content and the similar agronomic traits with Xiangyou 15 variety (Guan and Li, 2008). Several studies have been done in mature period of seeds, and some difference were found in FAD2 and FAD3 genes between high oleic acid rapeseed and Xiangyou 15 (Guan et al., 2006; Xiao et al., 2009; Guan et al., 2012).

A comparative proteomic approach based on 2-DE and mass spectrometry was adopted to identify proteins that changed in the 15-day-seedlings of M15 (oleic acid content is 78.9%) compared with Xiangyou 15 variety. And the gene levels of differentially expressed proteins were verified by qPCR analysis. The objective of these studies was to find out whether there has some remarkably differences between Xiangyou 15 variety and M15 in seedling period in seedling period.

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Correspondence: Chun-Yun GUAN
E-mail: guancy2011@aliyun.com

Materials and methods

Materials

The samples of Xiangyou 15 variety and M15 in this study were provided by Hunan Branch of National Oilseed Crops Improvement Center. And the two rapeseeds were planted in the same salver at 25°C for 15 days. The seedling of Xiangyou 15 variety and M15 has little difference. 1 g 15-day-seedlings were mixed together and briefly washed with sterile water thrice, then frozen in liquid nitrogen immediately and stored at − 80°C for protein extraction (Fig. 1).

Figure 1 The 15-day-seedling of different rapeseed. (A) The 15-day-seedling of Xiangyou 15 variety. (B) The 15-day-seedling of M15.

The determination of fatty acid content

The fatty acid content of rapeseed samples was determined by gas chromatograph. 1 mg rapeseed was dried at 80°C, crushed with high speed disintegrator, and then moved to 10 mL test tube, 1 mL 0.5 M KOH-CH_3OH and 2 mL aether and petroleum ether mixture with a volume rate of 1 to 1. And placed at 40°C for 24 h, then 2 mL H_2O was add to stratify, 1 μL upper sampling was extracted to determine with methyl heptadecanoate as the internal standard. And the gas chromatograph Agilent-6890A (Amercia), working conditions were: the inlet temperature of 270°C, the column temperature was 190°C with 5°C per min to 230°C, then holding 1 min, detector temperature was 260°C, nitrogen flow rate was 250 mL per min, hydrogen flow rate was 30 mL per min, air flow rate was 300 mL per min, split ratio was 40: 1, and column flow rate was 1 mL per min.

Protein extraction

Frozen seedling sample (1.0 g) was ground to powder in a mortar using liquid nitrogen precooled. The powders were displaced to a 10 mL centrifuge tube and then added with 10 volumes precooled (–20°C) sample buffer (8 M urea, 2% CHAPS). The mixture was vortexed, and then treated with sonicator in a beaker which contained with the mixture of ice and water. The swing was set to 21%. And the procedures were: cumulative ultrasounds for 60 s, ultrasound 0.1 s per 1 s.

The mixture was vortex extracted for 30 min at 25°C, centrifuged under 13 000 × *g* for 15 min at 4°C. The supernatant was transferred carefully to a new centrifugal tube, 4 volumes acetone (–20°C) was added, and then placed at –20°C overnight. The mixture was centrifuged under 13 000 g for 20 min at 4°C, the sediment was washed with –20°C acetone containing 0.07% DTT thrice,then dried using freeze-drying centrifuge (FDU-2100, EYELA, Japan). The powder was dissolved by rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% Bio-Lyte [pH 4–7], and 0.001% bromophenol blue) per 1 mg pellet. The content of protein was quantified using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Two-dimensional polyacrylamide gel electrophoresis

About 1.0 mg protein was dissolved in 450 μL rehydration buffer and applied to IPG strips (24 cm, pH 4–7, Bio-Rad). The IPG strip was rehydrated for 14 h in 400 μL rehydration buffer containing the protein sample. Then the isoelectric focusing (IEF) was performed at 20°C using a IPGphor II IEF system (Amersham Biosciences, Uppsala, Sweden) under the procedure listed in Table 1.

After IEF separation, the strips were equilibrated twice for 15 min each using equilibration buffer (6 M urea, 37.5 mM pH 8.8 Tris-HCl, 20% glycerol, and 2% DTT) with 1% DTT and 4% iodacetamide (IAA). The second dimension separa-

Table 1 Running conditions of isoelectric focusing

Step	Voltage (V)	Voltage increase model	Time
S1	50	Accelerated	12 h
S2	100	Accelerated	30 min
S3	250	Accelerated	30 min
S4	500	Accelerated	30 min
S5	1000	Accelerated	30 min
S6	4000	Linear	2 h
S7	10000	Linear	3 h
S8	10000	Linear	70000 VHr
S9	500	Accelerated	Any time

VHr: volt-hours.

tion was performed by SDS-PAGE on a vertical slab acrylamide gel (12% total monomer, with 2.6% crosslinker) using an Ettan Dalt SIX electrophoresis system (Amersham Biosciences, Uppsala, Sweden). The electrophoresis initiated with a constant electric current of 10 mA for 45 min and was switched to 20–30 mA until the bromophenol blue frontier reached the bottom of the gel. After SDS-PAGE, the gel was fixed for 1 h in a solution containing 40% methanol and 10% acetic acid, washed with deionized water for 15 min, and then stained in a staining buffer (0.12% Coomassie brilliant blue G-250, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol) for 24 h (Candiano et al., 2004), and then destaining by ddH_2O until the background was clear. The experiment was repeated in triplicate for each sample.

Gel image analysis

Images of stained gels were acquired by the image scanner Typhoon 9410 (Amersham Biosciences, Uppsala, Sweden) in a transmission mode. Image analysis was performed with PDQuest 8.0 software (Bio-Rad). After background subtraction and spot detection, spots were matched and normalized using the method of total density in the gel image. Only spots with statistically significance (Student's *t*-test, $p < 0.05$) and reproducible changes were considered, and the protein spots with an abundance ratio of at least two were selected as differentially expressed proteins.

Proteins identification

To identify proteins in protein spots using MS, protein spots were excised from 2-DE gels and washed with water twice, and digested as described by Katayama et al. (2001). Extracted lyophilized tryptic peptides were dissolved with 0.1% TFA in 50% acetonitrile, then 1 μL of the peptide mixture was mixed with an equal volume of saturated CHCA solution. Mass spectrometric analysis was performed with a MALDI-TOF/TOF mass spectrometer 4800 (Applied Biosystems, Foster City, CA, USA). Data were acquired in a positive MS reflector. Mass spectra were obtained from each sample spot by accumulating 900 laser shots in a mass range of 800–3500 Da. The 10 most abundant precursor ions per sample were selected for subsequent fragmentation. Both the MS and MS/MS data were interpreted and processed using Global Proteome Server (GPS) Explorer software (V3.6, AB Sciex) and Mascot2.1 (Matrix Science). NCBInr was selected as the database and taxonomy, respectively. Search parameters used fixed cysteine carbamidomethylation and variable methionine oxidation as modifications, trypsin of the digestion enzyme, one missed cleavage site, fixed modification of cysteine carboamidomethylation and partial modifications of Oxidation (M) and Acetyl (N–term), MS tolerance of 0.15 Da, and MS/MS tolerance of 0.25 Da. The protein score C.I.% had to be over the significance (> 95) threshold level ($p < 0.05$).

qPCR analysis

Total RNA for qPCR analysis was extracted from 15-day-seedlings using the Plant RNA Midi Kit (Omega, USA). cDNA was synthesized from the total RNA (≤5μg) with the Prime Script™ 1st strand cDNA Synthesis Kit according to the manufacturer's instructions. The primers were designed using Primer 5.0 software according to the cDNA sequences. Reactions were carried out with the CFX96TM Real-Time System (BIO-RAD, USA) using two-step cycling conditions of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s. The reaction mixture (20 μL) contained 1 μL cDNA solution, 10 μL SYB® Premix Ex Taq (Tli RNaseH Plus) (2 ×), 0.5 μL of each primer and 8 μL water. The reactions were performed in triplicate, and the results were averaged. UBC21 (ubiquitin-conjugating enzyme 21) was used as the reference gene, which is a stably expressed reference gene for vegetative tissues (Chen et al., 2010). And the primer pairs of UBC21 were: 5′-CCTCTGCAGCCTCCTCAAGT (F), and 5′-CATATCTCCCCTGTCTTGAAATGC (R). The relative gene expression was evaluated using the comparative cycle threshold method (Livak and Schmittgen, 2001). Statistical analysis was performed using ANOVA and Post Hoc test with a p value < 0.05 being accepted as significant. And the primer sequences used in qPCR were listed in Table 2.

Results

Fatty acid composition in rapeseed of Xiangyou 15 variety and M15

The M15 derived from Xiangyou 15 variety, therefore, there has little genetic background difference between them. The fatty acid composition of rapeseed was determined by gas chromatography (Zhang et al., 2009) with methyl heptadecanoate as internal standard compound (Table 3). And there has a obviously diference in oleic acid content.

Change in protein abundance between different samples

Two-dimensional electrophoresis analysis of the total protein in seedlings of M15 and Xiangyou 15 variety was performed at least in triplicate and showed a high level of reproducibility. Representative gels are shown in Fig. 2. Among all the tested samples, more than 9702 protein spots were reproducibly detected with PDQuest 8.0 software on CCB G-250-stained gels. Quantitative analysis revealed that 277 protein spots showed a significant ($p < 0.05$) change in intensity by more than 2-fold from M15 to Xiangyou 15 variety.

Identification of differentially expressed proteins

Fifty differentially expressed protein spots (the ratio of M15

Table 2 Primer sequences used in qPCR

Gene name	GI Number	Sequence (5′-3′)
β-glucosidase	gi1732572	F:GGACAAAGTGAAAAACTGGATTACG R: GTAGGCTGAACGGCTAAAGACCC
ATP synthase CF1 alpha subunit	gi7525018	F: ACCCGTGAGTGAGGCTTATTTGG R: CTGAAATCTTACCTCGACCATCA
Glutamine synthetase	gi12643761	F: ATCCTTTCCGTGGAGGCAATAAC R: GAAAATCTCAGCAGCTCTTGCACGT
Myrosinase	gi|127733, gi|56130949	F: GCGAACTCAATGCTACTGGCTAC R: TTTGTGGTAGTAATCAAGACCTCCT
Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|27752813	F: AGAGCTATATTTGCCAGAGAATTGG R: CTCGGCAATAATGAGCCAAACTA
Ribulose bisphosphate	gi|266893	F: GAAGTTCGTTGAGAGCCTTGGAGTT R: TCTCATAAGTCATCTCGGGTTGC
Rubisco ssu precursor	gi|17855	F: GTTGGAAAGAAGAAGTTTGAGACCC R: TTCGGTAAGGTCAGGAAGGTAAGAG
ATP synthase subunit beta	gi|75336517	F: ACAATGCTCTGGTGGTTAAGGGTC R: GCTCATAGCTACAGCTCTAACTCGG
Fructose-bisphosphate aldolase	gi|42573227	F: GTGTTGGACAAATCCGAATGGGTCA R: GGAGGACGACAGAAGCGGAAGAA
Putative chloroplast translation elongation factor EF-Tu	gi|23397095	F: AGATGAAGAGTCGAAGATGGTTATG R: CACCAACAGTCTTTCCTCCTTCT
Actin	gi|32186916	F: GTCCGTGACATAAAGGAGAAACT R: GAACCTCAGGACAACGGAATCTC
Rubisco activase	gi|23320705	F: TGTATGCTCCTTTAATTCGTGATGG R: CGTCACGAGTAGGTGCCCAGTAG
High chlorophyll fluorescence 136	gi|15237225	F: GGAACGAGACAGACTTTGTTAGAGA R: GTTTGCCAATAATCCATCCTTCT
Chlorophyll a/b binding protein	gi|18266039	F: TGGCTATTTGGGCTACTCAAGTGAT R: CCATCTCCTGCGACTCTGTAACCT
Chlorophyll a/b binding protein type I	gi|7271945	F: TGGCTATTTGGGCTACTCAAGTGAT R: CCATCTCCTGCGACTCTGTAACCT

Table 3 The fatty acid content of rapeseed

Samples	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Arachidonic acid
Xiangyou 15 variety	4.334%	1.939%	64.208%	19.707%	9.007%	0.805%
M15	3.631%	2.159%	80.935%	7.399%	4.636%	1.241%

to Xiangyou 15 variety or Xiangyou 15 variety to M15 from 3 to 5) were identified, 48 proteins were successfully identified, and the results were summarized in Table 4. Based on putative physiologic functions, the identified proteins can be classified as the following categories, carbohydrate and energy proteins (36 protein spots), stress and defense proteins (4 protein spots), photosynthesis (3 protein spots), protein metabolism (1 protein spot) and other proteins (4 protein spots). Most of the proteins involved in metabolism of carbohydrate and energy have higher expression in M15, which may reveal M15 has higher metabolism ability. But the corresponding genes has no relationship with FAD2 or FAD3 gene, the reason may be that there has no fatty acid synthesized in seedling period.

Gene expression analysis by qPCR

To investigate the changes in gene expression at the mRNA level, qPCR analysis of identified proteins was performed (Fig. 3). For several differentially expressed protein spots were identified as the same one protein, so there only 15 proteins were investigated. The results showed that, compared with Xiangyou 15 variety the transcripts of M15 of ATP synthesis CF1 alpha subunit (0.7 fold), glutamine synthetase (0.87 fold), Rubisco ssu precursor (0.73 fold), fructose-bisphosphate aldolase (0.71 fold), β-glucosidase (0.28 fold), Ribulose bisphosphate carboxylase/oxygenase activase (0.09 fold) and actin (0.01 fold) were downregulated, especially the expression of β-glucosidase (0.28 fold), Ribulose bisphosphate carboxylase/oxygenase activase (0.09 fold) and actin (0.01 fold) were significantly downregulated. While compared with Xiangyou 15 variety the transcripts of chlorophyll a/b binding protein (1.67 fold), Rubisco activase (1.72 fold), chlorophyll a/b binding protein type I (1.45 fold) and ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (1.78 fold) were significantly upregulated. The expres-

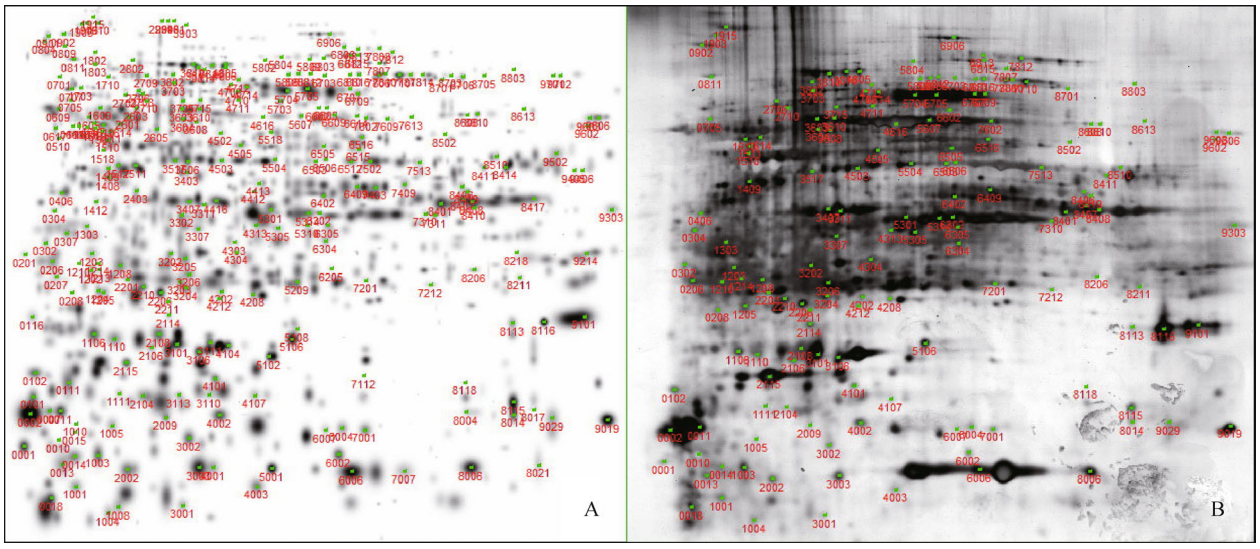

Figure 2 Two-dimensional electrophoresis gel of separated proteins. Proteins were separated in an IPG strip (24 cm, pH 4–7) and in the second dimension on a 12% gel. (A) Xiangyou 15 variety, (B) M15.

Table 4 Proteins identified by MS/MS

Spot No.	Protein name	Accession No.	The species category	Protein MW	Protein PI	Fold change (M15/Xiangyou 15 variety)
Carbohydrate and energy proteins						
8510	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	0.32
7513	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase large subunit	gi|27752813	*Arabidopsislyrata* subsp. lyrata	47433.9	6.13	3.45
2115	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase large subunit	gi|21634119	*Jacquemontia reclinata*	49905.1	6.71	0.31
3111	Ribulose 5-bisphosphate carboxylase large subunit	gi|11990183	*Diodia sarmentosa*	51527.9	5.96	0.24
8613	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase large subunit	gi|52001695	*Tristiropsis acutangula*	50582.4	6	0.33
8408	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase large subunit	gi|34015063	*Tristiropsis acutangula*	44167.2	6.12	3.44
1106	Ribulose-bisphosphate carboxylase large subunit	gi|17154704	*Tigridia pavonia*	49200.7	6.34	3.91
609	Ribulose bisphosphate	gi|266893	Cucumber	45681.2	7.57	4.04
3205	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase	gi|12274881	*Huperzia billardierei*	49929.2	6.73	3.62
9019	Ribulose-1,5-bisphosphate carboxy-lase/oxygenase large subunit	gi|13548758	*Dacrycarpus imbricatus*	47995.2	6.6	3.41
4107	Ribulosebiphosphate carboxylase large subunit	gi|1079594	Pentodon	53300.7	6.22	3.06
1111	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	4.54
1003	Rubisco ssu precursor	gi|17855	*Brassica napus*	20301.1	7.63	3.5
4002	Rubisco ssu precursor	gi|17855	*Brassica napus*	20301.1	7.63	3.18
9702	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	0.24
4304	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	0.33
4313	Ribulose 1,5-bisphospate carboxylase	gi|14670019	*Synandrodaphne paradoxa*	43687	6.25	3.87
6516	Ribulose-1,5-bisphosphate carboxylase	gi|1050738	*Pentodon pentandrus*	51722	6.04	3.68
6002	Rubisco ssu precursor	gi|17855	*Brassica napus*	20301.1	7.63	3.77
4003	Rubisco ssu precursor	gi|17855	*Brassica napus*	20301.1	7.63	4.76

(*Continued*)

Spot No.	Protein name	Accession No.	The species category	Protein MW	Protein PI	Fold change (M15/Xiangyou 15 variety)
5305	Ribulose bisphosphate carboxylase	gi|1346967	*Chloroplast Brassica oleracea*	52922.5	5.88	4.21
9405	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|34015063	*Tristiropsis acutangula*	44167.2	6.12	0.25
6709	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	4.4
1617	Rubisco activase	gi|23320705	*Medicago sativa*	29999.2	5.63	0.25
8414	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	0.31
7403	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	3.76
7201	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	3.66
10	Ribulose bisphosphate carboxylase	gi|1346967	Chloroplast *Brassica oleracea*	52922.5	5.88	2.01
5504	Ribulose bisphosphate	gi|266893	Cucumber	45681.2	7.57	4.09
809	ATP synthase CF1 alpha subunit	gi|7525018	*Arabidopsis thaliana*	55294	5.19	3.97
3802	ATP synthase subunit β-1	gi|26391487	*Arabidopsis thaliana*	59634	6.18	3.74
1710	ATP synthase subunit beta	gi|75336517	*Brassica napus*	53682.9	5.21	4.05
1610	ATP synthase subunit beta	gi|75336517	*Brassica napus*	53682.9	5.21	3.01
3113	Fructose-bisphosphate aldolase	gi|42573227	*Arabidopsis thaliana*	41318.5	9.07	0.24
8706	β-glucosidase	gi|1732572	*Brassica nigra*	50676	6.27	0.26
8705	β-glucosidase	gi|1732572	*Brassica nigra*	50676	6.27	0.28
Stress and defense proteins						
9701	Myrosinase	gi|127733	*Brassica napus*	62695.9	6.62	0.28
3816	Myrosinase	gi|56130949	*Brassica rapa* subsp. pekin	62926	6.65	4.39
5806	Myrosinase	gi|127733	*Brassica napus*	62695.9	6.62	3.36
7808	Myrosinase	gi|127733	*Brassica napus*	62695.9	6.62	3.38
Photosynthesis						
3204	Chlorophyll a/b binding protein	gi|18266039	*Brassica oleracea*	28184.1	5.17	4.13
1205	Chlorophyll a/b binding protein type I	gi|7271945	*Asarina barclaiana*	18689.6	5.6	3.08
2403	HCF136 (High chlorophyll fluorescence 136)	gi|15237225	*Arabidopsis thaliana*	44076.4	6.79	3.76
Protein metabolism						
1511	Glutamine synthetase, chloroplastic	gi|12643761	Brassica napus	47314.9	6.16	3.54
Other proteins						
2605	Actin	gi|32186916	Upland cotton	41700.9	5.31	4.43
8608	Putative chloroplast translation elongation factor EF-Tu	gi|23397095	*Arabidopsis thaliana*	51623.6	5.84	0.31
6815	OSJNBa0042N22.23	gi|32492228	*Oryza sativa*	84497	9.17	4.08
2002	Unknow protein	gi|55168344	*Oryza sativa*	123805.3	7.02	4.19

sion of ATP synthase subunit beta (0.91 fold), myrosinase (1.02 fold), high chlorophyll fluorescence 136(1.05 fold) and chloroplast translation elongation factor EF-Tu (0.96 fold) almost has no change. The mRNA level does not always correlate with the protein level in this study, the reason may be the post-transcriptional regulation (Yan et al., 2006).

Discussion

In this study, 15-day-seedlings of Xiangyou 15 variety and M15 were selected for proteomic analysis. These proteins were mainly involved in metabolism of carbohydrate and energy (75%), stress and defense (8.3%), photosynthesis (6.3%), protein metabolism (2.1%) and other proteins (8.3%). Several proteins present in multiple spots in this study, the reason may be potential protein isoforms derived from alternatively spliced mRNAs were dispersed in different spots (Jiang et al., 2007), or post-translational modifications, such as glycosylation and phosphorylation, which altered the molecular weight and/or charge of proteins.

The differences in physiological and chemical characteristics

The M15 derived from Xiangyou 15 variety has little genetic background difference. And the 15-day-seedling has little difference too. However, the fatty acid content has obviously

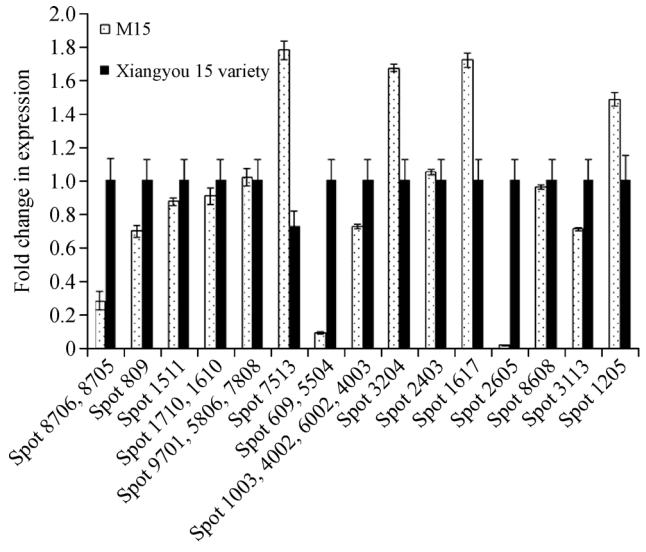

Figure 3 Real-time PCR analysis of the transcript levels of differentially expressed proteins. Spot 8706, 8705, β-glucosidase (gi|732572); Spot 809, ATP synthase CF1 alpha subunit(gi|7525018); Spot 9701, 5806, 7808, myrosinase (gi|127733); Spot 1511, myrosinase (gi|12643761); Spot 7513, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit(gi|27752813); Spot 609 and 5504, Ribulose bisphosphate(gi|266893); Spot 1003, 4002, 6002 and 4003, rubisco ssu precursor(gi|17855); Spot 1710, 1610, ATP synthase subunit beta(gi|75336517); Spot 3113, fructose-bisphosphate aldolase(gi|42573227); Spot 8608, putative chloroplast translation elongation factor EF-Tu(gi|23397095); Spot 2605, actin(gi|32186916); Spot 1617, rubisco activase(gi|23320705); Spot 2403, high chlorophyll fluorescence 136(gi|15237225); Spot 3204, chlorophyll a/b binding protein(gi|18266039); Spot 1205, chlorophyll a/b binding protein type I, (gi|7271945).

different, especially the oleic acid content, Xiangyou 15 variety was 64.208%, while the M15 was 80.935%.

Carbohydrate and energy proteins

Metabolisms of carbohydrate and energy are basic but essential activities in plant. Approximately 81.3% identified proteins were involved in the group of carbohydrate and energy metabolism. This suggests that altered carbohydrate and energy proteins play an important role in the growth of high oleic acid rapeseed seedling. Most of the identified proteins were associated with the metabolism of carbohydrate and energy (Table 3).

Rubisco activase is a member of the AAA + family (Archie et al., 2003), promote and maintain the catalytic activity of rubisco, which catalyzes the first major step of carbon fixation and regulates the activity of rubisco in response to light-induced changes in the redox potential (Nancy, 2002). It is a major rate-limiting factor of photosynthesis under present atmosphere (Portis, Parry, 2007). In rapeseed, seed oil content correlated well with rubisco activity in the silique wall but not in the leaf, a finding consistent with the photosynthesis results (Hua et al., 2012). And the activities of rubisco and phosphoribulokinase were sufficient to account for significant refixation of CO_2 produced during *B. napus* oil biosynthesis (Ruuska et al., 2004). And the qPCR analysis showed that the expression of rubisco activase was significantly higher in M15 seedling than Xiangyou 15 variety seedling.

Ribulose bisphosphate carboxylase, ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit, ribulose 1, 5-bisphospate carboxylase (spot 4313, 6516) and ribulose bisphosphate belongs to the rubisco. And we can see that the expression of different rubisco has the different fold change of high oleic acid rapeseed to Xiangyou 15 variety, the reason may be that different rubisco is incompatible with structural and biochemical information (Delwiche and Palmer, 1996). Rubisco catalyzes the first step in net photosynthetic CO_2 assimilation and photorespiratory carbon oxidation (Spreitzer and Salvucci, 2002), and it has the function of activation of ribulose-1, 5-bisphosphate carboxylase/oxygenase involves the ATP-dependent carboxylation of the epsilon-amino group of lysine leading to a carbamate structure. In developing embryos of *Brassica napus* L., rubisco acts without the Calvin cycle to increase the efficiency of carbon use during the formation of oil. In comparison with glycolysis, this metabolic conversion provided 20% more acetyl-CoA for fatty-acid synthesis and resulted in 40% less loss of carbon as CO_2 (Jörg et al., 2004).

β-glucosidase (EC 3.2.1.21) is a glucosidase enzyme that acts upon β-1,4 bonds linking two glucose or glucose-substituted molecules. A β-glucosidase was purified from seeds of *Brassica napus* L. (oilseed rape). β-glucosidase is encoded by a small gene family in B. napus, the genes are expressed in the seed, with a low degree of expression in other tissues. *In situ* hybridization also detected β-glucosidase mRNA in shoots, young roots, and the basal part of the hypocotyls (Falk and Rask, 1995). Spot 8705, 8706 were identified as β-glucosidase, and the qPCR analysis showed that the expression in M15 was significantly lower in Xiangyou 15 variety.

Fructose 1, 6-biphosphate aldolase (d-fructose-1, 6-bisphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) is a key enzyme in plants, which is involved not only in glycolysis and gluconeogenesis in the cytoplasm, but also in the Calvin cycle in plastids. Research on Fructose 1, 6-biphosphate aldolase in various organisms has been reported (Lu et al., 2012). And the spot 3113 was identified as fructose-bisphosphate aldolase in this study, the qPCR analysis showed that the expression of fructose-bisphosphate aldolase in M15 was lower in Xiangyou 15 variety. Eight Fructose 1, 6-biphosphate aldolase family genes (AtFBA1–8) were identified and analyzed in *Arabidopsis thaliana*, and most of the AtFBA genes, except for AtFBA3, were significantly responsive to drought stress in roots (Lu et al., 2012). But

there has no relatively study in rapeseed, and its function need to be further investigated.

Stress and defense proteins

Myrosinases (β-thioglucoside glucohydroase, E. C. 3.2.3.1) are thioglucosidases that hydrolyze the natural plant products glucosinolates. It is generally believed to be part of the plant's defense against insects, and possibly also against pathogens (Rask et al., 2000). Spot 9701, 5806, 7808 (gi|127733) and Spot 3816 (gi|56130949) were identified as glutamate synthase myrosinase. Oilseed rape contains a unique defense system known as the glucosinolate–myrosinase system. Myrosinase is present in specialist cells known as 'myrosin cells' and can also be known as toxic mines. The myrosin cell idioblasts of *Brassica napus* were genetically reprogrammed to undergo controlled cell death (ablation) during seed development (Ishita et al., 2011). Two different myrosinase proteins were found in oilseed rape (*Brassica napus* L. cv. Bienvenu) seedlings, they have similar kinetic characteristics, while the physical is different (David and John, 1991). The qPCR analysis showed there has little difference between two materials.

Photosynthesis

Chlorophyll a/b complexes associated with PSI and PSII in higher plants (Jansson, 1994), in addition to responsible for absorption and conversion of light energy in oxygen-evolving photosynthetic organisms (Green and Durnford, 1996), it plays an important role in the adaptation processes under excessive light conditions (Horton et al., 2005), furthermore, it fulfills multiple functions, such as light harvesting and energy dissipation under different illumination (Liu et al., 2008). Western blotting showed that light-harvesting chlorophyll a/b binding protein declined considerably during senescence, but on regreening it increased back to the levels seen in green leaves (Zavaleta et al., 1999). Spot 1205 and 3204 were identified as chlorophyll a/b binding protein. But there has no relatively study on rapeseed. The qPCR analysis showed that the expression of chlorophyll a/b binding protein in M15 seedling has 1.6 fold to Xiangyou 15 variety seedling. It indicates that chlorophyll a/b binding protein may be a promising protein for the further study of M15.

Protein metabolism

In higher plants, the GS/glutamate synthase cycle provides the only efficient pathway for the conversion of inorganic nitrogen to the organic form (Stewart et al., 1980). In this study, the spot 1511 was identified as glutamate synthase, the qPCR analysis showed that the expression of glutamate synthase was significantly lower in M15 seedling than Xiangyou 15 variety seedling. Glutamine synthetase gene has been cloned from *Brassica napus* (Ochs et al., 1993), and its overexpression can alter ammonia assimilation and the response to phosphinothricin (Downs et al., 1994).

Other proteins

The actin cytoskeleton mediates a variety of essential biologic functions in all eukaryotic cells. In addition to providing a structural framework around which cell shape and polarity are defined, its dynamic properties provide the driving force for cells to move and to divide. And the spot 2605 was identified as actin in this study, the qPCR analysis showed that the expression of actin was significantly lower in M15. This gene plays an important role in fiber elongation but not fiber initiation in cotton (Li et al., 2005). But there has no relatively literature on the study of actin in rapeseed.

Chloroplast translation elongation factor EF-Tu plays a functional role in heat tolerance by acting as a molecular chaperone, it can reduce thermal aggregation of rubisco activase (Zoran et al., 2007). This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis (Ferro et al., 2003). The spot 8608 was identified in this study. In pea, the expression of this gene stimulated by light and it may have an important role in plant adaptation to environmental stresses (Singh et al., 2004). The chloroplast translation elongation factor (EF-Tu) was upregulated under drought (Salekdeha et al., 2002; Kamal et al., 2012). But there has no study of chloroplast EF-Tu in rapeseed.

Conclusions

A comparative proteomic approach based on 2-DE and mass spectrometry was adopted to identify proteins that changed in the 15-day-seedlings of M15 compared with Xiangyou 15 variety. 48 spots which has 3.0-fold from M15 compared with Xiangyou 15 variety were successfully identified by MALDI-TOF/TOF MS. All identified proteins were mainly involved in metabolism of carbohydrate and energy (81.3%), stress and defense (8.3%), and other functions (10.4%). Then qPCR analysis was used to verify the expression levels of differentially expressed proteins, which not well agree with the proteomic results. In this work, the proteomic analysis revealed that some proteins, such as rubisco, chlorophyll a/b and rubisco activase may have important roles in the fatty acid synthesis of M15.

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Compliance with ethics guidelines

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References

Allman F M A, Gomes K, Favaloro E J, Petocz P (2005). A diet rich in high-oleic-acid sunflower oil favorably alters low-density lipoprotein cholesterol, triglycerides, and factor VII coagulant activity. J Am Diet Assoc, 105(7): 1071–1079

Archie R P Jr. (2003). Rubisco activase–Rubisco's catalytic chaperone. Photosynthesis Research,75: 11–27

Bradford M M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72(1–2): 248–254

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri G M, Carnemolla B, Orecchia P, Zardi L, Righetti P G (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis, 25(9): 1327–1333

Chen X, Truksa M, Shah S, Weselake R J (2010). A survey of quantitative real-time polymerase chain reaction internal reference genes for expression studies in Brassica napus. Anal Biochem, 405 (1): 138–140

David C J, John T R (1991). Development and characteristics of myrosinase in Brassica napus during early seedling growth. Physiol Plant, 82(2): 163–170

Delwiche C F, Palmer J D (1996). Rampant horizontal transfer and duplication of rubisco genes in eubacteria and plastids. Mol Biol Evol, 13(6): 873–882

Downs C G, Christey M C, Davies K M, King G A, Seelye J F, Sinclair B K, Stevenson D G (1994). Hairy roots of Brassica napus: II. Glutamine synthetase overexpression alters ammonia assimilation and the response to phosphinothricin. Plant Cell Rep, 14(1): 41–46

Falk A, Rask L (1995). Expression of a zeatin-O-glucoside-degrading β-glucosidase in *Brassica napus*. Plant Physiol, 108(4): 1369–1377

Ferro M, Salvi D, Brugière S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003). Proteomics of the chloroplast envelope membranes from Arabidopsis thaliana. Mol Cell Proteomics, 2(5): 325–345

Fuller G, Diamond M J, Applewhite T H (1967). High-oleic safflower oil. Stability and chemical modification. J Am Oil Chem Soc, 44(4): 264–266

Green B R, Durnford D G (1996). The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol, 47(1): 685–714

Guan C Y, Liu C L, Chen S Y, Peng Q, Li X, Guang M (2006). A mutant of winter rapeseed (*Brassica napus* L.) with high oleic acid composition. Acta Agonomica Sinica, 32: 1625–1629 (in Chinese)

Guan M, Li X (2008). The studies of agronom ic characteristics of high oleic acid lines on rapeseed (*Brassica napus*). Chin J Oil Crop Sci, 30: 25–28 (in Chinese)

Guan M, Li X, Guan C (2012). Microarray analysis of differentially expressed genes between *Brassica napus* strains with high- and low-oleic acid contents. Plant Cell Rep, 31(5): 929–943

Horton P, Wentworth M, Ruban A (2005). Control of the light harvesting function of chloroplast membranes: the LHCII-aggregation model for non-photochemical quenching. FEBS Lett, 579(20): 4201–4206

Hua W, Li R J, Zhan G M, Liu J, Li J, Wang X F, Liu G H, Wang H Z (2012). Maternal control of seed oil content in *Brassica napus*: the role of silique wall photosynthesis. Plant J, 69(3): 432–444

Ishita A, Birgit H B, Magnor H, Bjørn I H, Caroline M (2011). Oilseed rape seeds with ablated defence cells of the glucosinolate–myrosinase system. Production and characteristics of double haploid MINELESS plants of *Brassica napus* L. J Ex Bot, 62(14): 4975–4993

Iwona R, Catherine E R, Dilip KN, Jones P J H. (2006). Phytosterols mixed with medium-chain triglycerides and high-oleic canola oil decrease plasma lipids in overweight men. Metabolism, 55(3): 391–395

Jansson S (1994). The light-harvesting chlorophyll a/b-binding proteins. Biochim Biophys Acta, 1184(1): 1–19

Jiang Y, Yang B, Harris N S, Deyholos M K (2007). Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. J Exp Bot, 58(13): 3591–3607

Kamal A H M, Kim K H, Shin K H, Choi J S, Baik B K, Tsujimoto H, Heo H Y, Park C S, Woo S H (2010). Abiotic stress responsive proteins of wheat grain determined using proteomics technique. Aust J Crop Sci, 4: 196–208

Katayama H, Nagasu T, Oda Y (2001). Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom, 15(16): 1416–1421

Li X B, Fan X P, Wang X L, Cai L, Yang W C (2005). The cotton ACTIN1 gene is functionally expressed in fibers and participates in fiber elongation. Plant Cell, 17(3): 859–875

Liu C, Zhang Y, Cao D, He Y, Kuang T, Yang C (2008). Structural and functional analysis of the antiparallel strands in the lumenal loop of the major light-harvesting chlorophyll a/b complex of photosystem II (LHCIIb) by site-directed mutagenesis. J Biol Chem, 283(1): 487–495

Livak K J, Schmittgen T D (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25(4): 402–408

Lu W, Tang X, Huo Y, Xu R, Qi S, Huang J, Zheng C, Wu C A (2012). Identification and characterization of fructose 1,6-bisphosphate aldolase genes in *Arabidopsis* reveal a gene family with diverse responses to abiotic stresses. Gene, 503(1): 65–74

Mark A D, Jay T A, Michael D B (2001). Capturing value in the supply chain the case of high oleic acid soybeans. International Food and Agribusiness Management Review., 5: 87–103

Nancy A E (2002). Alternative splicing and the control of flowering time. Plant Cell, 14(4): 743–747

Ochs G, Schock G, Wild A (1993). Chloroplastic glutamine synthetase from *Brassica napus*. Plant Physiol, 103(1): 303–304

Portis A R Jr, Jr (2003). Rubisco activase—Rubisco's catalytic chaperone. Photosynth Res, 75(1): 11–27

Portis A R Jr, Parry M A J (2007). Discoveries in Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. Photosynth Res, 94(1): 121–143

Rask L, Andréasson E, Ekbom B, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol, 42: 99–113

Zoran R, Ivana M, Jianming F, Eduardo C, Benjamin P D (2007). Chloroplast protein synthesis elongation factor, EF-Tu, reduces thermal aggregation of rubisco activase. J Plant Physiol, 164(12): 1564–1571

Spreitzer R J, Salvucci M E (2002). Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol, 53(1): 449–475

Robert J N, Benjamin W, Thomas A W, Patrick S, Garry H, Robert F (2004). Decreased aortic early atherosclerosis and associated risk factors in hypercholesterolemic hamsters fed a high- or mid-oleic acid oil compared to a high-linoleic acid oil. J Nutr Biochem, 15(9): 540–547

Ruuska S A, Schwender J, Ohlrogge J B (2004). The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes. Plant Physiol, 136(1): 2700–2709

Salekdeha G H, Siopongcoa J, Wade L J, Ghareyazie B, Bennett J (2002). A proteomic approach to analyzing drought- and salt-responsiveness in rice. Field Crops Res, 76(2-3): 199–219

Jörg S, Fernando Gn, John B O, Yair S H. (2004). Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds. Nature, 432(7018): 779–782

Singh B N, Mishra R N, Agarwal P K, Goswami M, Nair S, Sopory S K, Reddy M K (2004). A pea chloroplast translation elongation factor that is regulated by abiotic factors. Biochem Biophys Res Commun, 320(2): 523–530

Stewart G R, Mann A F, Fentem P A (1980). Enzymes of gluiamate formation: glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. In: Zn B J Miflin, ed: The Biochemistry of Plants, l5. Academic Press, San Diego, CA, 271–327

Xiao G, Wu X M, Guan C Y (2009). Identification of differentially expressed genes in seeds of two *Brassica napus* mutant lines with different oleic acid content. Afr J Biotechnol, 8: 5155–5162

Yan S P, Zhang Q Y, Tang Z C, Su W A, Sun W N (2006). Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol Cell Proteomics, 5(3): 484–496

Zavaleta M H A, Thomas B J, Thomas H, Scott I M (1999). Regreening of senescent *Nicotiana* leaves. J Exp Bot, 50(340): 1677–1682

Zhang Z Q, Xiao G, Guan C Y (2009). Determining biodiesel yield by gas chromatography inner standard method. Journal of the Chinese Cereals and Oils Association., 24: 139–142 (in Chinese)

Zhang Z Q, Xiao G, Liu R Y, Tan T L, Guan C Y, Wang G H(2011). Efficient Construction of a normalized cDNA library of the high oleic acid rapeseed seed. African Journal of Agricultural Research., 6: 4288–4292