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Characterization and functional analysis of a plastidial *FAD6* gene and its promoter in the mesocarp of oil palm (*Elaeis guineensis*)

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ABSTRACT

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important oil crops in the tropics with special fatty acid compositions. In this paper, a cDNA sequence putatively encoding the plastidial ω6 fatty acid desaturase (FAD) which plays a key role in the linoleic acid biosynthesis, designated *EgFAD6*, was isolated from the mesocarp of oil palm. Sequence analysis indicated that it had an open reading frame (ORF) of 1320 base pairs (bp) encoding 349 amino acids (aa) of 51 KDa. The real-time fluorescent quantitative PCR results indicated that *EgFAD6* mRNA exhibited an extremely high expression level in the mature leaves, and it showed quite flat during the mesocarp ripening with a slight increase at the phase 2. The heterologous expression of *EgFAD6* in yeast accumulated a considerable amount of linoleic acid, while no significant difference in other fatty acids when compared with the control pYES2. Furthermore, the promoter of *EgFAD6* (*proEgFAD6*) with 1474 bp in length was isolated and used for functional analysis. The bioinformatics analysis indicated *proEgFAD6* contains several potential *cis*-acting elements, such as light and hormone-responsive elements. Expression analysis in transgenic *Arabidopsis* revealed that *proEgFAD6* could drive *β-glucuronidase* (*GUS*) reporter gene express in the leaf, silique coat, stem and flower. These results revealed the function of *EgFAD6* and the feature of its promoter, which will be useful for understanding the function and regulation mechanism of plastidial linoleic acid synthesis in other tropical palm plants.

1. Introduction

The oil palm (*Elaeis guineensis* Jacq.) derives from African and is known as the most efficient oil-bearing crop which is presently cultivated in many tropical areas (Damayant et al., 2014; Sowunmi, 1999). The oil palm produces two types of plant oil, crude palm oil and palm kernel oil, extracted from the seed endosperm and the fruit mesocarp where it can comprise up to 90% of the dry weight, respectively (Aba and Baiyeri, 2012). The kernel oil contains 60%–70% lauric acid and myristic acid, while the mesocarp oil contains 44% palmitic acid, 39% oleic acid and 10% linoleic acid (Oo et al., 1985). Palm oil is rich in palmitic and oleic acid, which are the preferred materials and the ratio between saturated and unsaturated fatty acids (UFAs) is close to unity. Given the great potential value of oil palm, in particular its oil and fatty acids, many researchers have shown increasing interest in its fatty acid metabolism and oil synthesis. Genetic engineering has become a new overview for plant improvement to improve valuable traits by producing novel plant varieties. The fine qualities endowed by high-oleic acid and high-linoleic acid prompt more and more efforts directing genetic improvement to achieve higher level of oleic acid (Cheath et al., 1995). Hence, knowledge of biosynthesis and regulation mechanism related with fatty acid desaturases (FADs) in oil palm mesocarp is of most importance.

In higher plants, polyunsaturated fatty acids (PUFAs) are synthesized by a variety of FAD. FAD is ubiquitous enzyme family and responsible for introducing double bonds into the hydrocarbon chains of fatty acids (Singh et al., 2002). They play an essential role in fatty acid metabolism and maintain biological membranes in most creatures. Plastid-soluble stearoyl-CoA desaturase implemented the first desaturation reaction and formed the oleoyl-ACP ($18:1^{\Delta 9}$-ACP). ω-6 FADs execute the second biochemical process to desaturate oleic acid to linoleic acid, which catalyzes the addition of one double bond to △-12-position of oleic acid. Linoleic acid ($18:2^{\Delta 9,12}$) is an essential fatty acid belonging to the ω-6 polyunsaturated fatty acid group and required for normal growth of all eukaryotes, as an integral component of cell membranes. Probably more importantly, linoleic acid is a precursor for synthesis of γ-linolenic acid ($18:3^{\Delta 6,9}12$) and other long-chain PUFAs (LCPUFAs). Recent studies indicated that γ-linolenic acid can alleviate

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the symptoms of kinds of diseases such as cancer, adipogenesis, cardiovascular disease and psoriasis. Being a kind of nutritionally and pharmaceutically important substance, γ-linolenic acid has become the hot spot both in academic and applied fields (Xian et al., 2002). Additionally, De novo biosynthesis of α-linolenic acid (ALA) is converted from linoleic acid by ω-3 FAD. Linoleic acid and α-linolenic acid are so-called essential fatty acid (EFAs) to human bodies because of their inability to synthesize these compounds (Kinney et al., 2002). Therefore, ω-6 FAD is the key enzyme for producing linoleic acid and is also the speed-limiting enzyme for routes of ω-6 and ω-3. Cloning and characterization of ω-6 desaturase gene will help understand the biosynthetic pathway of γ-linolenic acid and further enhance the percentage of γ-linolenic acid in total fatty acids.

The desaturation processes take place in both the plastidial membrane and the endoplasmic reticulum (ER) membrane through both prokaryotic (plastid) and eukaryotic (ER) pathways (Zhang et al., 2009a). The genes for ER- and plastid-derived ω-6 FADs whose genetic loci are *fad2* and *fad6*, respectively, have been characterized from different kinds of plant species including *Arabidopsis* (Falcone et al., 1994), spinach (Schmidt et al., 1994), soybean (Heppard et al., 1996), and cotton (Pirtle et al., 2001). Additionally, with significant health benefits of UFAs gain increased interesting, selecting a best promoter to optimize the expression of a specific desaturase producing distinctive UFAs in metabolic engineering has been studied in detail over the past few decades. These include *napin* promoter in the oilseed rape, phaseolin promoter, which promote the gene encoding β-phaseolin storage protein, linin promoter and conlinin promoter (Ezcurra et al., 1999). These four promoters have been discovered in various crops and presents specific to seed in their native species (Boothe et al., 2010). Most recently, some promoters has been determined led to higher accumulation of UFAs (Song et al., 2017; Dong et al., 2018). However, in oil palm, plastid- derived ω-6 desaturase gene (*EgFAD6*) and its promoter function have never being clarified (Sun et al., 2016).

In this paper, the full length of the gene encoding the *EgFAD6* was isolated from the oil palm and its function of the conversion from oleic acid to linoleic acid was identified by heterologous expression in yeast strain *Saccharomyces cerevisiae*. Additionally, the *EgFAD6* promoter region was isolated and assessed using the *β-glucuronidase* (*GUS*) reporter system through transgenic *Arabidopsis* plants. Therefore, these results revealed the function of *EgFAD6* elucidated the feature of its promoter. The obtained knowledge would be useful to understand the role of *EgFAD6* on fatty acid biosynthesis in oil palm fruits and other palm plants.

2. Materials and methods

2.1. Plant, strain and plasmid

Oil palm (*Elaeis guineensis* Jacq.) fruits, including five different developmental stages of 30–60 DAP (days after pollination, phase 1), 60–100 DAP (phase 2), 100–120 DAP (phase 3), 120–140 DAP (phase 4) and 140–160 DAP (phase 5), were harvested from the Coconut Research Institute, Chinese Agricultural Academy of Tropical Crops, Hainan in China. The auxotrophic *S.cerevisiae* strain INVSc1, *Agrobacterium tumefaciens* strain GV3101 and the high copy number shuttle vector pYES3, the plant expression vector pCAMBIA1300s-GUS were stored in our laboratory.

2.2. Isolation of the full-length EgFAD6 cDNA, the EgFAD6 promoter region sequence

Frozen oil palm fruits were ground to powder in liquid nitrogen. The extraction of total RNA was isolated based on the CTAB method according to the manufacturer's instructions. The total RNA was treated with DNaseI to remove the remaining genomic DNA. About 2 μg of purified total RNA was used as the template to synthesize the first-strand cDNA with the TIANScript One Step RT-PCR Kit. Based on the conserved regions of *EgFAD6* gene from several organisms, the forward primer (5′-GAGCTCATGGCGTGCCGGCTCTCCAAT-3′) and the reverse primer (5′-GAATTCTCATGTGTAATCAGGCATGAATTTTCG-3′), was designed to amplify the ORF of the *ω6FAD* gene from the mesocarp of oil palm fruits.

Total genomic DNA was extracted from the oil palm leaves based on the CTAB method. The promoter region sequence of the *EgFAD6* was amplified by the PCR using the FADP-F (GCGAAGCTTTCTACGAATTG ATATAAGCCTA) and the FADP-R (TATGGATCCCCGCTCGGGGGAAAC CAA) primers. The amplified products were purified by AxyPrep™DNA Gel Extraction kit (Axygen, China), cloned into pMD19-T Easy vector (TakaRa, Dalian, China), then transformed into *DH5α* competent cells and sequenced.

2.3. Quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) was performed using the SYBR® Premix Ex Taq™II (TaKaRa, Dalian, China). PCR reaction was performed in a total volume of 20 μL systems, including 10 μL of SYBR® Premix Ex Taq™II, 0.8 μL of each primer (10 mM), 2 μL of the cDNA and 6.4 μL of double distilled water. The relative transcript quantity of the *EgFAD6* was quantified in terms of comparative threshold cycle (Ct) using the $2^{-\Delta\Delta Ct}$ method. All data were given in terms of relative mRNA expression as means ± s.d. The statistical significance was analyzed by the SPSS 22.0. The *β-actin* gene was used as the reference gene. The primers were shown as follows: RTActin-F: 5′-TGGAAGCTGCTGGAAT CCAT-3′; RTActin-R: 5′-TCCTCCACTGAGCACAACGTT-3′; RTFAD-F: 5′-TTGGTGGCACAGTCCATTGT-3′; RTFAD-R: 5′-TGATGAGCTGCCTGCA AGTT-3′;

2.4. Bioinformatics analysis

The ORF of the cDNA was inferred by the online software ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/orffinder/). The homology searching and the theoretical physicochemical property of the deduced polypeptide were analyzed using NCBI Blast server and the ProtParam tool of the ExPASy (http://web.expasy.org/protparam/). The protein subcellular localization of the *EgFAD6* was predicted by the online prediction system LocTree3 (https://www.rostlab.org/services/loctree2/). The deduced amino acid sequences were aligned using the ClustalX program. The neighbor-joining (NJ) method in MEGA5 was used to construct the phylogenetic tree. The significance level of the NJ analysis was examined by bootstrap testing with 1000 repeats. The tree was described by Treeview software. The promoter prediction and the *cis*-acting elements were performed on the Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.5. Plasmid construction, yeast transformation and induction

The auxotrophic *S. cerevisiae* strain INVSc1 (His^-, Leu^-, Trp^-, Ura^-) is a fast-growing diploid strain ideal for expression. The pYES3 is a high copy, autonomously replicated *S. cerevisiae* and *E. coli* shuttle vector which provide ampicillin resistance to *E. coli* and uracil prototrophy to trp1 yeast. *EgFAD6* was cloned into the yeast expression vector pYES3. The recombinant yeast expression vector p*FAD6* was transformed into the defective mutant INVSc1 strain of *S. cerevisiae* (Gietz and Schiestl, 2007), and the transgenic yeasts were selected on the synthetic minimal medium plates lacking tryptophan (SC-Trp). All experiments were performed in triplicate.

2.6. Gas chromatographic analysis of fatty acids from yeast strains

After induction, the yeast cells were harvested by centrifugation, washed three times with sterile water to eliminate the influence of the residual medium and metabolites to the fatty acid analyses. The