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An analysis and research on biosynthesis pathway and gene regulation of fatty acid of *Camellia oleifera* seeds

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ABSTRACT

Taking fruit expanding period and oil synthetic peak period of Oiltea camellia (Camellia oleifera Abel.) seeds as materials to build transcriptome and expression profile database, it is indicated that there are totally 104 non-redundant gene sequences involved in fatty acid biosynthesis of Oiltea camellia and 14 main enzyme genes involved after a comprehensive analysis on transcriptome and expression profile database. Taking KEGG database as a reference, we analyzed the biosynthetic routes of fatty acid of Oiltea camellia and drawn its route graph, which revealed the regulation of key enzyme genes and the basic catalytic rule of fatty acid biosynthesis in Oiltea camellia, providing certain scientific basis for oil content increasing and fatty acid components by ways of genetic engineering.

Keywords: *Camellia oleifera* Abel., fatty acid biosynthesis, transcriptome, expression profile

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INTRODUCTION

As the world woody edible oil plants, Oiltea camellia(*Camellia oleifera* Abel.), which is equivalent to Elaeis quineensis Jacq., Olea europaea L. and Cocos nucifera L., is one of the most important and peculiar oil tree species in China[1]. Camellia oil, extracted from oiltea camellia seeds, containing rich unsaturated fatty acid to more than 90%, 10% higher than that of olive, is the most high-quality edible oil in the world. Since fat is the basic source for human life activity and the fatty acid can't be synthesized by human life activity, so fatty acid in camellia oil becomes an important source for human ingestion of essential fatty acid. With the national economic development, the demand of camellia oil rises day by day and the quality of it will be higher and higher. In addition, there is a great demand for special fatty acid in chemical and pharmaceutical industry [2, 3-5]. Thus, camellia oil output enhancement and quality improvement are the main goal of oiltea camellia breeding. It is hard to satisfy the modern breeding requirements by conventional breeding ways. By means of molecular breeding to regulate the biosynthetic routes of fatty acid of oiltea camellia over expression of key genes of fatty acid biosynthesis should be promoted not only to enhance the quality of oil and improve the composition of fatty acid, but also regulate the expression of some key gene to satisfy the demand for special fatty acid in chemical and pharmaceutical industry, so that the increased demand of camellia oil is satisfied and the value for nutrition and industry is achieved. Thus, this article is based on the transcriptome and expression profile database in fruit expanding period and oil synthetic peak period, using correlation theory of molecular biology to systematically analyze the synthetic pathway of fatty acid of oiltea camellia and investigate the concrete functions of key enzyme genes in the process of fatty acid biosynthesis, providing theoretic foundation and scientific basis for synthetic pathway regulation of fatty acid of camellia oil and components improvement of fatty acid by means of molecular breeding

EXPERIMENTAL SECTION

2.1 *Materials Selection*

Select the oiltea camellia breed branded 'Huashuo' in the last ten days of June, 2009 (fruit expanding period) and middle ten days of October of the same year (oil synthetic peak period) in oiltea camellia germplasm resource garden in Central South University of Forestry and Technology; remove the seeds after plucking the fruit and preserve them in liquid nitrogen; then go back to the lab to preserve the seeds in the refrigerator with ultra low temperature under -80℃.

2.2 *The Total RNA Extraction, RNA Concentration and Quality Inspection of Oiltea Camellia Kernel*

Take oiltea camellia breed branded 'Huashuo' in fruit expanding period and synthetic peak period respectively; extract the total RNA of the kernel specimen of these two periods respectively with Invigrogen's total RNA extraction kit; Extract and inspect the RNA integrity with electrophoresis; Determine the RNA concentration with nuclein/protein quantitative spectrophotometer.

2.3 *Database Building for Transcriptome and Expression Profile of Oiltea Camellia Seeds*

Preserve the above-mentioned two periods of RNA specimen with dry ice and send them to BGI for digital transcriptome and expression profile sequence; Install software to complete transcriptome data assembly, combination and de-redundancy to gain non-redundant Unigene sequence, which structures transcriptome and expression profile database of oiltea camellia seeds in the process of oil biosynthesis and regulation.

2.4 Analysis on Transcriptome and Expression Profile Database of Oiltea Camellia Seeds

Download the transcriptome and expression profile sequencing database from BGI and categorize the Unigenes in the database per metabolic pathway; analyze comprehensively the regulatory sites and functions of all genes in the process of oil formation of the oiltea camellia seeds; select sequences above 300bp among the Unigenes sequences whose information is inconsistent in Nr, SwissProt, KEGG, COG and other database and re-compare them with Blastx in GenBank. Meanwhile, compare the differential expression of all genes between fruit expanding period and maturity period; analyze and identify all genes' functions and their expression status in the process of seeds development to discover the basic rules of all metabolic pathways of oiltea camellia.

RESULTS AND DISCUSSION

3.1Functional Genes Involved in Synthetic Pathway of Fatty Acid of oiltea camellia Seeds

Annotate the genes of transcriptome sequence of oiltea camellia seeds, 18714 Unigene being annotated. Taking KEGG database as a reference, it is merged into 124 metabolic pathways, of which there are 104 Unigenes sequences involved in fatty acid biosynthesis, and 14 enzyme genes involved in fatty acid anabolism (table 1).

Table1. Functional Enzyme Genes Regulating Fatty Acid biosynthesis in the Transcriptome of Oiltea Camellia Seeds

No.	Names of Functional genes	Plants Comparison with Blastx	Value	Value of Expectation	Similarity
1	acetyl-CoA carboxylase carboxyl transferase alpha subunit (ACCase-α-CT)	*Glycine max*	208	1e-58	90%
2	acetyl-CoA carboxylase carboxyl transferase beta subunit (ACCase-β-CT)	*Camellia oleifera*	469	1e-161	94%
3	acetyl-CoA Carboxylase biotin carboxylase (ACCase-BC)	*Camellia sinensis*	531	2e-174	84%
4	acetyl-CoA carboxylase biotin carboxyl carrier protein (ACCase-BCCP)	*Camellia chekiangoleosa*	131	3e-35	100%
5	beta-ketoacyl-acyl-carrier-protein synthase III(KAS III)	*Camellia chekiangoleosa*	211	1e-64	98%
6	beta-ketoacyl-acyl-carrier-protein synthase II(KAS II)	*Camellia chekiangoleosa*	692	0.0	99%
7	beta-ketoacyl-acyl-carrier protein reductase (KAR)	*Arabidopsis thaliana*	197	9e-60	72%
8	beta-hydroxyacyl- acyl-carrier protein dehydrase (DH)	*Camellia chekiangoleosa*	207	8e-65	99%
9	Enoyl-acyl-carrier-protein reductase I (ENR I)	*Camellia chekiangoleosa*	457	5e-159	98%
10	Enoyl-acyl-carrier-protein reductaseII (ENR II)	*Ricinus communis*	197	1e-59	86%
11	malonyl coenzyme A-acyl carrier protein transacylase (MCAT)	*Capsicum annuum*	415	2e-140	93%
12	fatty acyl-ACP thioesterase B (Fat B)	*Camellia oleifra*	403	2e-135	100%
13	stearoyl -acyl-carrier protein desaturase (SAD)	*Camellia chekiangoleosa*	824	0.0	99%
14	fatty acyl-ACP thioesterase A (Fat A)	*Jatropha curcas*	296	4e-97	91%

After comparing the homology with BlastX in GenBank, it is shown in Table 1 that there are 9 enzymes whose gene order has a extremely high similarity (up to 90%) with camellia plants' (*Camellia oleifera*, *Camellia chekiangoleosa*, *Camellia sinensis*), and there are other 5 enzymes whose gene order has a high similarity with Glycine max, Arabidopsis thaliana, Ricinus communis, Capsicum annuum and Jatropha curcas. Thus, it is confirmed that these 14 enzymes are : Acetyl-CoA carboxylase carboxyl transferase α subunit (ACCase-α-CT), Acetyl-CoA carboxylase carboxyl transferase β subunit (ACCase-β-CT), Acetyl-CoA Carboxylase biotin carboxylase (ACCase-BC), Acetyl-CoA carboxylase biotin carboxyl carrier protein(ACCase-BCCP), β ketoacyl acyl carrier protein synthase III

(KAS III), β- ketoacyl acyl carrier protein synthase II(KASII), β ketoacyl acyl carrier protein reductase(KAR),β hydroxyacyl acyl carrier protein dehydrase (DH), Enoyl acyl carrier protein reductaseI (ENRI), Enoyl acyl carrier protein reductase II(ENRII), Malonyl coenzyme A-acyl carrier protein transacylase (MCAT), Fatty acyl-ACP thioesterase B (FatB), Stearoyl –acyl carrier protein desaturase (SAD), Fatty acyl-ACP thioesterase A (FatA). They showed obvious difference of expression abundance in fatty acid biosynthesis of oiltea camellia seeds. Among these genes, ACCase-α-CT, ACCase-BC, ACCase-BCCP and KAR refer to high abundance expression with 13-15 copy numbers, while ACCase-β-CT, KAS III, KAS II, ENRI, FatB and SAD refer to medium abundance expression with 5-10 copy numbers, and DH, ENR II, MCAT and FatA refer to low abundance expression with 2-3 copy numbers.

3.2Functional Gene Expression and Functional Features of Key Enzyme in Fatty Acid biosynthesis of Oiltea Camellia Seeds in Different Periods

Based on the analysis and annotation of relevant functional genes on expression profile data, it is found that expression of key functional genes show obvious difference in the pathway of fatty acid biosynthesis of oiltea camellia fruit. It is divided into two types according to different genes expression abundance at different periods: (1) up-regulation refers to expression of oil synthetic peak period compared with that of fruit expanding period; (2) down-regulation refers to expression of oil synthetic peak period compared with that of fruit expanding period.

3.2.1 Expression Analysis on Relevant Functional Gene Expressing as Up-regulation in Oil Synthetic Peak Period

In the synthetic pathway of fatty acid of oiltea camellia, about 78% key enzyme functional genes are expressed up-regulation in oil synthetic peak period. There are two types of Acetyl-CoA carboxylase (ACCase) in nature, one of which is heterogeneity of ACCase and the other of which is homogeneity of ACCase. The ACCase in oiltea camellia refers to the former type. 4 subunits are contained in heterogeneity of ACCase, i.e. biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and 2 subunits of α-CT and β-CT of carboxyltransferase. The two former subunits formed BC and BCCP domains, while the latter two formed CT catalytic domains. Heterogeneity of ACCase mainly catalyzes Malonyl-CoA, which is the first step of fatty acid biosynthesis. It is divided into two steps for the catalytic process of heterogeneity of ACCase. Firstly, biotin carboxylation is generated under the catalysis of BC domain and the involvement of adenosine triphosphate (ATP), which is a protein-combined biotin agon carboxylation. Then, under the catalysis of CT domain, carboxyl is transferred from biotin to Acetyl-CoA(AcCoA), forming Malonyl-CoA[6,7]. It is revealed from Table 2 that ACCase-α-CT, ACCase-β-CT and ACCase-BCCP show high expression abundance in the development process of oiltea camellia seeds, and up-regulation is expressed in the oil synthetic peak period compared with fruit expanding period. Since ACCase is a rate-limiting enzyme biosynthesized with fatty acid, overexpression of ACCase gene is favorable to enhance the seed oil content of the oil crops.

MCAT catalyzes the transacylation of ACCase's product malonyl-CoA to combine malonyl-CoA with the activated ACP to generate malonyl-ACP, i.e. the donor of carbon chain in the synthetic process of fatty acid [8]. Since MCAT is mainly expressed in seeds, it is found in Table 2 that MCAT genes show a high expression in fruit expanding period and oil synthetic peak period.

KASII is a key enzyme for the conversion process of catalyzing palmitic acid to stearic acid (from 16:0-ACP to 18:0-ACP), determining the ratio of 16-carbon fatty acid and 18-carbon fatty acid. In addition, it is shown that KASII has a close relationship with plant's low-temperature adaptability and plant's growth and development [9, 10]. Combing with the research on peanut's KASII genes, it is guessed that KASII plays an important role in the whole growth and development process of oiltea camellia seeds.

Table2. Difference Analysis of Up-regulation on Key Genes of Fatty Acid Biosynthesis of Oiltea Camellia Seeds in Oil Synthetic Peak Period

No.	Names of Key Genes	Expression in Fruit Expanding period	Expression in Oil Synthetic Peak Period	Times of Differential Expression	Value P	Value FRD
1	ACCase-α-CT	140	370	1.3718	0	0
2	ACCase-β-CT	20	32	0.6477	0.114	0.252
3	ACCase-BCCP	52	138	1.3774	0	0
4	MCAT	119	316	1.3788	0	0
5	KAS II	11	42	1.9028	0	0
6	KAR	274	1003	1.8418	0	0
7	DH	56	132	1.2065	0	0
8	ENR I	17	19	0.1298	0.791	0.851
9	ENR II	3	22	2.8435	0	0
10	SAD	57	75	0.3655	0.149	0.280
11	FatA	34	76	1.1298	0	0

KAR is the first reduction reaction when catalyzing synthetic pathway of fatty acid. NADPH is used as reducing agent for this reaction with its product of hydroxyl-acetyl-ACP. KAS genes are initially found in oiltea camellia, while in the later research it is regarded that KAR genes only have their auxiliary functions in the synthetic process of fatty acid [11]. Thus, the mechanism is not deeply researched. It is found in this data analysis of expression profile (Table 2) that KAR shows high expression abundance in the whole seed development process. It is suggested that KAR should be deeply researched in the following study.

DH is used to dehydrate and catalyze α and β carton atoms of β-hydroxy-butyl to generate trans△2-ene-butyryl. It connects two reduction reactions in these two fatty acid biosynthesis [12]. It is found from data analysis in Table 2 that DH genes show high expression abundance in the seed development process and up-regulation is expressed in oil synthetic peak period.

ENR catalyzed the second reduction reaction of fatty acid biosynthesis, also taking NADPH as reducing agent with the connecting ACP of fatty acid as products. Once again, it could get into the circle of carbon chain extension reaction of the fatty acid. Each circle extends two carbon atom units until it is hydrolyzed from ACP by thioesterase. It is indicated from a research on ENR genes of rapeseeds by Kater's team that ENR genes are mainly expressed in the process of seed development and this expression gets enhanced in the process of seed fat accumulation [13], which conforms to the analytic result shown in Table 2.

Existing in plastid matrix, SAD, as one kind of soluble enzyme, is the key enzyme for unsaturated fatty acid synthetic metabolism. It catalyzes desaturation of stearoyl-ACP and introduces an oleoyl-ACP reaction formed with double bonds between C9 and C10 of fatty acid chain. The main purpose is to dehydrogenate stearyl to form oleic acid. It determines the ratio of plant saturated fatty acid and unsaturated fatty acid. Meanwhile, it also has close relationship with plant temperature shift responding and low-temperature adaptability enhancement [14]. It is seen from Table 2 that SAD gene in oiltea camellia has a high expression. Thus, saturated fatty acid content in ripe fruit is relatively low. In future breeding, expression of SAD gene could be enhanced to optimize fatty acid composition.

FAT is a kind of plastid globulin with nucleus gene coding [15, 16]. It determines the chain length and types of free fat generated in plant cells. According to different specific of fatty acid length, FAT is divided into FatA and FatB. As FatA encodes C18 fatty acid specific enzyme, it is regarded as an important "housekeeping" enzymes in all plant cells owing to the reason that C18:1 fatty acid and their derivatives generally represent the generation of new biosynthesis of fatty acid carbon chain. FatA has high activity of 18:1-ACP, which determines the exporting ability of 18:1 inside plant to outside plastid [17]. It is found from the difference analysis on expression profile (Table 2) that FatA is expressed up-regulation in oil synthetic peak period due to the mass biosynthesis of unsaturated fatty acid.

3.2.2Expression Analysis on Relevant Functional Gene Expressing as Down-regulation in Oil Synthetic Peak Period

Acetyl-CoA Carboxylase biotin carboxylase (ACCase-BC) genes, beta ketoacyl acyl carrier protein synthase III (KAS III) genes and fatty acyl-ACP thioesterase B (FatB) genes are all expressed down-regulation in key enzyme functional genes of regulating fatty acid biosynthesis of oiltea camellia seeds. ACCase-BC is an important part of ACCase. With the existence of ATP, biotin carboxylase subunits (BC) partially catalyze biotins and transfer them to BCCP [18]. It is revealed in the data analysis (Table 3) that ACCase-BC shows medium abundance expression in fruit expanding period and down-regulation is expressed in oil synthetic peak period, which might result from the constant transfer of partial biotins to BCCP.

Table3. Difference Analysis of Down-regulation on Key Genes of Fatty Acid Biosynthesis of Oiltea Camellia Seeds in Oil Synthetic Peak Period

No.	Names of Key Genes	Expression in Fruit Expanding period	Expression in Oil Synthetic Peak Period	Times of Differential Expression	Value P	Value FRD
1	ACCase-BC	9	6	-0.6174	0.429	0.539
2	KAS III	77	37	-1.0869	0	0
3	FatB	111	73	-0.6348	0.003	0.014

KAS III is one kind of KAS genes among the three. In the process of carbon chain extension, carbon-carbon bond is formed by generating a β-ketoacyl-ACP. These polymerizations are catalyzed by three different kinds of KAS for completion. KAS III is the first step of catalysis, i.e. catalyze malonyl-CoA and acetyl-CoA to synthesize 3-ketobutyryl-ACP. It is found in the KAS genes research on tobacco that overexpression on KAS III genes would reduce the synthetic speed of fatty acid. The oil content of oiltea camellia of transgenosis (KAS III) is lower than that of wild type, and the oil synthetic speed in seeds is obviously slower than wild type's [19]. It is found from

Table 3 that the expression of KAS III tends to down-regulate in oil synthetic peak period, which is favorable to fatty acid biosynthesis.

FatB is a decisive composition for the synthesis of saturated fatty acid. In the process of plant growing and seed development, FatB could regulate oil content and composition. Palmitic acid level is regulated by changing the expression of palmitic acid acyl-ACP FatB genes and KAS II genes. That is to say overexpression of palmitic acid acyl-ACP FatB genes would restrict KAS II so that palmitic acid content in soybean oil would get raised. Therefore, in future molecular biotech breeding, we could control the oil content by regulating FatB expression. It is shown in Table 3 that the down-regulation of FatB expression in oil synthetic peak period results from the mass synthesis of unsaturated fatty acid in Oiltea Camellia.

3.2.3 The Regulation Process of Fatty Acid Biosynthesis of Oiltea Camellia Seeds

According to the above transcriptome data analysis on fatty acid biosynthesis of oiltea camellia seeds and the difference analysis on functional genes expression of fatty acid biosynthesis of oiltea camellia seeds in different periods, regulating route of fatty acid biosynthesis of oiltea camellia seeds is drawn (Fig.1).

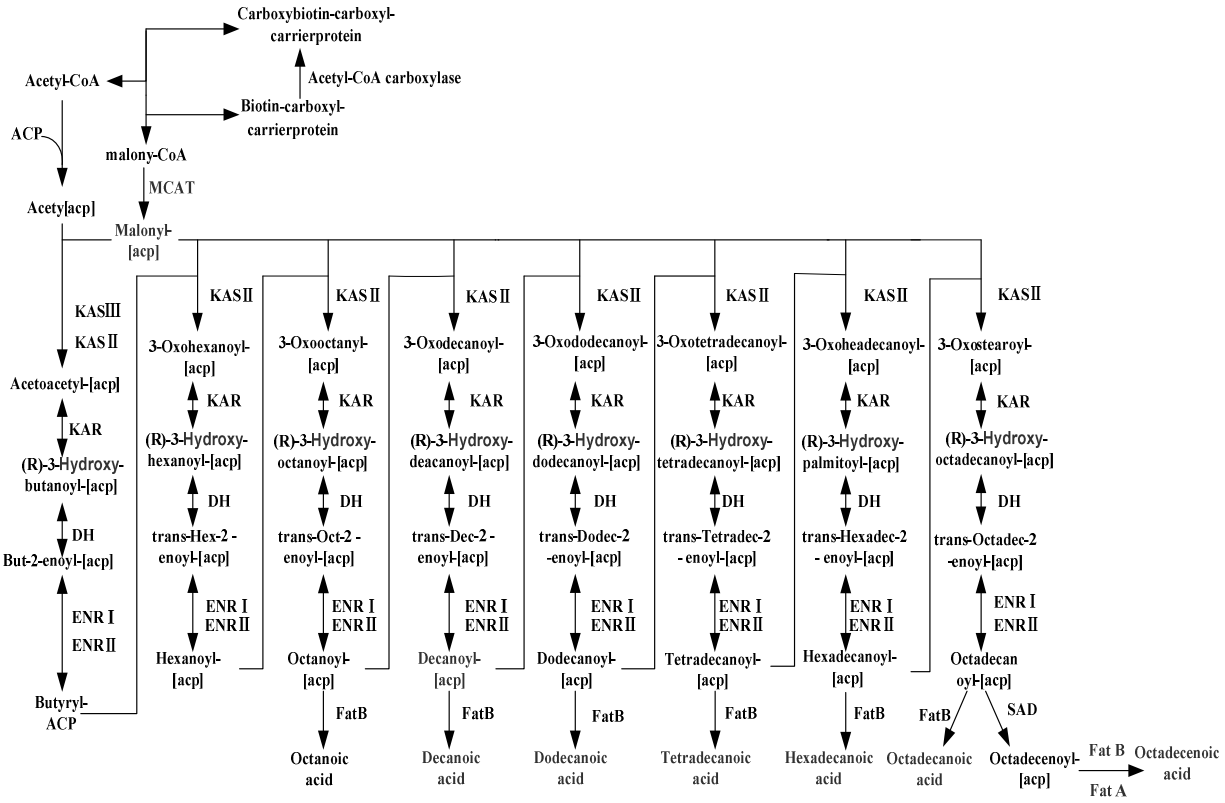

Fig. 1 The pathway of fatty acid biosynthesis in oiltea camellia seeds

CONCLUSION

Originated from China, oiltea camellia is a special woody edible oil plants whose product is acquired after squeeze is mainly camellia oil, which is a polymeric compound with a main composition of glyceride of higher fatty acid oleic acid. The unsaturated fatty acid content in camellia oil is very high, up to 90%, 10% higher than that in olive. It is mainly composed with oleic acid and linoleic acid with content of 75%～83% and 7.4%～13% respectively. A small amount of linolenic acid and other high valence unsaturated fatty acids are also contained in camellia oil. Long-time eating camellia oil is obviously helpful to improve diseases like hypertension, coronary disease, obesity, etc. The content and composition of fatty acid in camellia oil have a direct influence on its quality and health protection function. Therefore, high-quality camellia oil breeding and composition improvement are favorable to ensure the quality of edible oil and enhance its health protection value as well as satisfy the demand for special fatty acid [20]. From the above analysis on functional gene expression of key enzyme in the biosynthesis route of fatty acid of oiltea camellia and the regulating process of fatty acid biosynthesis, it is found that the oil content increases with the gradual maturity of oiltea camellia seeds. This mainly owes to the reason that the high abundance

expression of heterogeneity of ACCase accelerates the biosynthesis of fatty acid. It is obviously indicated that ACCase is the key target for gene regulation for oil content in oiltea camellia. Meanwhile, MCAT, KAS, KAR, DH and ENR compose fatty acid synthase FAS and such continuous polymerization as start, loading, condensation, reduction, dehydration and second reduction reaction are completed based on malonyl-ACP as the substrate. All of these genes have their expression in the whole synthetic route of fatty acid of oiltea camellia, indicating that they are the essential key enzyme genes for fatty acid biosynthesis. In this process, MCAT accelerates the generation of malonyl-ACP so that donor of carbon chain in the synthetic process of fatty acid is provided. Therefore, MCAT is the essential key enzyme gene for fatty acid biosynthesis. KAS II, which determines the ratio of 16-carbon fatty acid and 18-carbon fatty acid, is a key enzyme to catalyze palmitic acid to stearic acid. Enhancing the expression of KAS II could restrain the activity of palmitoyl thioesterase and raise the content of stearoyl ACP so that the content of unsaturated fatty acid could be raised. This conclusion is proved by Bleibaum's team's KAS II research on oiltea camellia seeds. In the process of fatty acid biosynthesis, palmitic acid and stearic acid are generated after 7 cyclic polymerizations and then stearoyl is dehydrogenated to form oleic acid through SAD. Thus, SAD directly determines the ratio of saturated fatty acid and unsaturated fatty acid. The enhancement of SAD expression could accelerate the transformation of saturated fatty acid to unsaturated fatty acid. In this synthetic process of fatty acid of oiltea camellia, fatty acid is released finally from ACP to form oleic acid under the catalysis of FAT (FatA and FatB). FatA has a high activity of 18:1-ACP, which determines the exporting ability of 18:1 inside plant to outside plastid. The overexpression of FatB in oil synthetic peak period is favorable to the mass biosynthesis of unsaturated fatty acid. Though partial genes relating to fatty acid biosynthesis of oiltea camellia have managed to be cloned and developed, their functions still need further verification on transgenic plant. What's more, fatty acid composition needs to be regulated through genetic engineering technology. This article provides theoretic foundation and scientific basis for oiltea camellia breeding with high content of oil and improvement of fatty acid composition by means of molecular breeding.

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