Developing functional markers for vitamin E biosynthesis in oil palm

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Abstract

Vitamin E is essential for human health and plays positive roles in anti-oxidation. Previously, we detected large variation in vitamin E content among 161 oil palm accessions. In this study, twenty oil palm accessions with distinct variation in vitamin E contents (171.30 to 1,258.50 ppm) were selected for genetic variation analysis and developing functional markers associated with vitamin E contents. Thirty-seven homologous genes in oil palm belonging to vitamin E biosynthesis pathway were identified via BLASTP analysis, the lengths of which ranged from 426 to 25,717 bp (average 7,089 bp). Multiplex PCR sequencing for the 37 genes found 1,703 SNPs and 85 indels among the 20 oil palm accessions, with 226 SNPs locating in the coding regions. Clustering analysis for these polymorphic loci showed that the 20 oil palm accessions could be divided into five groups. Among these groups, group I included eight oil palm accessions whose vitamin E content (mean value: 893.50 ppm) was far higher than other groups (mean value 256.29 to 532.94 ppm). Correlation analysis between the markers and vitamin E traits showed that 134 SNP and 7 indel markers were significantly (p < 0.05) related with total vitamin E content. Among these functional markers, the indel EgTMT-1-24 was highly correlated with variation in vitamin E content, especially tocotrienol content. Our study identified a number of candidate function associated markers and provided clues for further research into molecular breeding for high vitamin E content oil palm.

Introduction

Oil palm (Elaeis guineensis, 2n = 32) is the most efficient oil crop in the world, which has the highest oil yield per unit area of all oil crops [1, 2]. Oil palm occupies about eight percent of total plantation area of oil crops worldwide [3], and the palm oil contributes more than 30% of total vegetable oil production in the world [4, 5]. Palm oil obtained from the oil palm mesocarp is rich in vitamins, especially for vitamin A and E [6, 7]. In nature, vitamin E is comprised of four tocopherol types (α, β, γ, and δ) and four tocotrienol types (α, β, γ, and δ) [8–10].
Also, the raw data of multiple PCR sequencing of 20 oil palm individuals have also been uploaded to ENA (accession number PRJEB38216, https://www.ebi.ac.uk/ena/browser/view/PRJEB38216).

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Markers for vitamin E in oil palm

Tocopherols are mainly found in the leaves and seeds of dicotyledonous plants [11, 12], whereas tocotrienols are found in the seed and endosperm tissues of monocotyledonous plants, especially in cereal grain crops such as rice, maize, and barley [8, 13, 14]. Previous research had shown that palm oil had high vitamin E content (approximately 600–1000 ppm), which is comprised of α-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol [15, 16]. Of these components, α-tocopherol occupies a small percentage of total vitamin E content (10%–30%), while the tocotrienols are the major components (70%–90%) [7]. In fact, palm oil has the highest content of tocotrienols in all major types of edible oil [17, 18]. Vitamin E is a powerful anti-oxidant, tocotrienols in which are proven to have 40–60 times more potency than α-tocopherol [19, 20]. Due to this property, palm oil has excellent stability at high temperature and the food prepared with palm oil can have long shelf life [21]. Besides anti-oxidant [22], tocotrienols also have the functions of neuroprotection [23], anti-cancer [24], cardio-protective [25], anti-inflammatory [26], anti-diabetic [6], and inhibition of cholesterol synthesis [27], which make palm oil beneficial for human health. Vitamin E in oil palm has been extensively studied for its nutritional and health properties, attributed largely to its high tocotrienols content [28]. Our previous study has detected large phenotypic variation for vitamin E content in 161 oil palm accessions and the average value of total vitamin E content varied from 172.45 to 1287.96 ppm [16], which could provide genetic resources for identifying elite alleles associated with high vitamin E content [16]. However, as a perennial tree, oil palm requires 10–19 years for phenotypic selection by traditional breeding method [29]. Molecular breeding uses traits linked markers for seedling pre-selection of desired vitamin E phenotypes, which has a potential to greatly shorten the breeding cycle and reduce costs.

Single nucleotide variations in coding sequence (CDS) regions can result in missense and nonsense mutation, causing influencing on gene function and variations in agronomic traits [30–33]. For example, four non-synonymous substitutions for soybean Terminal Flower 1 (GmTfl1) lead to changes in stem growth habit and improvement of soybean yield [34, 35]. A single nucleotide polymorphism SNP in a C2H2-type transcription factor increased broad-spectrum blast resistance in rice [36]. Two nucleotide mutations in a serine hydroxymethyltransferase (SHMT) gene improved soybean resistance to cyst nematode [37]. Polymorphic sites within genes that are related to trait variation could be used to develop functional markers, which are beneficial to marker-assisted selection [38, 39].

Approaches to develop functional markers in plant species include polymerase chain reaction (PCR) based markers, such as simple sequence repeats (SSRs) [40], sequence tagged site (STS) [41], expressed sequence tag-derived microsatellite (EST-SSR) [42, 43], and intron sequence amplified polymorphism (ISAP) [44, 45]. Recently, a single-PCR-based approach was used to develop single nucleotide polymorphism (SNP) markers by performing PCR amplification for specific gene locus from different accessions [46–48]. However, this approach is inefficient and high cost, for obtaining genotypic information for one locus at a time. Multiplex PCR sequencing offers a better solution to produce markers from multiple loci, in which, pairs of primers covered different genic regions are used in amplification and PCR products are sequenced using next-generation sequencing to identify SNP markers [49]. This technology has been validated as a powerful tool to develop molecular markers for targeted sequences.

Functional markers development relies on the knowledge of genes with assigned functions [38]. Extensive research had revealed the vitamin E biosynthesis pathways (Fig 1), where vitamin E is mainly synthesized via shikimate pathway and methylyerythritol phosphate (MEP) pathway [10]. A phytol recycling pathway for phytol-PP production from chlorophyll degradation also partakes [9]. The genes encoding the respective enzymes of these pathways have been identified and cloned in Arabidopsis thaliana [8, 50–52] and some other plant species [53, 54]. The functions of most genes in biosynthesis pathway also have been verified by transgenic
technology, such as HPPD [55, 56], HPT [57, 58], HGGT [59, 60], MPBQ-MT [61], TC [62] and γ-TMT [63], which are conducive to finding candidate genes belonging to vitamin E biosynthesis pathways in oil palm.

In recent years, genetic variations associated with total vitamin E or component contents have been identified, which could be used for markers development and molecular breeding. Fritsche et al. [64] identified two SNPs in MPBQ-MT (VTE3) gene associated with γ-tocophorol content and α-tocopherol content separately, as well as one SNP in HPT (VTE2) gene associated with γ-tocopherol content in rapeseed. Two markers—InDel7 and InDel118—derived from insertion/deletions (Indels) polymorphism located respectively within the 5’ UTR region and the promoter region of Zea mays γ-TMT (VTE4) gene were significantly associated with α-tocopherol levels in maize kernels [65]. The effects of these two causative indels in ZmVTE4 gene were validated by haplotype analysis [66]. However, few researches were reported on the genetic dissection of vitamin E biosynthesis in oil palm. Kong et al. [13] successfully cloned HGGT and HPT genes from two oil palm species and found that these two genes had a high expression level in oil palm mesocarp. Meanwhile, EgHGGT gene was validated to be associated with the biosynthesis of α-tocotrienol and total vitamin E content [16].

Fig 1. Biosynthesis pathway of vitamin E and the expression pattern of candidate genes involved in the pathway. The heatmaps of candidate genes were visualized by the MeV software. The expression levels were calculated by using Log2(FPKM+1). Abbreviations: CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; HGGT, Homogentisate geranylgeranyltransferase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPT, homogentisate phytyltransferase; IDI, isopentenyl diphosphate isomerase; ISPF, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; GGPS, geranylgeranyl pyrophosphate synthase; GGR, geranylgeranyl reductase; MPBQ-MT, 2-methyl-6-phytyl-1,4-hydroquinone methyltransferase; PK, phytol kinase; TAT, tyrosine aminotransferase; TC, tocopherol/tocotrienol cyclase; TMT, tocopherol/tocotrienol methyltransferase.

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Based on the large phenotypic variations of vitamin E content and the known candidate genes involved in vitamin E biosynthesis in oil palm, it is possible to develop functional markers associated with vitamin E content. In this study, 20 oil palm accessions with distinct variation of vitamin E content (171.30 to 1258.50 ppm) were selected from the 161 accessions employed by Luo et al. [16] to develop trait related functional markers. Subsequently, we analyzed the correlation between these functional markers and vitamin E content, providing functional markers for further molecular breeding of oil palm cultivars with high vitamin E content.

Materials and methods

Plant materials, vitamin E investigation and DNA extraction

In previous studies, the vitamin E content of 161 oil palm accessions with three biological and technical replicates per sample were measured by high-performance liquid chromatography (HPLC) analysis [16]. In this study, ten top oil palm accessions with high total vitamin E content (more than 650 ppm) and ten top accessions with low total vitamin E content (less than 500 ppm) were selected from the 161 oil palm accessions. The 20 oil palm accessions were planted in 2008 in the oil palm germplasm collection of the Coconut Research Institute of Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan, China. The average temperature and humidity were approximately 23.9˚C and 89%, respectively. Detailed information for these 20 oil palm accessions is listed in S1 Table.

The spear leaves of the 20 oil palm accessions were sampled for DNA extraction using a modified cetyltrimethyl ammonium bromide (CTAB) protocol [67]. The procedure was as follows: about 0.2 g of leaf tissue was ground into a fine powder with liquid nitrogen using a mortar and pestle. The pulverized tissue was immediately transferred to a 2 mL tube with 1 mL extraction buffer (119.8 g/L sucrose; 0.1 mol/L Tris-HCl; 5 mmol/L EDTA; 20 g/L PVP 40) and 30 μL β-mercaptoethanol. This mixture was then vortexed vigorously and centrifuged for 5 minutes at 8 000 rpm at 4˚C, and the supernatant was discarded. Further, 1 mL of 65˚C lysis buffer (0.1 mol/L Tris-HCl; 81.8 g/L NaCl; 20 mmol/L EDTA; 20 g/L CTAB; 20 g/L PVP 40) was added in each tube, the sample was vortexed thoroughly, incubated in 65˚C water bath for 60 minutes and shook once every ten minutes, then centrifuged for 10 minutes at 8 000 rpm at room temperature. The supernatant was transferred to a new 2 mL tube, mixed with an equal volume of chloroform and isoamyl alcohol solution (24:1, v/v), vortexed gently, centrifuged at 12 000 rpm for 18 minutes, and the aqueous phase (top layer) was transferred into a new tube. This step was repeated twice. After that, 1/3 volume of 3 mol/L sodium acetate (pH = 5.2) and double volume of chilled isopropanol were added, mixed by inverting the tube 20–30 times. DNA precipitation could be seen in the form of a cottony white colored mass in the solution, then incubated at -20˚C overnight. The next day, the DNA was pelleted by centrifugation at 8 000 rpm for 3 minutes and washed twice with 1 mL of 70% ethanol. After removing all residual ethanol, the pellet was air-dried for 20–30 minutes and the obtained DNA was dissolved with 300 μL TE buffer. The concentration and quality of the 20 oil palm DNA samples was examined using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quantified DNA was diluted to 100 ng/μL for PCR amplification.

Identification of candidate genes involved in vitamin E biosynthesis

All protein-coding gene sequences of *E. guineensis*, *A. thaliana*, and *Z. mays* were downloaded from the National Center for Biotechnology Information website (NCBI). Putative genes belonging to the vitamin E biosynthesis pathway from *A. thaliana* and *Z. mays* were aligned against the oil palm protein database with a cut-off e-value of 1e-10 [68–71] to identify the
best-hit homologous genes in oil palm. The conserved domains of these homologous genes were predicted by aligning with the pfam database (http://pfam.xfam.org/search). Gene structures were predicted by aligning protein sequences with the E. guineensis genome and drawn using the software GSDS 2.0 (http://gsds.cbi.pku.edu.cn).

FPKM calculations for transcriptomes obtained from different developmental stages of oil palm mesocarps

A total of 5 transcriptomes (single-end, 454 GS FLX Titanium), including 15-week-old mesocarp (SRR190698), 17-week-old mesocarp (SRR190699), 19-week-old mesocarp (SRR190700), 21-week-old mesocarp (SRR190701), and 23-week-old mesocarp (SRR190702) [2], were downloaded from the NCBI website. FPKM (fragments per kilobase of exon model per million mapped reads) was calculated to measure gene expression level by using the following formula [72, 73]:

\[ FPKM = \frac{10^6 C}{NL/10^3} \]

where C is the number of fragments which exclusively aligned to one expressed sequence; N is the total number of reads which are aligned to all expressed sequences and L is the basic number in the CDS of the corresponding expressed sequence.

Primer design and PCR amplification

A total of 328 primer pairs were designed to cover the complete genomic region of the candidate genes in the vitamin E biosynthesis pathway by using the software Primer Premier 5.0. These primer pairs were used to amplify intronic and exonic regions with amplicons ranging in length from 377 to 1 709 bp. The primer sequences are listed in S2 Table. We used a 20 μL PCR reaction system that contained 3 μL DNA (50 ng/μL), 2 μL pfu PCR Buffer (10 ×), 0.4 μL dNTP (10 mM), 0.4 μL forward primer (10 mM), 0.4 μL reverse primer (10 mM), 0.4 μL pfu DNA polymerase, and 13.4 μL ddH₂O. The PCR program was set as follows: 94˚C for 30 s, 30 cycles of 94˚C for 10 s, 50–60˚C for 30 s, and 72˚C for 60 s, and then 72˚C for 10 minutes. The PCR products were electrophoretically visualized on a 1% agarose gel.

DNA library construction and illumina sequencing

PCR products per sample were mixed and fragmented with a Bioruptor Pico Sonication Device (Diagenode, Liege, Belgium). The fragmented DNA was detected by electrophoresis and targeted sizes (500 bp) were selected for DNA library construction which was conducted with TrueLib DNA Library Rapid Prep Kit (Excell Biotech, Taicang, Jiangsu, China, catalogue number NGS00-1063). End Rapid Repair Reaction Buffer (EB buffer) was used to resolve these short fragments for end reparation and poly (A) addition in a 50 μL reaction system that contained 1 μg fragmented DNA, 10 μL 5 × EB buffer, 3 μL end prep enzyme mix and ddH₂O. These treated DNA fragments were used for the following PCR amplification using a two-step PCR program: 22˚C for 10 minutes and 72˚C for 10 minutes. The sequence adaptors were linked to two ends of short DNA sequences and the adaptor-ligated DNA fragments were purified using AMPure XP beads (Beckman Coulter Inc, Brea, CA, USA, catalogue number A63881). The purified products were selected as templates for PCR amplification using ExHiFi 2x PCR Master Mix, Universal primer and Index primer in a 50 μL reaction system. After PCR products were purified (AMPure XP beads), the concentration and quality of DNA library were assessed with Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA) and
Agilent Bioanalyzer 2100 system separately. Finally, the prepared DNA library was sequenced on an Illumina Hiseq 4000 platform (Illumina Inc., San Diego, CA, USA) and paired-end reads were generated by sequencing. The sequence quality of raw reads was checked using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were filtered with the Trimmomatic software [74] to obtain clean reads.

**SNP and indel genotyping**

The genome sequence of *E. guineensis* was downloaded from the NCBI website (RefSeq: GCF_000442705.1). Clean reads were mapped onto the reference genome and selected to assemble gene sequences by using the software Vevet [75]. The scaffolds mapped to the candidate gene regions were further assembled via CAP3 [72] and manually checked in MEGA 7 [76]. Coverage ratios of assembled sequences mapped on reference sequences were calculated. SNPs and indels were manually identified after multiple sequence alignments for candidate gene sequences from the 20 oil palm accessions via MEGA 7. The exons sequences of the assembled genes were extracted and translated into amino acid sequences, and then the amino acid variations were manually counted.

The Pearson’s correlation coefficient and significance between markers and vitamin E content was performed by using the software IBM SPSS Statistics Version 20–32 bit.

**Clustering analysis**

The representive sequence for each gene with SNP and indel variations from each oil palm accession was combined into a single sequence for cluster analysis. Phylogenetic analysis was performed according to the procedures described by Bast [77] using the software MEGA 7, where the combined sequences were subjected to multiple sequence alignments by ClustalW [78]. Phylogenetic tree was generated by maximum likelihood method based on general time reversible (GTR) model [79] with 500 bootstrap repetitions. Pairwise distances between sequences were inferred under the maximum composite likelihood (MCL) approach. Meanwhile, the heatmap of vitamin E content was drawn using the software MeV 4.8 [80].

**RNA extraction, transcriptome sequencing and expression analysis**

Eight oil palm individuals (three biological replicates) with distinct variation of vitamin E contents were subjected to transcriptome sequencing, including R17 (ERR4091630, ERR4091631, ERR4091632), R15 (ERR4091624, ERR4091625, ERR4091626), R12 (ERR4091618, ERR4091619, ERR4091620), R06 (ERR4091609, ERR4091610, ERR4091611), R04 (ERR4091606, ERR4091607, ERR4091608), R07 (ERR4091612, ERR4091613, ERR4091614), R09 (ERR4091615, ERR4091616, ERR4091617), and R14 (ERR4091621, ERR4091622, ERR4091623). Mature mesocarps were sampled from these eight oil palm accessions (three replicates per sample) and immediately frozen in liquid nitrogen. Total RNA was extracted separately using the MRIP method described by Xiao et al. (2012) [81]. RNA degradation and contamination, especially for DNA contamination, was detected by 1.5% agarose gels. RNA concentration and purity were measured using the NanoDrop 2000 Spectrophotometer. RNA integrity was assessed by using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). mRNA was enriched by Oligo(dT) magnetic beads and then rRNA was removed. The purified mRNA was fragmented with divalent cations under increased temperature. These short fragments were taken as templates to synthesize the first-strand cDNA using random hexamer primers and superscript™ III (Invitrogen™, Carlsbad, CA, USA). Second-strand cDNA was then synthesized in a solution containing buffer, dNTP, RNaseH and DNA polymerase I, and subsequently purified using a QiaQuick PCR extraction
kit (Qiangen). EB buffer was used to resolve these short fragments for end reparation and poly (A) addition. The sequence adaptors were linked to two ends of short cDNA sequences and suitably sized (100–200 bp) cDNA fragments were selected out for PCR amplification based on the agarose gel electrophoresis results. Finally, the library established was sequenced with an Illumina Hiseq™ 2000 (Illumina Inc., San Diego, CA, USA). The paired-end library was developed according to the protocol of the Paired-End Sample Preparation Kit (Illumina, USA). FPKM values were calculated to investigate the expression levels of the EgTMT-1 gene in different oil palm individuals. Pearson’s correlation coefficient between FPKM values and vitamin E traits were calculated using the software IBM SPSS Statistics.

Results

Classification of 20 oil palm accessions based on total vitamin E content

According to the vitamin E contents determined by Luo et al. [16], the selected 20 oil palm accessions were classified into four groups based on their total vitamin E content (Fig 2). Six oil palm accessions (H group) showed high vitamin E content, ranging from 1 031.40 to 1 258.50 ppm with an average value of 1 122.67 ppm, including R10, 08, 07, 11, 09 and 14. Four oil palm accessions (M group) showed medium vitamin E content, ranging from 656.46 to 929.35 ppm with an average value of 771.66 ppm, including R03, 04, 02, and 19. Moreover, five oil palm accessions (L group) showed low vitamin E content, ranging from 308.19 to 422.62 ppm with an average value of 373.87 ppm. The remaining five oil palm accessions (EL group) showed extremely low vitamin E content, ranging from 171.30 to 282.58 ppm with an average value of 244.05 ppm, including R20, 17, 13, 05, and 16. The average vitamin E content of these 20 oil palm accessions was 645.61 ± 376.41 ppm (mean ± SD).

Fig 2. Variations of vitamin E content in 20 oil palm accessions. The vitamin E contents in 20 oil palm accessions (three biological and technical replicates per sample) included δ-tocotrienol, γ-tocotrienol, α-tocotrienol, α-tocopherol, total tocotrienol, and total vitamin E.

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Identification of candidate genes related to vitamin E biosynthesis

Thirty-seven candidate genes involved in vitamin E biosynthesis were identified by BLAST analysis, where the best-hit genes were found by blasting the protein database of *E. guineensis* against the *Arabidopsis* and *Z. mays* genes belonging to vitamin E biosynthesis pathway (Table 1). A total of 17 *EgTAT* genes were identified that catalyze tyrosine into p-hydroxyphenyl-pyruvate. Compared to the three *TAT* genes present in *Arabidopsis*, a significant expansion of *EgTAT* copies (up to 17) was detected in the genome of *E. guineensis*. One *EgHPPD* gene was identified, which converses p-hydroxyphenyl-pyruvate to HGA. Meanwhile, one *EgHGGT*

Table 1. Predicted candidate genes involved in vitamin E biosynthesis in *E. guineensis*.

| Gene name  | Best-hit gene in *A. thaliana* | E-value | Chromosome | Strand | Gene ID          | Gene length (bp) | Protein ID    | Protein length (aa) |
|------------|--------------------------------|---------|------------|--------|------------------|------------------|--------------|---------------------|
| EgCMK-1    | AtCMK                          | 0       | chr5       | +      | LOC105045599     | 7 589            | XP_010922252.1 | 397                 |
| EgCMK-2    | AtCMK                          | 2E-151  | chr10      | -      | LOC105052446     | 5 083            | XP_029122665.1 | 397                 |
| EgHPPD     | AtHPPD                         | 2E-80   | Scaffold   | -      | LOC105035707     | 5 743            | XP_019702972.1 | 200                 |
| EgHPT-1    | AtHST                          | 2E-180  | chr6       | -      | LOC105046456     | 7 259            | XP_010923356.1 | 402                 |
| EgHPT-2    | AtHPT1                         | 4E-173  | chr8       | +      | LOC105050383     | 18 852           | XP_001291355.1 | 400                 |
| EgHGTT     | AtHGTT                         | 2E-125  | chr10      | -      | LOC105052944     | 7 191            | XP_010932321.1 | 462                 |
| EgIDI      | AtIDI2                         | 7E-46   | Scaffold   | +      | LOC105061124     | 940              | XP_010943386.1 | 104                 |
| EgGGG     | AtGGG                          | 9E-107  | Scaffold   | -      | LOC105032472     | 1 592            | XP_010905229.1 | 413                 |
| EgGGPS-1   | AtGGPS2                        | 6E-28   | Scaffold   | +      | LOC105057082     | 426              | XP_010911086.1 | 141                 |
| EgGGPS-2   | AtGGPS1                        | 2E-38   | chr1       | -      | LOC105048867     | 11 386           | XP_010926643.1 | 395                 |
| EgGGPS-3   | AtGGPS1                        | 3E-130  | chr3       | +      | LOC105041405     | 3 041            | XP_010916141.1 | 349                 |
| EgGGPS-4   | AtGGPS1                        | 6E-144  | chr15      | +      | LOC105057980     | 1 755            | XP_010939037.1 | 369                 |
| EgISPF-1   | AtISPF                         | 3E-102  | chr4       | +      | LOC105043318     | 6 681            | XP_010919124.2 | 236                 |
| EgISPF-2   | AtISPF                         | 9E-105  | chr11      | +      | LOC105054207     | 3 774            | XP_010933967.1 | 208                 |
| EgMPBQ-MT  | AtMPBQ-MT                      | 0       | chr1       | +      | LOC105055609     | 6 402            | XP_010935791.1 | 340                 |
| EgPK-1     | AtPK                           | 3E-112  | Scaffold   | +      | LOC105034444     | 4 138            | XP_010906565.1 | 323                 |
| EgPK-2     | AtPK                           | 3E-70   | Scaffold   | -      | LOC105036025     | 6 539            | XP_010910062.1 | 307                 |
| EgTAT-2    | AtTAT2                         | 5E-22   | Scaffold   | -      | LOC105035000     | 2 850            | XP_010905916.1 | 474                 |
| EgTAT-3    | AtTAT2                         | 2E-16   | chr1       | +      | LOC105050532     | 9 153            | XP_010928898.1 | 481                 |
| EgTAT-4    | AtTAT2                         | 2E-24   | chr2       | -      | LOC105039748     | 4 940            | XP_010911890.1 | 412                 |
| EgTAT-5    | AtTAT2                         | 0       | chr3       | +      | LOC105040940     | 5 094            | XP_010916004.1 | 425                 |
| EgTAT-6    | AtTAT2                         | 2E-14   | chr3       | -      | LOC105042048     | 10 327           | XP_010914992.1 | 396                 |
| EgTAT-7    | AtTAT1                         | 2E-18   | chr3       | +      | LOC105041987     | 8 295            | XP_019704422.1 | 477                 |
| EgTAT-8    | AtTAT2                         | 5E-15   | chr4       | +      | LOC105043536     | 3 010            | XP_010919415.1 | 492                 |
| EgTAT-9    | AtTAT3                         | 6E-18   | chr7       | -      | LOC105047871     | 15 209           | XP_010925286.1 | 396                 |
| EgTAT-10   | AtTAT2                         | 5E-17   | chr7       | -      | LOC105048002     | 2 155            | XP_010925479.1 | 441                 |
| EgTAT-11   | AtTAT3                         | 8E-16   | chr8       | +      | LOC105049882     | 10 084           | XP_010927963.1 | 481                 |
| EgTAT-12   | AtTAT2                         | 1E-16   | chr11      | -      | LOC105045176     | 5 290            | XP_010939324.1 | 530                 |
| EgTAT-13   | AtTAT2                         | 3E-14   | chr13      | +      | LOC105055886     | 2 139            | XP_010936199.1 | 460                 |
| EgTAT-14   | AtTAT2                         | 1E-18   | chr13      | +      | LOC105056662     | 7 017            | XP_010937250.1 | 526                 |
| EgTAT-15   | AtTAT3                         | 4E-17   | chr15      | -      | LOC105058291     | 2 319            | XP_010939473.1 | 468                 |
| EgTAT-16   | AtTAT2                         | 5E-28   | chr15      | -      | LOC105058225     | 11 240           | XP_010939394.1 | 464                 |
| EgTAT-17   | AtTAT3                         | 4E-17   | chr15      | +      | LOC105058582     | 7 350            | XP_010938849.1 | 475                 |
| EgTC       | AtTC                           | 0       | chr3       | -      | LOC105040851     | 19 129           | XP_010915874.1 | 492                 |
| EgTMT-1    | AtTMT                          | 3E-153  | Scaffold   | -      | LOC105033221     | 25 717           | XP_010906230.1 | 327                 |
| EgTMT-2    | AtTMT                          | 2E-12   | chr4       | +      | LOC105042568     | 1 649            | XP_010918151.1 | 369                 |
| EgTMT-3    | AtTMT                          | 1E-15   | chr6       | -      | LOC105046511     | 11 273           | XP_010923409.1 | 342                 |

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and two $EgHPT$ were identified. The protein sequences of $EgHGGT$ and $EgHPT-2$ had higher similarity (56%), although the two types of genes have different functions in vitamin E biosynthesis. Moreover, one $MPBQ-MT$ was identified, which converts 2-methyl-6-genralylgeranylbenzoquinol (MGGBQ) and 2-methyl-6-phytylbenzoquinol (MPBQ) to 2,3-dimethyl-5-genralylgeranylbenzoquinol and 2,3-dimethyl-5-phytylbenzoquinol, respectively. Only one $EgTC$ gene (the final step in the biosynthesis of $\gamma$-tocotrienol, $\delta$-tocotrienol, $\gamma$-tocopherol, and $\delta$-tocopherol) was identified. In addition, three $EgTMT$ genes (involved in the production of $\alpha$-tocotrienol, $\beta$-tocotrienol, $\alpha$-tocopherol, and $\beta$-tocopherol) were identified.

The lengths of candidate genes involved in vitamin E biosynthesis varied from 426 to 25717 bp, with an average length of 7089 bp per candidate gene. Their protein sequences varied from 104 to 530 amino acids (aa), with an average length of 388 amino acids per gene.

**Candidate gene structures and conserved domains**

Gene structures of the 37 candidate genes were visualized by the software GSDS (Fig 3). The exon numbers of these candidate genes varied from 1 to 15, among which, $EgTAT$ contained the highest amount of exons (15), while $EgTMT-2$ contained only one exon. $EgTAT$ had 17 gene copies, some of which shared the same amount of introns and exons. For example, five $EgTAT$ copies ($EgTAT-8$, 10, 12, 13, 15) contained three introns and four exons. Meanwhile, two $EgCMK$, two $EgISPF$, and two $EgPK$ also had the same amount of introns and exons.

Conserved gene structures indicated that noticeable gene structure variations were existence in different $EgTMT$ copies: $EgTMT-1$ only contained one exon; $EgTMT-2$ had 10 exons, and $EgTMT-3$ had 13 exons. This gene structure variation may result in the divergence of $EgTMT$ gene function. Some $EgTMT$s may be responsible for the transformation from $\gamma$-tocopherol and $\delta$-tocopherol to $\alpha$-tocopherol and $\beta$-tocopherol, while others may be responsible for the transformation from $\gamma$-tocotrienol and $\delta$-tocotrienol to $\alpha$-tocotrienol and $\beta$-tocotrienol. Meanwhile, $EgHGGT$ and two $EgHPT$ copies have similar protein sequence and showed different function, which also showed high variation in gene structure.

The functional domains analysis demonstrated that most candidate genes contained the corresponding conserved functional domain, except for $EgPK-1$ and $EgPK-2$. Detailed information on candidate gene functional domains is listed in S3 Table.

**Expression pattern of candidate genes related to vitamin E biosynthesis in different developmental stages of mesocarp**

In order to detect the expression pattern of candidate genes in different stages of oil palm mesocarp, we calculated the FPKM values based on transcriptoms derived from different developmental stages of mesocarps, including mesocarp 15 weeks (SRR190698), 17 weeks (SRR190699), 19 weeks (SRR190700), 21 weeks (SRR190701), and 23 weeks (SRR190702). Sixteen $EgTAT$ genes showed different expression levels in the oil palm mesocarp. Among them, $EgTAT5$ have high expression level in different stages of oil palm mesocarp, which also has high similarity (E-value = 0) with Arabidopsis $TAT$ gene. $EgHPPD$ and $EgHGGT$ have higher expression level in mesocarp 21 weeks and 23 weeks than in other earlier stages of mesocarp. The two $EgHPT$ showed different expression summits among mesocarp development: $EgHPT-1$ has highest expression level in mesocarp 15 weeks, whereas $EgHPT-2$ has highest expression level in mesocarp 21 weeks and 23 weeks. Meanwhile, Three $EgTMT$ genes have all high expression level in any five mesocarp developmental stages. Moreover, the expression level of $EgTMT-1$ was gradually decreased from mesocarp 15 weeks to 23 weeks, and $EgTMT-2$ and $EgTMT-3$ was gradually increased from mesocarp 15 weeks to 23 weeks. The expression patterns of all candidate genes involved in vitamin E biosynthesis were showed in Fig 1.
Developing functional molecular markers from the 20 oil palm accessions

PCR products per oil palm accession were produced and sequenced using the Illumina sequencing platform at an average sequencing depth of 906×. In total, the number of clean reads generated from the 20 DNA libraries ranged from 3,305,260 to 5,346,160, with an average of 4,268,221 ± 472,954 (mean ± SD) of per sample, and GC contents changed from 38.69% to 42.85%. Q30 values of clean reads were all above 95%. After assembly, 485,051 to 1,034,321 bp scaffolds were mapped to the oil palm reference genome. Subsequently, the scaffolds were mapped to the corresponding target genes involved in vitamin E biosynthesis and further assembled. Five genes (EgGGR, EgGGPS-1, EgGGPS-3, EgGGPS-4, EgTMT-2) were not sequenced well in all 20 oil palm accessions and was not further investigated in the present study. The amplified regions of the remaining 32 genes covered between 59.21% and 100% of...
the corresponding targeted regions and the assembled fragments covered between 21.99% and 100% of amplified regions with an average of 14.02% missing (S4 Table).

The sequence alignment and analysis found 1,788 polymorphic sites (1,703 SNPs and 85 indels) among the 20 oil palm accessions. The density of SNP and Indel (the average length of fragments divided by the number of variations) of every gene was calculated and an average density of 1 SNP/179 bp and 1 indel/273 bp were observed (S4 Table). Of these, a large proportion of SNPs (1,410) and indels (74) were distributed in the intron regions of these candidate genes, while 67 SNPs and 11 indels were located in the untranslated regions and 226 SNPs in the coding sequence (CDS) regions (S5 and S6 Tables). Of the 226 SNPs within CDS regions, a total of 96 single nucleotide variations resulted in amino acid changes, which were mapped into 25 candidate genes. Meanwhile, among 96 SNPs, 56 were mapped into conserved domains of 16 candidate genes, including EgHGGT, EgTAT1, EgTAT2, EgTAT4, EgTAT5, EgTAT6, EgTAT7, EgTAT8, EgTAT9, EgTAT10, EgTAT12, EgTAT14, EgTAT15, EgTAT16, EgMPBQ-MT, EgTC (S1 Fig). No polymorphic sites caused frame-shift mutations in these genes among the 20 oil palm accessions. Among all candidate genes, EgTMT-1 was the most polymorphic (295 SNPs and 20 indels), followed by the EgTC gene which harbored 142 SNPs and 7 indels.

The association between genetic variants in vitamin E synthesis genes and vitamin E content

A total of 1,788 variations from 32 candidate genes in each oil palm accession were combined into a sequence, and the length of the combined sequence was 3,391 bp. The 20 oil palm accessions were divided into five clusters (groups I to V) on the bases of the pairwise sequence similarity over 70% [82] and bootstrap values higher than 50% [83]. Group I included the most oil palm accessions (8) and had the highest vitamin E content (274.05 ppm to 1,147.77 ppm, average 893.50 ppm), which also included the above high vitamin content group (5, H group) and middle vitamin E content group (2, M group) except R05 (Fig 4). The four components of vitamin E in group I also showed higher levels when compared to other groups. Group III was comprised of seven oil palm accessions, 1 from the H group, 1 from the M group, 3 from the L group and 2 from the EL group. The total vitamin E content in group III ranged from 171.30 to 1,258.50 ppm with an average of 532.94 ppm per accession. Both of group IV and V included two oil palm accessions and have an average of 543.23 and 345.49 ppm of total vitamin E content, respectively. Group II only contained one oil palm accession and the total vitamin E content was 256.29 ppm.

The correlation relationship between all markers and vitamin E content were analyzed. A total of 134 SNP and 7 indel markers showed significant ($p < 0.05$) correlation relationship with total vitamin E content. The associated SNPs were located onto 16 candidate genes involved in the vitamin E biosynthesis, including EgCMK-1 (2 SNP markers), EgHGGT (3), EgHPPD (1), EgGGPS-2 (1), EgTAT-3 (2), EgTAT-4 (3), EgTAT-5 (2), EgTAT-7 (3), EgTAT-8 (6), EgTAT-9 (16), EgTAT-14 (6), EgTAT-16 (4), EgMPBQ-MT (10), EgTC (5), EgTMT-1 (67) and EgTMT-3 (3) (S5 Table). The significantly associated indels only located onto EgTAT16 (1) and EgTMT-1 (6) (S6 Table). A large number of SNP (67) and indel (6) markers in EgTMT-1 gene were significantly associated with the variations of vitamin E content.

PCR products amplified by EgTMT-1-24 (EgTMT-1) primers contained two distinct alleles with amplicon sizes of 682 and 1,001 bp (Fig 5A). Sequencing results showed that the variation in the EgTMT-1-24 locus was caused by a 319 bp indel in an intron region of EgTMT gene. While all 20 oil palm accessions contained the 682 bp allele, the other allele (1,001 bp) was detected in only eight oil palm accessions (R3, 4, 5, 7, 8, 9, 10, and 11), five of which (83%)
belonged to the H group (Fig 5B). In order to further validate the association between the EgTMT-1-24 marker and vitamin E content, 53 oil palm accessions were amplified for this intron region with EgTMT-1-24 primer pairs. Significant association relationships between EgTMT-1-24 marker and total vitamin E content ($r = 0.504$), δ-tocotrienol (0.661), γ-tocotrienol (0.511) and total tocotrienol content (0.506) were detected ($p$-value less than 0.001).

**The relationship between EgTMT-1 expression and vitamin E content**

Based on transcriptome data, the FPKM values of EgTMT-1 gene were calculated in different oil palm individuals, including R17, R15, R12, R06, R04, R7, R9 and R14. The analysis showed...
that FPKM values in low vitamin E content individuals were 96.45 in R17, 80.10 in R15, 59.63 in R12, 61.37 in R06, and 63.01 in R04. While FPKM values in high vitamin E content individuals were 129.42 in R07, 106.01 in R09, and 131.82 in R14. The FPKM values of EgTMT-1 had significant positive correlation with the total vitamin E contents \((r = 0.742)\), \(\gamma\)-tocotrienol \((0.737)\) and total tocotrienol content \((0.737)\) at \(p\)-value less than 0.05. However, the correlation between FPKM values and \(\delta\)-tocotrienol was not significant \((r = 0.291 \text{ and } p = 0.485)\).

Discussion

Palm oil is derived from the fresh oil palm fruit and widely used in food and oleochemical industry [84, 85]. Vitamin E is an essential component in palm oil which has powerful antioxidant activity and is useful for improving oil nutritional value [10, 86]. Consequently, it will be important to understand the vitamin E biosynthesis pathway, find key genes and identify functional markers for further developing improved oil palm varieties. By far, genes involved in vitamin E biosynthesis have been characterized and functionally validated in some higher plants, such as *A. thaliana* [8, 51, 87–90], *Oryza sativa* [91–93], *Glycine max* [94, 95], and *Z. mays* [96, 97]. However, the molecular basis of vitamin E biosynthesis in *E. guineensis* is relatively unknown. In this study, we identified more candidate genes for vitamin E biosynthesis in *E. guineensis* (37) than in *A. thaliana* (18), *O. sativa* (23), and *Z. mays* (25). However, similar numbers of genes related to vitamin E biosynthesis were identified between *E. guineensis* (37) and *G. max* (39), which is interesting as both species have high vitamin E content. In *E. guineensis*, a significant expansion of the EgTAT gene family (17 copies) was detected relative to *A. thaliana* (3) [98], *O. sativa* (3), *Z. mays* (3), and *G. max* (5), which may suggest a higher efficiency of tyrosine transformation to \(p\)-hydroxyphenyl-pyruvate in oil palm.

Meanwhile, we identified one copy of EgHGGT and two copies of EgHPT that are crucial enzymes for tocotrienol and tocopherol biosynthesis, respectively. HGGT catalyzes HGA and GGDP into MGGBQ which is the precursor of tocotrienols, while HPT catalyzes HGA and PDP into MPBQ, which is the precursor of tocopherols [8]. Since tocotrienol comprises approximately 90% of the total vitamin E content in oil palm, EgHGGT should have a higher expression level or more gene copies in the genome of *E. guineensis*, which was not in accordance with our results. Therefore, it is possible that EgHPT genes can also catalyze HGA and GGDP to form MGGBQ in *E. guineensis*. TMT also showed higher copy number in *E. guineensis* (3) and *G. max* (3) [94, 95] compared to *A. thaliana* (1) [63, 99] and *O. sativa* (1) [92] and *Z. mays* (1) [97].

We also identified a large range in vitamin E content in the set of 20 oil palm accessions, with the total vitamin E content varying from 171.30 to 1258.50 ppm, far larger than the variation observed in other species [100, 101]. Hence, we set out to ascertain gene sequence variation among these 20 oil palm accessions as an explanatory factor for this large variation in vitamin E content. Although most genetic variants (82% of SNPs and 89% of indels) were found in the intron regions of candidate genes, 226 SNPs were found in exons of candidate genes, and 56 of these SNPs changed amino acids in conserved protein domains. Research has shown that SNPs detected in the CDS and promoter sequences could cause noticeable phenotypical variation. In rice, one single nucleotide changes in reduced expression of C2H2-type transcription factor enhanced disease resistance [36]. The single nucleotide variation in SHELL gene, encoding a homologue of SEEDSTICK, influenced the oil yield in *E. guineensis* [102]. A single base substitution in BATH/AMADH, which causes amino acid change in a highly conserved motif, had lead to the variation of the existence of fragrance [103]. Therefore, these 56 SNPs may lead to the change of amino acid sequence in conserved domain and influence enzymatic activity and subsequent vitamin E biosynthesis.
Conclusions

In summary, thirty-seven candidate genes involved in vitamin E biosynthesis were identified in oil palm. Gene structure, conserved domains and expression pattern of these candidate genes were analyzed. A total of 1,788 polymorphic sites (1,703 SNPs and 85 indels) were found among these 37 genes by Multiplex PCR sequencing and sequence alignment. Phylogenetic analysis based on all identified markers divided the 20 oil palm accessions into five groups which were related to the vitamin E content. Then the relationship between markers and vitamin E traits were further analyzed and a total of 141 function associated markers (134 SNP and 7 indel) were found. Among these markers, the 319 bp EgTMT-1-24 indel located in an intron region of EgTMT gene was easily detected by PCR amplification and showed significant relationship with vitamin E content, especially tocotrienol content, which was validated in a total of 53 oil palm individuals. Expression analysis on the base of eight different oil palm individuals also revealed that the FPKM values of EgTMT gene had significant positive correlation with the vitamin E contents. These identified vitamin E functional markers are useful for further study of the key genes which could regulate vitamin E biosynthesis in oil palm mesocarp and may have potential application in marker-assisted selection breeding for high vitamin E content oil palm germplasm.

Supporting information

S1 Fig. SNP distributions across the CDS regions of the 37 candidate genes involved in vitamin E biosynthesis. In the gene structure diagram, red blocks represent untranslated regions (UTR); pink blocks represent the conserved domain of the candidate genes; and the black lines represent SNP locations among the 37 candidate genes. Meanwhile, nucleotide and amino acid conversions were marked above the corresponding SNP markers. (TIF)

S1 Table. The detailed information of 20 oil accession used in the study. (XLSX)

S2 Table. Primers sequences used in the study. (XLSX)

S3 Table. Conserved domains of 37 candidate genes involved in vitamin E biosynthesis. (XLSX)

S4 Table. Target gene length, amplified gene region, number of variations, total fragment length and coverage ratio of vitamin E biosynthesis genes. (XLSX)

S5 Table. SNP markers across the 37 candidate genes involved in vitamin E biosynthesis and correlation coefficients with vitamin E content. (XLSX)

S6 Table. The indels located in the 37 candidate genes involving vitamin E biosynthesis and correlation coefficients with vitamin E content. (XLSX)

S1 Raw images. The original image of Fig 5A. (PDF)
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References
1. Barcelos E, Rios SA, Cunha RN, Lopes R, Motoike SY, Babychuk E, et al. Oil palm natural diversity and the potential for yield improvement. Front Plant Sci. 2015; 6:190. https://doi.org/10.3389/fpls.2015.00190 PMID: 25870604
2. Bourgis F, Kilaru A, Cao X, Ngando-Ebongue GF, Drira N, Ohrogge JB, et al. Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. Proc Natl Acad Sci USA. 2011; 108(30):12527–32. https://doi.org/10.1073/pnas.1106502108 PMID: 21709233
3. Wahid MB, Abdullah SNA, Henson IE. Oil Palm—Achievements and potential. Plant Prod Sci. 2005; 8 (3):288–97. https://doi.org/10.1626/pps.8.288
4. Murphy DJ. Oil palm: future prospects for yield and quality improvements. Lipid Technology. 2009; 21 (11–12):257–60. https://doi.org/10.1002/lite.200900067
5. Sharma M, Gupta SK, Mondal AK. Production and trade of major world oil Crops. Technological Innovations in Major World Oil Crops, Volume 1. New York: Springer; 2012. p. 1–15. https://doi.org/10.1007/978-1-4614-0356-2_1
6. Loganathan R, Selvaduray KR, Nesaretnam K, Radhakrishnan AK. Health promoting effects of phytonutrients found in palm oil. Mal J Nutr. 2010; 16(2):309–22. PMID: 22691935.
7. Sundram K, Sambanthamurthi R, Tan YA. Palm fruit chemistry and nutrition. Asia Pac J Clin Nutr. 2003; 12:355–62. PMID: 14506001.
8. Hunter SC, Cahoon EB. Enhancing vitamin E in oilseeds: unraveling tocopherol and tocotrienol biosynthesis. Lipids. 2007; 42(2):97–108. https://doi.org/10.1007/s11745-007-3028-6 PMID: 17393215
9. Muñoz P, Munné-Bosch S. Vitamin E in plants: biosynthesis, transport, and function. Trends Plant Sci. 2019; 24(11):1040–51. https://doi.org/10.1016/j.tplants.2019.08.006 PMID: 31608282
10. Wang L, Wang H, Lai Q, Li T, Fu X, Guo X, et al. The dynamic changes of ascorbic acid, tocopherols and antioxidant activity during germination of soybean (Glycine max). Int J Food Sci Technol. 2015; 50 (11):2367–74. https://doi.org/10.1111/jifs.12902
11. Matthias B, Vosmann K, Pharm LQ, Alzetmüller K. FA and tocopherol composition of Vietnamese oilseeds. J Am Oil Chem Soc. 2003; 80:1013–20. https://doi.org/10.1007/s11716-003-0813-y.
12. Seker M, Gul MK, Ipek M, Toplu C, Kaleci N. Screening and comparing tocopherols in the rapeseed (Brassica napus L.) and olive (Olea europaea L.) varieties using high-performance liquid chromatography. Int J Food Sci Nutr. 2008; 59(6):483–90. https://doi.org/10.1080/09637480701539484 PMID: 19086241
13. Kong SL, Abdullah SNA, Ho CL, Amiruddin MD. Molecular cloning, gene expression profiling and in silico sequence analysis of vitamin E biosynthetic genes from the oil palm. Plant Gene. 2016; 5:100–8. https://doi.org/10.1016/j.plgene.2016.01.003
14. Zhang G, Liu R, Zhang P, Xu Y, Zhu J, Gu M, et al. Variation and distribution of vitamin E and composition in seeds among different rice varieties. Acta Agronomica Sinica. 2012; 38(1):55–61. https://doi.org/10.1007/s1875-2780(11)60098-9
15. Choo YM, Ma AN, Chuah CH, Hurr TK, Bong SC. A developmental study on the appearance of tocopherols and tocotrienols in developing palm mesocarp (Elaeis guineensis). Lipids. 2004; 39 (6):561–4. PMID: 15554155.
Li W, Zhu Z, Chern M, Yin J, Yang C, Ran L, et al. A natural allele of a transcription factor in rice confers broad-spectrum blast resistance. Cell. 2017; 170(1):114–26. https://doi.org/10.1016/j.cell.2017.06.008 PMID: 28666113

Bakir DS, Yalcin G, Cucu AK. Isolation and determination of tocopherols and tocotrienols from the seed of Capparis Ovata grown in Turkey by reversed-phase high-performance liquid chromatography. Chromatographia. 2019; 83(1):77–86. https://doi.org/10.1007/s10337-019-03816-8

Pham AT, Lee JD, Shannon JG, Bilyeu KD. Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait. BMC Plant Biol. 2010; 10:195. https://doi.org/10.1186/1471-2229-10-195 PMID: 20828382

Ahsan H, Ahad A, Siddiqui WA. Pharmacological potential of tocotrienols: a review. Nutr Metab (Lond). 2014; 11(1):52. https://doi.org/10.1186/1743-7075-11-52 PMID: 25435896

Sen CK, Khaonna S, Roy S. Tocotrienols in health and disease: the other half of the natural vitamin E family. Mol Aspects Med. 2007; 28(5–6):692–728. https://doi.org/10.1016/j.mam.2007.03.001 PMID: 17507086

Luo T, Xia W, Gong S, Mason AS, Li Z, Liu R, et al. Identifying vitamin E biosynthesis genes in Elaeis guineensis by genome-wide association study. J Agric Food Chem. 2020; 68(2):678–85. https://doi.org/10.1021/acs.jafc.9b03832 PMID: 31858793

Vasanthi HR, Parameswari RP, Das DK. Multifaceted role of tocotrienols in cardioprotection supports their structure: function relation. Genes Nutr. 2012; 7(1):19–28. https://doi.org/10.1007/s12263-011-0227-9 PMID: 21604025

Liu B, Kanazawa A, Matsumura H, Takahashi R, Harada K, Abe J. Genetic redundancy in soybean number of seeds per pod in soybean. Plant Cell. 2012; 24(12):4807–18. https://doi.org/10.1105/tpc.1186/1471-2229-10-195 PMID: 20828382

Ghosh N, Das A, Khanna S. Vitamin E: Tocopherols and tocotrienols and their role in health and disease. Essential and Toxic Trace Elements and Vitamins in Human Health. Pittsburgh: Academic Press; 2020. p. 283–93.

Gupta AK, Cucu AK. Isolation and determination of tocopherols and tocotrienols from the seed of Capparis Ovata grown in Turkey by reversed-phase high-performance liquid chromatography. Chromatographia. 2019; 83(1):77–86. https://doi.org/10.1007/s10337-019-03816-8

Liu B, Kanazawa A, Matsumura H, Takahashi R, Harada K, Abe J. Genetic redundancy in soybean number of seeds per pod in soybean. Plant Cell. 2012; 24(12):4807–18. https://doi.org/10.1105/tpc.1186/1471-2229-10-195 PMID: 20828382

Jeong N, Suh SJ, Kim MH, Lee S, Moon JK, Kim HS, et al. Lr1 is a key regulator of leaflet shape and number of seeds per pod in soybean. Plant Cell. 2012; 24(12):4807–18. https://doi.org/10.1105/tpc.112.104968 PMID: 23243125

Liu B, Kanazawa A, Matsumura H, Takahashi R, Harada K, Abe J. Genetic redundancy in soybean number of seeds per pod in soybean. Plant Cell. 2012; 24(12):4807–18. https://doi.org/10.1105/tpc.112.104968 PMID: 23243125

Pham AT, Lee JD, Shannon JG, Bilyeu KD. Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait. BMC Plant Biol. 2010; 10:195. https://doi.org/10.1186/1471-2229-10-195 PMID: 20828382

Jeong N, Suh SJ, Kim MH, Lee S, Moon JK, Kim HS, et al. Lr1 is a key regulator of leaflet shape and number of seeds per pod in soybean. Plant Cell. 2012; 24(12):4807–18. https://doi.org/10.1105/tpc.112.104968 PMID: 23243125

Ahn SJ, Ahad A, Siddiqui WA. A review of characterization of tocotrienols from plant oils and foods. J Chem Biol. 2015; 8(2):45–59. https://doi.org/10.1037/s12154-014-0127-8 PMID: 25870713

Bakir DS, Yalcin G, Cucu AK. Isolation and determination of tocopherols and tocotrienols from the seed of Capparis Ovata grown in Turkey by reversed-phase high-performance liquid chromatography. Chromatographia. 2019; 83(1):77–86. https://doi.org/10.1007/s10337-019-03816-8

Matthäus B. Use of palm oil for frying in comparison with other high-stability oils. Eur J Lipid Sci Technol. 2007; 109(4):400–9. https://doi.org/10.1002/ejlt.200600294
37. Liu S, Kandoth PK, Warren SD, Yeckel G, Heinz R, Alden J, et al. A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature. 2012; 492(7428):256–60. https://doi.org/10.1038/nature11651 PMID: 2235880

38. Andersen JR, Lubberstedt T. Functional markers in plants. Trends Plant Sci. 2003; 8(11):554–60. https://doi.org/10.1016/j.tplants.2003.09.010 PMID: 14607101

39. Liu Y, He Z, Appels R, Xia X. Functional markers in wheat: current status and future prospects. Theor Appl Genet. 2012; 125(1):1–10. https://doi.org/10.1007/s00122-012-1829-3 PMID: 22366867

40. Zhou L, Yarra R, Zhao Z, Jin L, Cao H. Development of SSR markers based on transcriptome data and association mapping analysis for fruit stalk thickness associated traits in oil palm (Elaeis guineensis Jacq.). Biotech. 2020; 10(6):260. https://doi.org/10.31205-020-02249-9 PMID: 32537380

41. Wang L, Li G, Peña RJ, Xia X, He Z. Development of STS markers and establishment of multiplex PCR for Glu-A3 alleles in common wheat (Triticum aestivum L.). J Cereal Sci. 2010; 51(3):305–12. https://doi.org/10.1016/j.jcbs.2010.01.005

42. Chen C, Bowman KD, Choi YA, Dang PM, Rao MN, Huang S, et al. EST-SSR genetic maps for rice (Oryza sativa L.). Theor Appl Genet. 2007; 110(7):107–18. https://doi.org/10.1007/s00122-003-1223-2 PMID: 12712246

43. Xia W, Xiao Y, Liu Z, Luo Y, Mason AS, Fan H, et al. Development of gene-based simple sequence repeat markers for association analysis in rice (Oryza sativa L.). Mol Breeding, 2014; 34(2):525–35. https://doi.org/10.1007/s11032-014-0555-x

44. Lu C, Yu S, Yu J, Fan S, Song M, Wang W, et al. Development and appraisement of functional molecular marker: intron sequence amplified polymorphism (ISAP). Hereditas. 2008; 30(9):1207–16. https://doi.org/10.3724/sp.1005.2008.01207 PMID: 18779181

45. Xiong F, Liu J, Zhong R, Jiang J, Han Z, He L, et al. Intron targeted amplified polymorphism (ITAP), a new sequence related amplified polymorphism-based technique for generating molecular markers in higher plant species. Plant Omics. 2013; 6(2):128–34.

46. Paux E, Sourdille P, Salse J, Saintenac C, Choulet F, Leroy P, et al. A physical map of the 1-gigabase bread wheat chromosome 3B. Science. 2006; 322(5898):101–4. https://doi.org/10.1126/science.11161847 PMID: 18832645

47. Ravel C, Prud S, Canaguier A, Dufour P, Giancola S, Balfourier F, et al. DNA sequence polymorphisms and their application to bread wheat quality. Euphytica. 2006; 132(3):695–704. https://doi.org/10.1007/s10681-006-9288-z

48. Zhang W, Gianibelli MC, Ma W, Rampling L, Gale KR. Identification of SNPs and development of allele-specific PCR markers for gamma-gliadin alleles in Triticum aestivum. Theor Appl Genet. 2003; 107(1):130–8. https://doi.org/10.1007/s00122-003-1223-2 PMID: 12712246

49. Liu Y, He Z, Appels R, Xia X. Functional markers in wheat: current status and future prospects. Theor Appl Genet. 2012; 125(1):1–10. https://doi.org/10.1007/s00122-012-1829-3 PMID: 22366867

50. Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. 2009; 27(11):1025–31. https://doi.org/10.1038/nbt.1583 PMID: 19881494

51. Liu S, Kandoth PK, Warren SD, Yeckel G, Heinz R, Alden J, et al. A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. Nature. 2012; 492(7428):256–60. https://doi.org/10.1038/nature11651 PMID: 2235880

52. Andersen JR, Lubberstedt T. Functional markers in plants. Trends Plant Sci. 2003; 8(11):554–60. https://doi.org/10.1016/j.tplants.2003.09.010 PMID: 14607101

53. Liu Y, He Z, Appels R, Xia X. Functional markers in wheat: current status and future prospects. Theor Appl Genet. 2012; 125(1):1–10. https://doi.org/10.1007/s00122-012-1829-3 PMID: 22366867

54. Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. 2009; 27(11):1025–31. https://doi.org/10.1038/nbt.1583 PMID: 19881494

55. Chen C, Bowman KD, Choi YA, Dang PM, Rao MN, Huang S, et al. EST-SSR genetic maps for rice (Oryza sativa L.). Theor Appl Genet. 2007; 110(7):107–18. https://doi.org/10.1007/s00122-003-1223-2 PMID: 12712246

56. Liu Y, He Z, Appels R, Xia X. Functional markers in wheat: current status and future prospects. Theor Appl Genet. 2012; 125(1):1–10. https://doi.org/10.1007/s00122-012-1829-3 PMID: 22366867

57. Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, et al. Microdroplet-based PCR enrichment for large-scale targeted sequencing. Nat Biotechnol. 2009; 27(11):1025–31. https://doi.org/10.1038/nbt.1583 PMID: 19881494

58. Ming T, Zhao L, Zhang L, Wang Y, Cui L, Tang Y, et al. Molecular cloning and characterization of 4-hydroxyphenylpyruvate dioxygenase gene from Lactuca sativa. J Plant Physiol. 2011; 168(10):1076–83. https://doi.org/10.1016/j.jplph.2010.12.017 PMID: 21349599
[57.] Crowell EF, McGrath JM, Douches DS. Accumulation of vitamin E in potato (Solanum tuberosum) tubers. Transgenic Res. 2008; 17(2):205–17. https://doi.org/10.1007/s11248-007-9091-1 PMID: 17415670

[58.] Lee K, Lee SM, Park S, Jung J, Moon JM, Cheong JJ, et al. Overexpression of Arabidopsis homogentisate phytyltransferase or tocopherol cyclase elevates vitamin E content by increasing γ-tocopherol level in lettuce (Lactuca sativa L.). Mol Cells 2007; 24:301–6. PMID: 17978586.

[59.] Cahoon EB, Hall SE, Ripp KG, Ganzke TS, Hitz WD, Coughlan SJ. Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. Nat Biotechnol. 2003; 21(9):1082–7. https://doi.org/10.1038/nbt853 PMID: 12897790

[60.] Zhang G, Liu R, Zhang P, Xu G, Zhu J, Liang G, et al. Increase of tocotrienol content by overexpression of OsHGGT gene in transgenic rice. Biotechnol Bulletin. 2012;(8):64–70.

[61.] Xu M, Zhou J, Zhang L, Fan Y, Wang L. Cloning of γ-tocopherol methyltransferase (GmVTE3) gene and engineering vitamin E content in seed of transgenic tobacco. Scientia Agricultura Sinica. 2010; 43(10):1994–9. https://doi.org/10.3864/j.issn.0578-1752.2010.10.003

[62.] Sattler SE, Cahoon EB, Coughlan SJ, DellaPenna D. Characterization of tocopherol cyclases from higher plants and cyanobacteria. Evolutionary implications for tocopherol synthesis and function. Plant Physiol. 2003; 132(4):2184–95. https://doi.org/10.1104/pp.103.024257 PMID: 12913173

[63.] Bergmüller E, Porfirova S, Dörmann P. Characterization of an Arabidopsis mutant deficient in γ-tocopherol methyltransferase. Plant Mol Biol. 2003; 52:1181–90.

[64.] Fritzche S, Wang X, Li J, Stich B, Kopisch-Obuch FJ, Endrigkeit J, et al. A candidate gene-based association study of tocopherol content and composition in rapeseed (Brassica napus). Front Plant Sci. 2012; 3:129. https://doi.org/10.3389/fpls.2012.00129 PMID: 22740840

[65.] Li Q, Yang X, Xu S, Cai Y, Zhang D, Han Y, et al. Genome-wide association studies identified three independent polymorphisms associated with alpha-tocopherol content in maize kernels. PLoS One. 2012; 7(5):e36807.

[66.] Xiao Y, Yu Y, Li G, Xie L, Guo X, Li J, et al. Genome-wide association study of vitamin E in sweet corn kernels. The Crop Journal. 2020; 8(2):341–50. https://doi.org/10.1016/j.cj.2019.08.002

[67.] Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 1980; 8:4321–6. https://doi.org/10.1093/nar/8.19.4321 PMID: 7433111

[68.] Christie N, Tobias PA, Naidoo S, Kulheim C. The Eucalyptus grandis NBS-LRR gene family: physical clustering and expression hotspots. Front Plant Sci. 2015; 6:1238. https://doi.org/10.3389/fpls.2015.01238 PMID: 26793216

[69.] Hirsch CN, Foerster JM, Johnson JM, Sekhon RS, Muttoni G, Vaillancourt B, et al. Insights into the maize pan-genome and pan-transcriptome. Plant Cell. 2014; 26(1):121–35. https://doi.org/10.1105/tpc.113.119992 PMID: 24488960

[70.] Prosdocimi F, Faria-Campos AC, Peixoto F, Pena SDJ, Ortega JM. Clustering of Schistosoma mansoni mRNA sequences and analysis of the most transcribed genes: implications in metabolism and biology of different developmental stages. Mem Inst Oswaldo Cruz. 2002; 97:61–9. https://doi.org/10.1590/s0074-02762000000900014 PMID: 12426597

[71.] Radakovits R, Jinkerson RE, Fuerstenberg SI, Tae H, Settlage RE, Boore JL, et al.Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropsis gaditana. Nat Commun. 2012; 3:686. https://doi.org/10.1038/ncomms1688 PMID: 22353717

[72.] Conesa A, Madrigal P, Tarazona S, Gomez-Cabrer o D, Cervera A, McPherson A, et al. A survey of best practices for RNA-seq data analysis. Genome Biol. 2016; 17:13. https://doi.org/10.1186/s13059-016-0881-8 PMID: 26813401

[73.] Zheng X, Xu H, Ma X, Zhan R, Chen W. Triterpenoid sapo nins biosynthetic pathway profiling and candidate gene mining of the Ilex asprella root using RNA-Seq. Int J Mol Sci. 2014; 15(4):5970–87. https://doi.org/10.3390/ijms15045970 PMID: 24722569

[74.] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30(15):2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24965404

[75.] Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008; 18(5):821–9. https://doi.org/10.1101/gr.074492.107 PMID: 18349386

[76.] Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

[77.] Bast F. Sequence similarity search, multiple sequence alignment, model selection, distance matrix and phylogeny reconstruction. Nature Protocol Exchange. 2013. https://doi.org/10.1038/protex.2013.065
Markers for vitamin E in oil palm

78. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007; 23(21):2947–8. https://doi.org/10.1093/bioinformatics/btm404 PMID: 17846036

79. Nei M, Kumar S. Phylogenetics and population genomics. Molecular evolution and phylogenetics. New York: Oxford University Press; 2000. p. 249–53.

80. Howe EA, Sinha R, Schlauch D, Quackenbush J. RNA-Seq analysis in MeV. Bioinformatics. 2011; 27(22):3209–10. https://doi.org/10.1093/bioinformatics/btr490 PMID: 21976420

81. Xiao Y, Yang Y, Cao H, Fan H, Ma Z, Lei X, et al. Efficient isolation of high quality RNA from tropical palms for RNA-seq analysis. Plant Omics. 2012; 5(6):584–9.

82. Giegerich R, Wheeler D. Pairwise sequence alignment. Sequence Comparison Computational Biology. 7. London: Springer; 2009. p. 35–62. https://doi.org/10.1007/978-1-84800-320-3

83. Liao W, Yang Y, Li Y, Wang G, Peng M. Genome-wide identification of cassava R2R3 MYB family genes related to abscission zone separation after environmental-stress-induced abscission. Sci Rep. 2016; 6:32006. https://doi.org/10.1038/srep32006 PMID: 27573926

84. Basiron Y. Palm oil production through sustainable plantations. Eur J Lipid Sci Tech. 2007; 109(4):289–95. https://doi.org/10.1002/elt.200600223

85. Ogan IM, Marie-Josée D, Michael N. Palm oil: processing, characterization and utilization in the food industry—A review. Food Bioscienc. 2015; 10:26–41. https://doi.org/10.1016/j.fbio.2015.01.003

86. Siles L, Cela J, Munne-Bosch S. Vitamin E analyses in seeds reveal a dominant presence of tocotrienols over tocopherols in the Arecaceae family. Phytochemistry. 2013; 95:207–14. https://doi.org/10.1016/j.phytochem.2013.07.008 PMID: 23920227

87. Riewe D, Kooi M, Liese J, Pfeiffer M, Lippmann R, Schmeichel J, et al. A tyrosine aminotransferase involved in tocopherol synthesis in Arabidopsis. Plant J. 2012; 71(5):850–9. https://doi.org/10.1111/j.1365-313X.2012.05035.x PMID: 22540282

88. Vidi PA, Karnwischer M, Baginsky S, Austin JR, Csucs G, Dormann P, et al. Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J Biol Chem. 2006; 281(16):12255–34. https://doi.org/10.1074/jbc.M511939200 PMID: 16414959

89. Wang M, Toda K, Block A, Maeda HA. TAT1 and TAT2 tyrosine aminotransferases have both distinct and shared functions in tyrosine metabolism and degradation in Arabidopsis thaliana. J Biol Chem. 2010; 285(10):7563–76. https://doi.org/10.1074/jbc.RA110.006539 PMID: 20630953

90. Zhang C, Cahoon RE, Hunter SC, Chen M, Han J, Cahoon EB. Genetic and biochemical basis for alternative routes of tocotrienol biosynthesis for enhanced vitamin E antioxidant production. Plant J. 2013; 73(4):628–39. https://doi.org/10.1111/tpj.12067 PMID: 23137278

91. Chaudhary N, Khurana P. Vitamin E biosynthesis genes in rice. Molecular characterization, expression profiling and comparative phylogenetic analysis. Plant Sci. 2009; 177(4):628–39. https://doi.org/10.1016/j.plantsci.2009.07.014

92. Matsuzuka K, Kimura E, Nakagawa K, Murata K, Kimura T, Miyazawa T. Investigation of tocotrienol biosynthesis in rice (Oryza sativa L.). Food Chem. 2013; 140(1–2):91–8. https://doi.org/10.1016/j.foodchem.2013.02.058 PMID: 23578619

93. Wang X, Song Y, Li J. High expression of tococromanol biosynthesis genes increases the vitamin E level in a new line of giant embryo rice. J Agric Food Chem. 2013; 61(24):5860–9. https://doi.org/10.1021/jf401325e PMID: 23738742

94. Hu L, Lv S, Xu H, Li L, Wang P, Li Y. Genome-wide identification and analysis of genes involved in vitamin E biosynthesis pathway in Soybean. International Conference on Remote Sensing, Environment and Transportation Engineering; Nanjing, China 2011. p. 7610–3.

95. Vinutha T, Bansal N, Kumari K, Prashat GR, Sreevathsra R, Krishnan V, et al. Comparative analysis of tocopherol biosynthesis genes and its transcriptional regulation in soybean seeds. J Agric Food Chem. 2017; 65(50):11054–64. https://doi.org/10.1021/acs.jafc.7b03448 PMID: 29121768

96. Hunter CT, Saunders JW, Magallanes-Lundback M, Christensen SA, Willett D, Stinard PS, et al. Maize wz disrupts homogentisate solanesyl transferase (ZmHst) and reveals a plastoquinone-9 independent path for phytone desaturation and tocopherol accumulation in kernels. Plant J. 2018; 93(5):799–813. https://doi.org/10.1111/tpj.13821 PMID: 29315977

97. Xie L, Yu Y, Mao J, Liu H, Hu JG, Li T, et al. Evaluation of Biosynthesis, Accumulation and antioxidant activity of vitamin E in sweet corn (Zea mays L.) during kernel development. Int J Mol Sci. 2017; 18(12):2780. https://doi.org/10.3390/ijms18122780 PMID: 29261149

98. Sandorf I, Hollander-Czytko H. Jasmonate is involved in the induction of tyrosine aminotransferase and tocopherol biosynthesis in Arabidopsis thaliana. Planta. 2002; 216(1):173–9. https://doi.org/10.1007/s00425-002-0888-0 PMID: 12430028
99. Shintani D, DellaPenna D. Elevating the vitamin E content of plants through metabolic engineering. Science. 1998; 282(5396):2098–100. https://doi.org/10.1126/science.282.5396.2098 PMID: 9851934

100. Iqbal SZ, Mustafa HG, Asi MR, Jinap S. Variation in vitamin E level and aflatoxins contamination in different rice varieties. J Cereal Sci. 2014; 60(2):352–5. https://doi.org/10.1016/j.jcs.2014.05.012

101. Muzhingi T, Palacios-Rojas N, Miranda A, Cabrera ML, Yeum KJ, Tang G. Genetic variation of carotenoids, vitamin E and phenolic compounds in Provitamin A biofortified maize. J Sci Food Agric. 2017; 97(3):793–801. https://doi.org/10.1002/jsfa.7798 PMID: 27173638

102. Singh R, Low ET, Ooi LC, Ong-Abdullah M, Ting NC, Nagappan J, et al. The oil palm SHELL gene controls oil yield and encodes a homologue of SEEDSTICK. Nature. 2013; 500(7462):340–4. https://doi.org/10.1038/nature12356 PMID: 23883930

103. Yundaeng C, Somta P, Tangphatsomruang S, Chankaew S, Srinives P. A single base substitution in BADH/AMADH is responsible for fragrance in cucumber (Cucumis sativus L.), and development of SNAP markers for the fragrance. Theor Appl Genet. 2015; 128(9):1881–92. https://doi.org/10.1007/s00122-015-2554-5 PMID: 26081947