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*Full Length Research Paper*

# Identification of differentially expressed genes in seeds of two *Brassica napus* mutant lines with different oleic acid content

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**The regulation of seed oleic acid synthesis in rapeseed is largely unknown. In this study, gene expression pattern during seed development between two *Brassica napus* mutants was compared. Using immature seeds 27 days after pollination, differentially expressed cDNA clones were identified by subtractive suppression hybridization (SSH). A total of 480 cDNA clones corresponding to 88 genes were found up-regulated and 18 genes down-regulated in seeds with high oleic acid content. Most of the differentially expressed genes are related to metabolism and regulation. The possible role of these genes in seeds was discussed. Further analysis of the function of these genes may provide novel targets for manipulation of fatty acid composition in rapeseed.**

**Key words:** *Brassica napus*, differential gene expression, oleic acid content, seed development, subtractive suppression hybridization.

## INTRODUCTION

Oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are major components of fatty acids in vegetable oils. The standard rapeseed oil in China contains about 6% palmitic acid, 5% stearic acid, 61% oleic acid, 20% linoleic acid and 8% linolenic acid. Oils with higher content of oleic acid are of interest for nutritional and industrial purposes (McVetty et al., 2002). Therefore, high oleic acid and low linoleic acid content is one of major objectives for rapeseed breeders.

The endoplasmic reticulum-bound oleate desaturase FAD2 is the key enzyme responsible for the conversion of oleic acid to linoleic acid in non-photosynthetic tissues in plants (Okuley et al., 1994) and *fad*2 is considered as a candidate gene controlling the oleic acid content in rapeseeds (Stoutjesdijk et al., 2002; Hu et al., 2006). Genes coding for FAD2s have been cloned and characterized from plants such as *Arabidopsis thaliana* (Okuley et al., 1994), soybean (Heppard et al., 1996), *Brassica napus* (Scheffler et al., 1997), sunflower (Hongtrakul et al., 1998), peanut (López et al., 2000) and olive (Hernández et al., 2005).

Schierholt et al., (2001) reported that two loci associated with the oleic acid content in a high oleic acid mutant. It has been suggested that oleic acid content in *B. napus* is affected by one major gene and seveal minor genes (Guan, 2006).

Our knowledge of fatty acid metabolism has benefited considerably from the investigations of several *Arabidopsis* T-DNA tagged mutants (Hugly et al., 1989; Kunst et al., 1989; Hugly and Somerville 1992). The composition of fatty acids synthesized in plants is primarily determined by thioesterases, condensing enzymes and desaturases. Manipulation of the thioesterases and desaturases for modifying unsaturation levels of fatty acids in transgenic rapeseed has made great success (Kinney, 1994; Ohlrogge, 1994; Topfer et al., 1995).

The suppression subtractive hybridization (SSH) technique is a method based on a specific form of PCR that permits exponential amplification of cDNAs which differ in abundance, whereas amplification of sequences of identical abundance in two populations are suppressed

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(Diatchenko et al., 1996). SSH has been widely used in the study of gene expression differentiation in animals and plants (Li et al., 2004; Bouton et al., 2005).

In this study, we identified genes differentially expressed in two *B. napus* mutant lines with different oleic acid content by SSH. The two mutant lines derived from the same parent, therefore, all different sequence data and information caused by different genetic background can be deduced directly. This study cataloged temporal changes in gene expression between two mutant lines. It provides insights into the regulation net works responsible for seed fatty acid synthesis.

## MATERIALS AND METHODS

### Plant materials

Two *B. napus* mutant lines that differ in seed oleic acid content (HO/LO) were used in this study. The two lines were derived from an EMS-mutagenized (1%, v/v) population of *B. napus* double low cv Xiangyou 15 with moderate oleic acid content (64%). Individuals in mutagenized population were selfed for four times. For each generation, selfed seeds of each individual were analysed by GC, individuals with higher and lower oleic acid content were selected for next selfed. Ultimately, two lines with oleic acid content 71.7 and 55.3%, respectively, were selected. No significant difference between the pair of mutant lines in the investigated traits of agronomic importance except seed fatty acids composition (see Results). To reduce possible environmental effects, both HO and LO mutant lines were grown in the same greenhouse. Siliques of HO and LO individual plant were harvested at 10, 15, 20, 25, 30, 35, 40 and 45 days after pollination (DAP), and immature selfed seeds were separated from silique coats and subject for lipid analysis, mRNA extraction and subsequent SSH library construction.

### Lipid analysis

The total lipids were extracted using Soxhlet extraction. 5 g samples of the dry seed powder were weighed and added into Whatman Cellulose extraction thimbles for Soxhlet extraction and followed by gravimetric estimation of extractable lipid content (Coonrod et al., 2008). Thimbles were packed with glass wool and placed in a soxhlet extraction apparatus. Petroleum ether (b. p. 40 - 60°C with 0.01% (w/v)) was added to each boiling flask containing teflon boiling chips. The samples were refluxed at 7 refluxes per hour for 48 h by adding petroleum ether as needed. Extracted lipids were rinsed with petroleum ether from the apparatus into preweighed beakers. The petroleum ether was evaporated until no petroleum ether odor was detected. Residual petroleum ether was removed by vaccum pump and the oil content of the seed was determined by weight of lipid/dry seed (w/w).

The contents of each fatty acid composition in leaves, roots, stems and seeds of the two mutants were measured by gas chromatography (GC). Fatty acid methyl esters (FAME) were prepared by transesterification with methanolic sodium methoxide. The temperature was increased from 190 to 210°C at 2.75°C per min and held at the final temperature for 8 min. The temperatures of injector and flame ionization detector (FID) were held at 220 and 240°C, respectively. Samples of FAME (1.0 µl) were injected using an autosampler at a split ratio of 50:1. Peaks were identified by comparison of their retention times with those of the authentic standard (OMEGA).

### RNA isolation, RT-PCR, Northern blot analysis

Total RNA was extracted from plant tissues using a Plant RNA Kit (OMEGA) according to the manufacturer's protocol. For RT-PCR, first-stand cDNA synthesis was perfomed with 1 µg total RNA using ImProm-II™ Reverse Transcriptase (Promega). The quantity of products was normalized using *B. napus β-actin* as a control (Yan et al., 2008). The semi-quantitative RT-PCR was used to monitor the changes in mRNA transcript levels of these genes and the primers were listed in table 1. Northern blots were performed with Digoxin labeled probes prepared from the cDNA fragments isolated from the SSH library using a Digoxin Random Prime Labeling Kit (Innogent Bioscience Inc.).

### Subtractive suppression hybridization

For subtractive suppression hybridization, polyA$^+$ RNAs were isolated from the total RNA of HO and LO mutant lines using the E.Z.N.A mRNA Enrichment Kit (OMEGA) and the PCR select™ cDNA subtraction Kit (Clontech) according to the manufacturer's protocols. To detect the gene expression in HO, both forward subtraction and reverse subtraction were carried out. In the forward subtraction, cDNA from HO was served as tester and the cDNA from LO was served as driver. In the reverse subtraction, the forward tester cDNA from HO used as reverse driver and forward driver used as the reverse tester. Each tester or driver cDNA sample was generated from 2 µg polyA$^+$ RNA.

### cDNA subtraction library construction

The products of the second PCR containing enriched differentially expressed transcripts, were inserted into pMD18-T cloning vector (TAKARA) and transformed to the TOP10 *Escherichia coli* competent cells. The TOP10 *E. coli* was plated onto LB medium plates containing ampicillin, X-gal and IPTG. White colonies were selected and used for amplification by inoculating them to 96-well microtitre plate containing ampicillin LB medium and cultured for 9 h with shaking. The library was replicated and added equal volume 30% (v/v) glycerol, then stored at -70°C.

### Reverse Northern hybridization screening

Some researchers found that the subtracted library is likely to contain a significant number of false positives when compared by SSH between two materials with similar genetic background. So we did reverse Northern hybridization screening for the subtracted libraries to eliminate false positives. 1 µl of the growing culture of the library was used as template for PCR amplification as described by Diatchenko et al., (1996). After amplification, 5 µl PCR products were loaded onto 1.5% gels, positive clones were selected for further analysis. Another 5 µl PCR products were combined with 5 µl 0.6 N NaOH and 1 µl of this alkali denatured PCR products was transferred to positively charged nylon membrane (OSMONICS) and immobilized via UV cross-linking. A set of two identical membranes were prepared. Probes were synthesized and the second-strand cDNA of was labeled using Digoxin Random Prime Labeling Kit (Innogent Bioscience Inc.). The forward- and reverse-subtracted probes were digested by *Rsa* I to remove the adaptor sequences, then, they were hybridized with nylon membrane respectively. hybridization was carried out at 68°C for 16 h. They were washed twice with 2 × SSC and 0.1% SDS solution at 68°C for 20 min and followed by washing three times with 0.1 × SSC and 0.1% SDS solution at 68°C for 20 min. The chemiluminescence signal was detected using Chemiluminescent Nucleic Acid Detection Kit (Innogent Bioscience Inc.) company by exposing the membrane to X-ray film (BioMax light film, Kodak) for 20 min at room temperature.

**Table 1.** Oligonucleotide primer sequences designed for amplification of 30 RT-PCR products isolated from cDNA subtraction library.

| RT-PCR product | Forward primer(5′-3′) | Reverse primer(5′-3′) |
|---|---|---|
| B4 | ACAAAAGAAACCACCACC | GAGTAAAACAATGCAGGAGA |
| B5 | TCACATACCGCAAAGATTTC | GCGTCACTTTCCTGTAGCA |
| B13 | TCTGATTCTTTCGATACACG | GCACAAGTTTTAAGGGACA |
| B43 | AAGAAGGGTAAAGCCAAATG | CGAGAAGGTTAATCCAAGTT |
| B50 | CCTCTAAGAAAGAACCCAT | TCGCTTGTAAACTCACTCA |
| B58 | ATACAAAACGAGACGACGAT | TAGGGAGACAACAAGCACC |
| B115 | GTCTGAACAACAGCAAGGA | GAAGAAGCGTATGAGGAGG |
| B156 | CTATCTTCCCTACATTAACAC | CTTTGCATCTCAACAGTCC |
| B157 | TCATCCAGGACAAGCAACA | GTACGCGGGGATTGATTTA |
| B200 | TTGACAGAAACGGAGCATC | AGTACGCATTAGGCCACAG |
| B201 | ATACTCTAGCCACGGCACT | GGTCAACTCAAAATTCGTTC |
| B209 | GCGGGGACTCAAACAATT | ACAACGGCCAGAACTACA |
| B256 | TGGTGGTGATTGCTGTTG | TGATGAGGGTTTGTCTCACCACATT |
| B276 | TGCGTTTTACTGCCTTACT | AGGTTACACGGGGAGATTA |
| B281 | AACTTTGACAGAAACGGAG | AGGCATCTTAAACCCACT |
| B290 | GGGATGCCTTATCAACTG | CCAAATAGAAGACGCTGT |
| B292 | AAAAGCAGTAGGAACGAG | GTACGCGGGGACGATGAA |
| B295 | CGGCGTAATAATGACAAC | TCTACAATAAGATTAAGGGAGA |
| B307 | TACGCGGGGACAACAAAAT | CCAACCCTTAAATCACCAG |
| B311 | CGAAAGTGAGCCAAGACA | GCAGGTACTAGCCACAGA |
| B312 | GGGTTCATTCAAGTCTGC | GTTTATGGTTTTGGTGGC |
| B319 | AGAAGGGTAAAGCCAAAT | CGAGAAGGTTAATCCAAGT |
| B332 | GAGATGATCGGGATTTATT | ATGGAGATTTGCGTATTT |
| B345 | GTTGGTTTGCGTATGACTG | AGAAGCAGATTCGGAACA |
| B361 | ATCGTAATACTGGGCATAC | CTTAGGGGTTTAGTGTTTG |
| B370 | TAATACAGTGCCAGGGAA | TGAATAACAATATCGGAGC |
| B398 | GTGTCTGAACAACAGCAAG | AGAAGCGTATGAGGAGGT |
| B412 | AAGCCAAGATTCTCCTCC' | CACTCTGACAACCCACAAG |
| B420 | AAAGGGAGTGAGCAGAAC | CCAAGTGGAAGAAGAAGC |
| B478 | TCTTCTCGTGCGTTCCAT | CGATTGTTGCTTGTCCTG |

**Sequence analysis**

Positive clones were selected and sent directly to Invitrogen Biological Engineering Technology and Services CO., Ltd (Shanghai, China) for sequencing. The sequence data were analyzed by BLASTX and BLASTN for DNA and protein homologies (National Center for Biotechnology Information).

## RESULTS

### Accumulation of fatty acids in two rapeseed mutants

The major objective of this study was to identify genes that are differentially expressed in developing seeds of rapeseed mutants with different oleic acid content. The fatty acid composition in leaves, roots and stems are similar between the two mutants (date not show). Whereas, in seeds, oleic acid content is 71.7% of total oil in HO, while 55.3% in the LO, in contrast, HO seeds have less linoleic acid and linolenic acid than LO seeds (Table 2). Seed development from pollination to mature requires more than 40 days in rapeseed under normal conditions. We compared the time courses of fatty acids accumulation at developing seeds between two lines and found that the significant change of oleic acid content begin at 25 DAP (Figure 1), we therefore selected the 27 DAP seeds as the experimental materials for analysis.

### Library construction and screening

A total of 518 individual recombinant clones were obtained, 480 of them contain inserted fragment (Figure 2). In reverse Northern hybridization screening, all these 480 clones were hybridized with the forward- and reverse-subtracted probes. Among them, 106 clones showed obvious expression differentiation. 88 clones were found to be up-regulated in HO line and 18 clones showed

**Table 2.** Fatty acid compositions in the two lines of *B. napus* seeds (%).

| Sample | % of total fatty acids | |
|---|---|---|
| | HO line | LO line |
| Palmitic acid | 4.48 ± 0.2 | 6.58 ± 0.2 |
| Stearic acid | 3.47 ± 0.3 | 4.45 ± 0.3 |
| Oleic acid | 71.71 ± 0.5 | 55.26 ± 0.5 |
| Linoleic acid | 14.71 ± 0.3 | 20.12 ± 0.3 |
| Linoleic acid | 6.27 ± 0.2 | 12.35 ± 0.2 |
| Erucic acid | 0.15 ± 0.3 | 0.13 ± 0.2 |
| oil (% of dry weight) | 40.3 ± 0.5 | 40.5 ± 0.5 |

Measurement of total triacylglycerols and each fatty acid contents was carried out by methods as described in Materials and Methods. Date were presented as the mean±SD (n = 3).

down-regulation (indicated by the arrows in Figure 3). There are a significant number of false positives in our library that are compatible with other similar researches (Li et al., 2006), it may be due to the two mutant lines derived from the same parent and are in the same genetic background.

### Single-Pass sequencing and identification of differentially expressed genes

All 106 clones were sequenced and 88 uniESTs were obtained. The average length was about 350 bases (after removal of vector, poor quality and polyA sequences). Sequence analysis showed that 73 uniESTs have been submitted at GenBank, 19 of the 73 uniESTs matched previously described genes in *Arabidopsis* or *Brassica* (Table 3), which mainly shared significant similarity to metabolic enzymes and regulatory protein. 15 uniESTs have no match with entries in GenBank, they may be novel genes or derived from the variable 3′-terminal of full-length cDNA.

### RT-PCR pattern analysis of the differential expression mRNAs

To evaluate the result of the subtractions, 30 up-regulated genes in HO line were selected for further semi-quantitative RT-PCR confirmation, including 15 genes with known function and 15 genes with unknown function. Based on their sequence, we designed specific primers for each gene and amplified by RT-PCR. As shown in Figure 4, all genes showed differential expression in 27 DAP seeds of the two lines. Overall, the results of RT-PCR are consistent with the data from reverse Northern hybridization screening analysis, indicating that the application of SSH in this study was successful.

## DISCUSSION

### Differentially expressed gene related to fatty acid synthesis

Stearoyl-acyl-carrier-protein-desaturase (S-ACP-DES) gene (X63364) is the only differentially expressed gene related to fatty acid synthesis in our SSH library. In plants, C18:1 formation is catalyzed by the soluble S-ACP-DES. The members of S-ACP-DES are specific for particular substrate chain length and introduce double bond between specific carbon atoms. The SACP-DES has been purified from several plants and the genes characterized from several different species (Shanklin and Somerville, 1991; Thompson et al., 1991; Cahoon et al., 1996, 1998; Whittle et al., 2005). Since S-ACP-DES is the only plant enzymes which introduces the first double bond at carbon 9 and forms oleoyl-ACP, their activity primarily determines the ratios of unsaturated FAs (including oleic acid, linoleic acid, linolenic acid and erucic acid) to the total FAs. Difference in the activity of S-ACP-DES resulted in different unsaturated FAs content in seeds (Knutzon et al., 1992; Schnurbusch et al., 2000). Figure 5 showed the ratios of unsaturated to the total FAs in HO and LO lines at different stages. The date showed that there is more unsaturated FAs content in HO than in LO lines. This suggests different activity of S-ACP-DES in the two lines at the different stages. This result was confirmed by both SSH and RT-PCR analyses.

### Regulatory factors

In addition to thioesterases, condensing enzymes and desaturases, the regulation of fatty acids metabolism is shared by other factors. Studies on promoters of fatty acid metabolism genes identified some transcription factors that may play a regulatory role (Ohlrogge, 1997). By analogy to the study of other organisms, the control of expression of plant fatty acid synthetase genes is likely to involve a complex set of *cis* and *trans* acting factors. For example, promoters of animal fatty acid biosynthetic genes are regulated by hormones such as insulin (Moustaid et al., 1994), by dietary fatty acids (Roder et al., 1994), by glucose levels (Pripbuus et al., 1995).

There are many regulatory protein in our SSH list, such as TCP family transcription factor (B4), transcription factor (B16), auxin-induced protein (B156), zinc finger (C2H2 type) family protein (B201), calmodulin binding protein (B420), glucose regulated repressor protein (B49), defender against apoptotic death protein (B305) and signal peptidase (B319). TCP transcription factor works as Transcriptional activator and play a role in cell differentiation and cell growth in plants (Riechmann et al., 2000; Kosugi and Ohashi, 2002). Auxin is essential to the control of plant growth and development, profoundly affects turgor, elongation, division and cell differentiation.

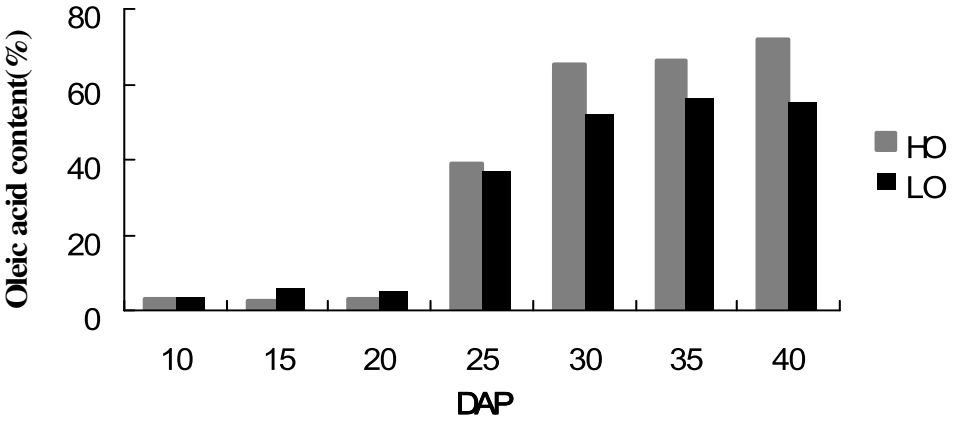


**Figure 1.** Comparison of accumulation oleic acids between HO and LO mutant lines.

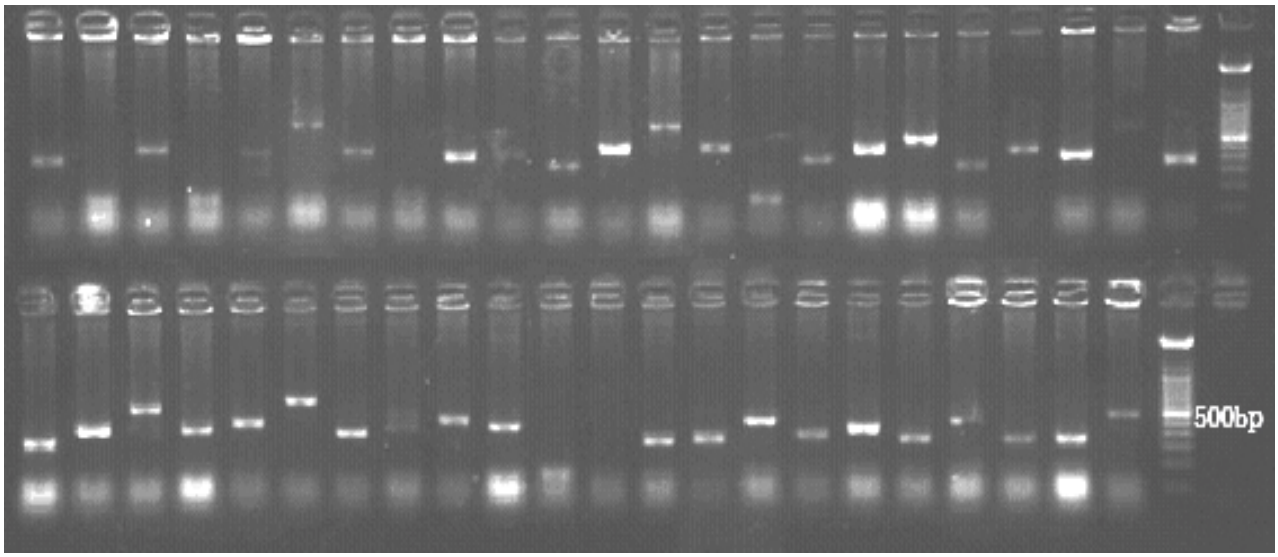


**Figure 2.** Primary screen of the subtracted cDNA library by colony PCR (partly). Colony PCR was performed as described and the products were resolved on 1.5% agarose gels.

These are major driving and shaping forces in morphogenesis and oncogenesis (Steffen and Athanasios, 1996). Several reports suggested that auxin can change membrane lipid composition, especially the amounts of the various unsaturated fatty acids (Moore et al., 1983; Goldberg et al., 1983; Liu et al., 1995). Auxin may change fatty acid composition of storage lipid in similar way. Polyunsaturated fatty acid, such as linolenic acid, arachidonic acid, eicosapentaenoic acid and decosahexaenoic acid are suggested to induce apoptotic death of tumour cells (Sessler and Ntambi, 1998). Therefore defender protein against apoptotic death may be related to fatty acid metabolism. Zinc finger (C2H2 type) family protein, calmodulin binding protein, glucose regulated repressor protein are common regulatory genes and involve in various regulatory pathways in plant (Whittle et al., 2005).

## Genes of unknown-function

Beside genes of known function, our library contains a high proportion of uniESTs of unknown function (78.4%). A number of these uniESTs may be novel genes. Since lipid synthesis and regulation of fatty acid metabolism involve different pathways in plant, it is not surprising that novel genes have been identified in our experiment. Our next objective is to analyze the function of these differential expression genes by knocking out their function in *B. napus* using RNAi method.

## Conclusion

In conclusion, we had identified a number of differentially expressed genes between two mutant *B. napus* lines with

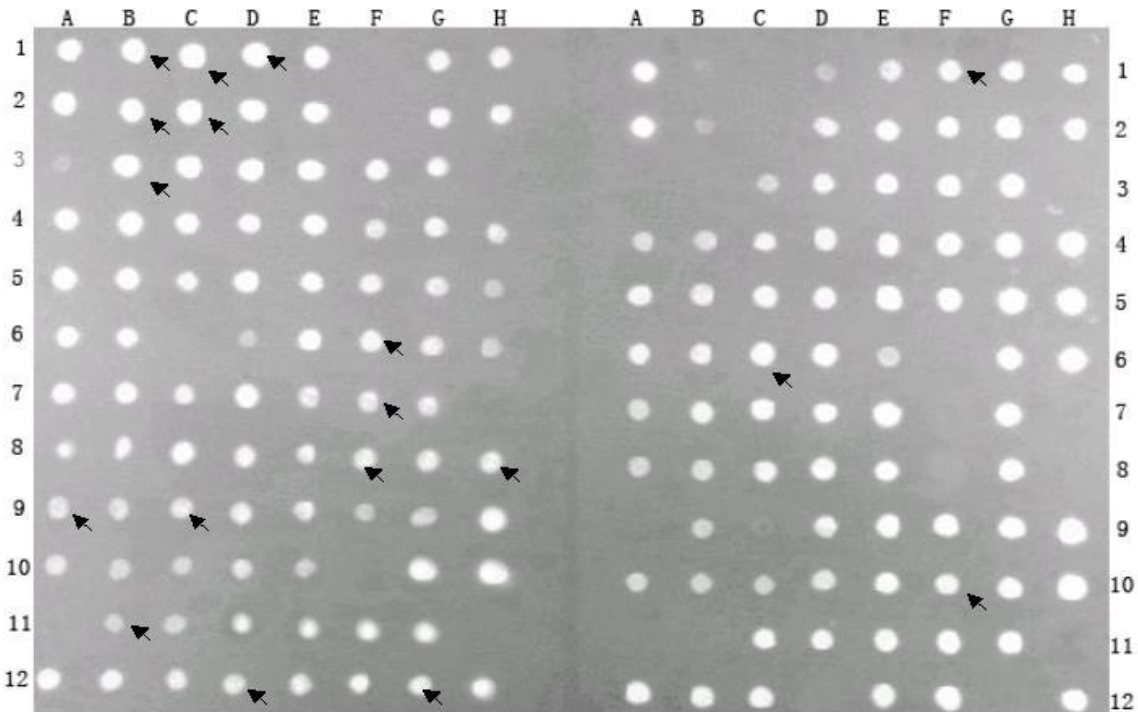


**Figure 3.** Reverse northern hybridization screening of cDNA subtracted libraries. The PCR products of cDNA clones from the subtracted libraries were blotted on two nylon membranes at same locations and two membranes were, respectively, hybridized with forward-(left) and reverse-(right) subtracted cDNA probes. Arrows indicate the representatives of the differentially expressed clones.

**Table 3.** Selected differential expressed genes in subtracted library.

| Clone | Blast homology | Source | GenBank match | E-value |
|---|---|---|---|---|
| B4 | TCP family transcription factor | *A. thaliana* | NP_197719 | 1e-31 |
| B16 | transcription factor | A. thaliana | NP_181834 | 3e-155 |
| B49 | glucose regulated repressor protein | *A. thaliana* | BAF01819 | 7e-74 |
| B156 | IAA 12(auxin-induced protein 12), transcription factor | *A. thaliana* | NP_171949 | 1e-146 |
| B200 | membrane protein | *B. juncea* | AAT38818 | 1e-126 |
| B201 | zinc finger (C2H2 type) family protein | *A. thaliana* | NP_565684 | 4e-115 |
| B276 | invertase/pectin methylesterase inhibitor family protein | *A. thaliana* | NP_199730 | 8e-45 |
| B281 | defensin-like protein | *B. napus* | EF182821 | 3e-79 |
| B292 | senescence-associated protein sen1-like protein | *A. thaliana* | BAD95161 | 4e-119 |
| B305 | defender against apoptotic death protein | *B. rapa* | NP_174500 | 2e-56 |
| B307 | ribosomal protein | *B. rapa* | CAA80864 | 1e-55 |
| B319 | eukaryotic translation initiation factor -5A | *B. napus* | AAR91929 | 3e-63 |
| B326 | heterogeneous nuclear ribonucleoprotein | *A. thaliana* | NM_180208 | 1e-42 |
| B339 | aspartic protease | *B. oleracea* | X77260 | 8e-44 |
| B361 | inorganic pyrophosphatase family protein | *A. thaliana* | NM_121002 | 4e-98 |
| B412 | ATP-dependent Clp protease proteolytic subunit | *A. thaliana* | NP_563836 | 3e-59 |
| B420 | calmodulin binding prorein/ translation elongation factor | *A. thaliana* | NP_001030993 | 1e-33 |
| B461 | stearoyl –ACP desaturase | *B. napus* | CAA44964 | 1e-149 |
| B478 | signal peptidase | *A. thaliana* | NP_175669 | 4e-104 |

different oleic acid content by using the SSH method. Our data show an additional level of complexity in the regulation of fatty acids metabolism, which may be spread out and coordinated among the many enzymes

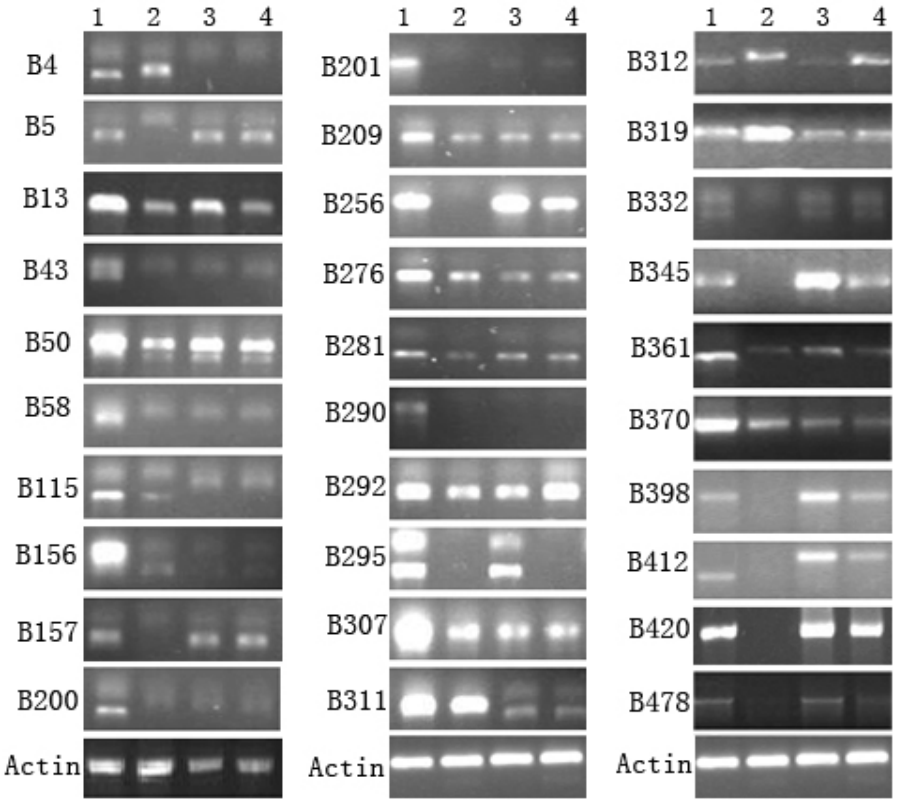


**Figure 4.** Semi-quantitative RT-PCR analysis of differential expression of genes in seeds and leaves of two mutants. The clone ID is listed on the left side. The names of corresponding homologue genes are listed in Tables 2. Lane 1, seeds of HO; Lane 2, seeds of LO; Lane 3, leaves of HO; Lane 4, leaves of LO.

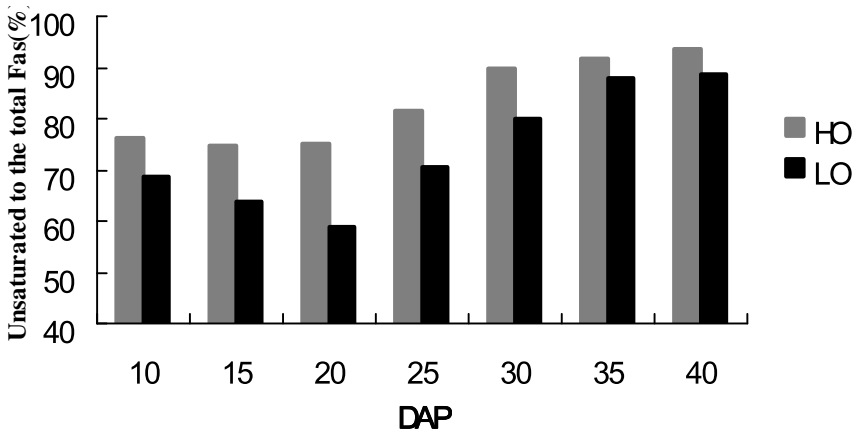


**Figure 5.** Comparison of total polyunsaturated fatty acids between HO and LO.

and regulatory factors involved in the pathway. Experiments are underway at present to determine whether knockout of these genes or regulatory factors in transgenic plants can yield instructive or useful changes in seed fatty acids metabolism. Anyway, the information generated by this study will facilitate the manipulation of the quality of oils produced in seeds of oil crops.

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