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Research article

Transcriptional profiling by DDRT-PCR analysis reveals gene expression during seed development in *Carya cathayensis* Sarg.

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ABSTRACT

Hickory (*Carya cathayensis* Sarg.) seed has one of the highest oil content and is rich in polyunsaturated fatty acids (PUFAs), which kernel is helpful to human health, particularly to human brain function. A better elucidation of lipid accumulation mechanism would help to improve hickory production and seed quality. DDRT-PCR analysis was used to examine gene expression in hickory at thirteen time points during seed development process. A total of 67 unique genes involved in seed development were obtained, and those expression patterns were further confirmed by semi-quantitative RT-PCR and real time RT-PCR analysis. Of them, the genes with known functions were involved in signal transduction, amino acid metabolism, nuclear metabolism, fatty acid metabolism, protein metabolism, carbon metabolism, secondary metabolism, oxidation of fatty acids and stress response, suggesting that hickory underwent a complex metabolism process in seed development. Furthermore, 6 genes related to fatty acid synthesis were explored, and their functions in seed development process were further discussed. The data obtained here would provide the first clues for guiding further functional studies of fatty acid synthesis in hickory.

1. Introduction

Hickory (*Carya cathayensis* Sarg.) is a well-known nut tree in Eastern China. It is very important economically, since its seed possesses more than 70% lipid in which polyunsaturated fatty acids (PUFAs) as major components benefit human health (Huang et al., 2006). Hickory becomes one of the highest oil content trees reported by far. Despite its obvious scientific and economic interest, literature and molecular resources available for hickory remain scarce (Zheng et al., 2009). Presently, knowledge of lipid accumulation in plants was based almost entirely on studies of oil seeds. Several authors (Norton and Harris, 1975; Baud et al., 2002; Giovannoni, 2004; Lu et al., 2006; Bourgis et al., 2011; Troncoso-Ponce et al., 2011) reported morphological and physiological changes in the seed development of fruit trees. Norton and Harris (1975) reported that the growth and composition of siliquas and seeds of oilseed rape was followed over 12 weeks from shortly after anthesis to maturity. Seed development could be divided into three phases. The neutral lipids content increased from 20% of the total lipids in Phase I to 93% at maturity. The proportion of structural lipids declined as that of storage lipids increased. In Phase I the fatty acid (FA) composition of the lipid resembled that of photosynthetic tissue. In Phase II, VLPUFAs storage triglycerides ($C_{20:1}$; $C_{22:1}$) appeared, MUFA ($C_{18:1}$) transitorily increased, but two PUFAs (i.e., $C_{18:2}$ and

Abbreviations: BAP, BCR-associated protein; C4H, cinnamate 4-hydroxylase; DAP, days after pollination; DDRT-PCR, differential display reverse transcriptase polymerase chain reaction; FA, fatty acid; FAD, fatty acid desaturase; FAH12, fatty acid hydroxylase 12; GO, gene ontology; MES9, methyl esterase 9; MUFA, monounsaturated fatty acid; NAD1, NADH dehydrogenase subunit 1; PUFAs, polyunsaturated fatty acids; SAD, stearoyl-ACP desturases; SUCLG, succinyl-CoA ligase; TDFs, transcript-derived fragments; TUB, tubulin; TFs, transcription factors; TAG, triacylglycerol; VLPUFAs, very long polyunsaturated fatty acids.

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$C_{18:3}$) fell dramatically. In Phase III, the content of $C_{22:1}$ continued to rise, but the proportions of the other FAs remained constant. Baud et al. (2002) reported that the seed development in *Arabidopsis thaliana* also could be divided into three stages. During early embryogenesis, seed weight and lipid content were low whereas amount of starch was transiently accumulated. In the second stage, i.e. maturation phase, a rapid increase of dry weight in seeds was observed while storage oils and proteins were accumulated in large quantities. During the last stage, seed dry weight remained constant while an acute loss of water took place in the seed.

More attention was gradually paid to gene expression and regulation in recent years. Giovannoni (2004) reported the molecular basis of ethylene signaling in tomato adapted to promote the successful fruit development and seed dispersal. Lu et al. (2006) reported that additional genes were needed for high production in transgenic plants. Then they used the model plant *A. thaliana* expressing castor fatty acid hydroxylase (FAH12) to identify genes which could boost hydroxy fatty acid accumulation in transgenic seeds. Bourgis et al. (2011) reported that the high oil content in oil palm was associated with much higher transcript levels of all fatty acid synthesis enzymes. Synthesis of fatty acids and supply of pyruvate in the plastid was a major control over the storage of oil in the mesocarp of oil palm. Troncoso-Ponce et al. (2011) made comparisons of gene expression across multiple oil species: *Ricinus communis*, *Brassica napus*, *Euonymus alatus* and *Tropaeolum majus*. Analysis of EST levels from these oil seeds revealed both conserved and distinct species-specific expression patterns for genes involved in the synthesis of glycerolipids and their precursors. Independent of the species and tissue type, ESTs for core fatty acid synthesis enzymes maintained a conserved stoichiometry and a strong correlation in temporal profiles throughout seed development. More than 350 genes encoding enzymes and proteins involved in lipid metabolism were obtained. Bryant et al. (2011) presented the identities of 119 nuclear genes encoding chloroplast-localized proteins required for embryo development in Arabidopsis. Borisjuk et al. (2004) reported that energy metabolism changed during seed development. Seed development was also regulated by photohormones. Sucrose represented a signal for differentiation and up-regulated storage-associated gene expression. It suggested many genes for nuclear metabolism and signal transduction played indispensable roles in seed development.

In hickory, researchers focused mainly on the morphology, physiology and biochemistry of nut development (Tian et al., 1986; Liu et al., 2006; Xie et al., 2008; Zhang et al., 2012). Tian et al. (1986) studied the lipid conversion of hickory in the process of nut development and found that the stage of lipid accumulation was between late July and mid-September. The main fatty acid was composed of oleic acid, palmitic acid, stearic acid, linoleic acid and linolenic acid. The saturated fatty acids gradually decreased along with the nut ripening, whereas the unsaturated ones increased, especially oleic acid. The acid value also decreased gradually from the early stage of lipid formation to the stage of maturity. Liu et al. (2006) tested the proteins and amino acids contents in hickory kernel, and concluded that the proteins and amino acids contents were average 113.0 mg g^{-1} and 117.1 mg g^{-1}, respectively. Xie et al. (2008) explored the nut's growth process in order to obtain the pattern of oil accumulation. The results showed that the development of the *C. cathayensis* nut throughout its growing season was divided into two distinct phases. Phase I occurred from early May to early August. Phase II occurred from early August until harvest in early September, wherein the kernel developed and filled out with nutrients accumulating and transforming. Zhang et al. (2012) analyzed the oil content and fatty acid composition in the nuts of hickory and the results showed that the oil contents varied from 51.0% to 63.9%. The main fatty acids were palmitic acid, palmitoleic acid, stearic acid, and so on. So an investigation of the regulation of genes in seed development would provide insight into the molecular mechanism of this process in hickory.

Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) is an efficient, sensitive and reproducible technology that offers several advantages over other methods of gene expression analysis (Vogeli-Lange et al., 1996; Hill et al., 2003; Meng et al., 2012). This technique has been utilized widely as a simple method for identification and clone of differentially-expressed genes at different developmental stages in a plant without previous genomic information. In this study, we used the RNA finger-printing technique of DDRT-PCR to identify transcript-derived fragments (TDFs) in hickory at thirteen time points during seed development process. The functions of the 67 inducible unique genes in the seed development process in hickory were further discussed.

2. Materials and methods

2.1. Plant material and RNA extraction

The experimental materials were collected from five clones of 40-year-old hickory trees in the fields of Lin'an City (30°N, 119°W), Zhejiang Province, China. Based on our physiochemical analysis and morphological observations of hickory nuts, the nuts were harvested at different developmental stages, including 94, 97, 100, 102, 104, 106, 109, 111, 113, 116, 119, 123, 128 days after pollination (DAP) (Xie et al., 2008). A total of 30–50 nuts each time were immediately frozen in liquid nitrogen. For RNA extraction, each frozen sample was ground in a stainless steel blender and subsequently in a stainless steel grinder to fine powder at the 13 indicated time points, respectively. Equal amounts of ground sample from the five trees were mixed in a centrifuge tube. RNA extraction was performed as described by Wang et al. (2000). After deoxyribonuclease I treatment, the quantification and purity of RNA samples were assessed using ultraviolet spectrophotometry. The integrity was verified by electrophoresis on 1% agarose gels stained with ethidiumbromide. The RNA pellets were stored at −80 °C until needed. Total RNA (20 μg) was treated with 10 units RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) in a 25 μl reaction volume at 37 °C for 30 min. The RNA pellets were washed in 70% ethanol and suspended in 10 μl DEPC-treated ddH_2O just before use.

2.2. First-strand cDNA synthesis

The first strand of cDNA was synthesized following the manufacturer's recommendations for TransScript First-Strand cDNA Synthesis SuperMix (Transgene, SP, China), using 1 μl of μg ml^{-1} oligo (dT) primer AP in a 20 μl reaction. The cDNA was diluted 1:10 and stored at −20 °C for subsequent PCR.

2.3. Differential display RT-PCR

Differential display RT-PCR (Liang andPardee,1992) was performed on single-stranded cDNA using combinations of 4 random primers (ARP1–ARP4) (Table S2) and 3 anchored primers (AP2, AP7, and AP10) (Table S3). The cDNA samples from different time points (94, 97, 100, 102, 104, 106, 109, 111, 113, 116, 119, 123, and 128 DAP respectively) were synthesized appropriately in equal amounts for use in DDRT-PCR experiments. The PCR mixture contained 40 ng cDNA, 2 μM HAP primers, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 1 unit of AmpliTaq DNA polymerase (Perkin–Elmer) in a final volume of 20 μl. A 45-cycle PCR reaction (30 s at 95 °C, 60 s at 40 °C, 30 s at 72 °C followed by 5 min at 72 °C) was used. A 519-bp fragment of the barley *alpha-tubulin*