simpósio estadual de agroenergia
V reunião técnica de agroenergia - RS

EXPRESSION PROFILE OF OIL GENES IN TUNG SEEDS

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INTRODUCTION

As a result of increasing population, reducing petrochemical resources and environmental consciousness, there will be a worldwide increasing demand of any renewable energy supply that would not cause adverse environmental impacts and do not compete with food supply. Tung oil, the major product of tung tree (*Vernicia fordii)* seeds, is considered one of the highest quality oils. It is widely used in paints, high quality printing, plasticizers, medicine, and in chemical reagents. Moreover, because tung seeds accumulate high content of oil (approximately 50 %), it has being recently considered for use in biodiesel production (SHANG et al., 2010).

However, making it an ideal biodiesel crop requires genetic manipulations for increased oil yield and modified oil composition using the genes that are involved in oil biosynthesis pathway. Nowadays, the information regarding gene expression in tung tree is limited, which has hampered breeding approaches. In this study we have analyzed the expression profile of a subset of oil genes in samples from different stages of seed development.

MATERIAL AND METHODS

The transcripts potentially involved in lipid metabolism were searched in an *in house* transcriptome through the annotation against the KEGG (http://www.genome.jp/kegg/) database using Blastall software. For RT-qPCR analysis, three replicates of RNA samples from seeds of tung fruits from 20 (S1), 35 (S2), 50 (S3), 80 (S4) and 100 (S5) DAF, as presented in Figure 1, were extracted using the Trizol reagent (Invitrogen). The RNA quality was accessed by electrophoresis on a 1 % agarose gel. Total RNA (1 µg) was digested with 1U DNAse I and reverse transcribed using the M-MLV enzyme and oligo-24TV primers, according to manufacturer's instructions (Invitrogen). Specific primers for the amplification of the selected genes were designed using Vector NTI10 software (Invitrogen). The cDNAs were amplified by RT-qPCR in a final volume of 20 µL containing 1 µL cDNA, 10 µL of Platinum Sybr green UDG (Invitrogen), and 2-5 ρmol of

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each primer. Amplification was standardized in a 7500 Real time Fast thermocycler (Applied Biosystems) using the following conditions: 50 °C for 20 sec, 95 °C for 10 min followed by 45 cycles of 15 sec at 95 °C and 60 sec at 60 °C. The PCR products for each primer set were subjected to melt curve analysis in order to verify the presence of primer dimmers or nonspecific amplicons. Genes coding for *actin*, *ubiquitin* and *tubulin* were used as internal controls. The relative expression data was calculated according to the $2^{-\Delta\Delta Cq}$ method and presented as fold change. Statistical analyzes were performed using the computer program SAS System for Windows version 9.1.3. Data were subjected to variance analysis ($p \leq 0.05$). In case of statistical significance, the relative expression among stages of seed development was compared by Tukey test ($p \leq 0.05$).

Figure 1. The five stages of fruit development used to obtain seeds for RT-qPCR analysis.

RESULTS AND DISCUSSION

The first committing step in the fatty acid biosynthesis (FAS) is the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by acetyl CoA carboxylase (ACCase) (Figure 2A). There are two types of ACCase in higher plants, the homomeric and the heteromeric ACCase. The RT-qPCR analysis from different stages of tung seed development shows that the homomeric ACCase (HomoACC) is more expressed in S3 and S4 stages, while the BCCP (biotin carboxyl carrier protein), a subunit of the heteromeric ACCase, is more expressed in S4 and S5 stages (Figure 2B). A similar expression pattern was observed in the *Jatropha curcas* (XU et al., 2011), oil palm (NAKKAEW et al., 2008) and castor bean (CHEN et al., 2007) seeds, suggesting that both enzymes play a role in the oil accumulation in seeds. Since ACCs expression was correlated with the oil content in oil palm seeds (NAKKAEW et al., 2008), it is probably a rate-limiting step in fatty acid biosynthesis, and may be useful as a molecular marker to assist the selection of high oil productive varieties.

The synthesis of fatty acids may be accomplished by producing the 16:0-ACP fatty acids, which are hydrolyzed by acyl-ACP thioesterases (FATA and FATB) that release fatty acids from the ACP molecule to be transported to the endoplasmic reticulum (ER). However, the 18:0-ACP

generated in the FAS may be desaturated to produce unsaturated fatty acids before being released from the ACP and transported to ER. Desaturases play a pivotal role in fatty acid desaturation during fatty acid and lipid biosynthesis (Figure 2A). The RT-qPCR results show that tung stearoyl ACP desaturase (SAD) has a bell-shaped expression pattern, with the highest expression at S2, S3 and S4 stages of seed development (Figure 2B). A similar expression pattern was observed for other oilseed crops with high content of unsaturated and conjugated fatty acids, such as physic nuts and castor bean (CHEN et al., 2007). In accordance, the expression of delta-12-fatty acid desaturase (FAD2) and omega-3-fatty acid desaturase (FAD3) was greatly increased in the end of seed development, which are associated with the high content of polyunsaturated fatty acids in tung seeds produced from the C18:1 fatty acids generated earlier by SAD. This meant that the expression of these genes at the transcription level was closely correlated with TAG accumulation within tung developing seeds. FAD3 precursor was constantly expressed during seed development; however, the increase of almost 15-fold of the expression of FAD9 in the mature seed compared to seeds from the stage 1 indicates that FAD9 may also play an important role in the accumulation of tung oil or in the conversion of specific double bounds in fatty acids of tung seeds; therefore, *V. fordii* FAD9 must be further investigated in detail. The expression of tung FATA is constant in all stages with a sharp reduced expression at S3 and S5 stages (Figure 2B). It is possible that FATA has a housekeeping function, providing constant demands of fatty acids for membrane lipid biosynthesis, in addition to the assembly into TAG.

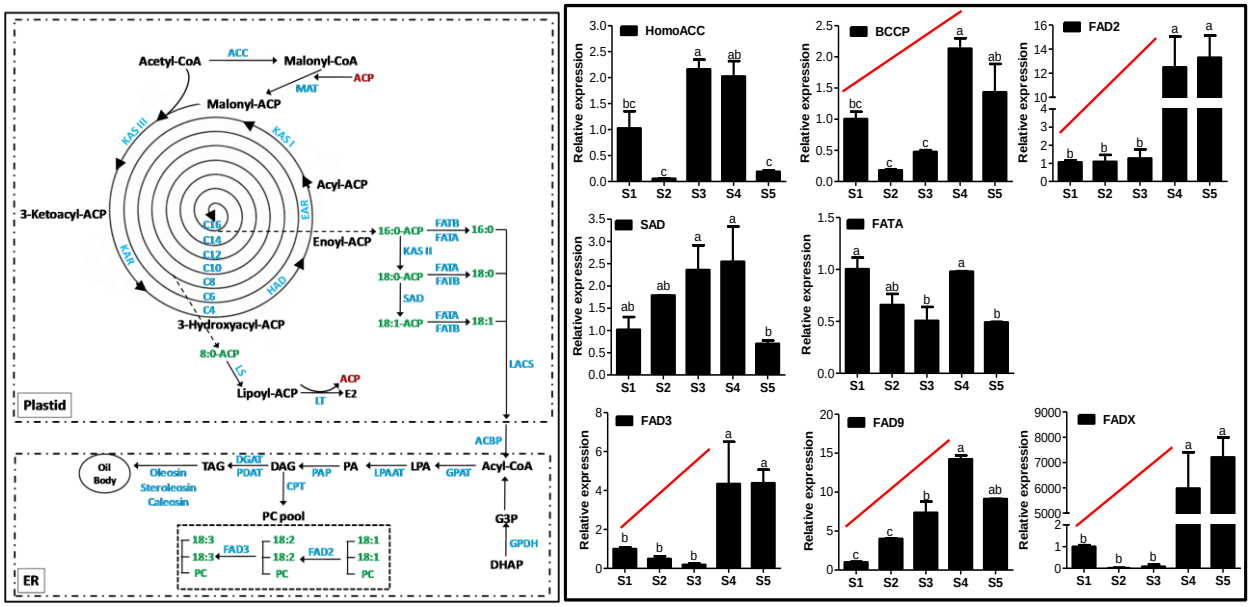

Figure 2. Oil biosynthetic pathway in tung seeds (A), and expression profile of genes related to lipid

accumulation in tung seeds (B).

In tung seeds, the most common fatty acids (more than 80 %) are the conjugated fatty acids, such as α-eleostearic acid (18:3^{9cis,11trans,13trans}). The enzymes capable to synthesize conjugated fatty acids are called conjugases and are closely related in terms of their amino acid identity to the FAD2 family. According to the RT-qPCR results, the expression of the tung conjugase FADX increases more than 7000-fold in mature seeds compared to seeds from stage 1 (Figure 2B), confirming its importance in tung seeds. The high content of unsaturated fatty acids in the oil is undesirable for biodiesel production because unsaturated fatty acids affect oxidative stability and ignition quality of the biodiesel (KNOTHE, 2005). Therefore, the desaturases and conjugase identified in the present study are potential candidates for RNAi constructs for efficiently modify fatty acids composition in tung seed oil in order to improve its fuel properties to be used as biodiesel.

CONCLUSIONS

In the present work we analyzed the expression of a subset of tung oil genes. These information will be useful to develop approaches that may be applied in breeding programs and to engineer the entire oil synthesis pathway of tung seeds. It may be used to increase the expression of enzymes related to oil synthesis, or change the expression of enzymes related to the accumulation of unsaturated and unusual fatty acids in tung seeds or other plants. This approach provided a valuable source of genes involved in the seed oil biosynthesis that will be usefull to breed tung tree for higher fruit yield and for modified oil properties to be used as biodiesel.

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