# NOVEL PLANT TRANSFORMATION VECTORS CONTAINING *γ-TMT* AND *FAD2* GENE

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## ABSTRACT

*Fatty acids are the main groups of components of plant membrane lipid and seed storage lipid, and the major source of energy in plant. Tocopherols are essential micronutrients for humans and animals, with several beneficial effects in plants. According to bioinformation analysis of the cDNA sequence, a specific fragment of FAD2 from immature maize embryos and γ-TMT gene from Perilla frustescens leaf were isolated by RT-PCR. Results of sequence analysis indicate that FAD2 fragments are contain open reading frame of 1,236 bp long coding for a 46 kD polypeptide, and γ-TMT fragments are contain open reading frame of 894 bp long coding for a 34 kD polypeptide. In this study, we developed two plasmids designated pBI121-TMT and pCAMBIA1301S-FAD2 that incorporate modified and improved expression omega-3 and vitamin E content in seeds of the plant transformation. The FAD2 and γ-TMT genes of each plasmid were driven by the constitutive CaMV 35S promoter which is mostly used for driving trangene expressions in both* monocot *and* dicot plant transformation.

***Keywords:*** *FAD2* gene; *Perilla frustescens*; *γ-TMT*

## INTRODUCTION

Vitamin E is an essential nutritional element for both humans and animals and therefore must be adequately provided by foods or other supplements. Vitamin E is a collective term for unsaturated α-,β-,γ- and δ-tocopherols and tocotrienols (Eitenmiller 1997). α-tocopherol is considered to be the most important form of vitamin E for human or animal health (Traber and Sies, 1996) since α-tocopherol has the highest vitamin E activity among tocopherols. Tocopherol has received little attention since the discovery of vitamin E. Recently more studies indicate that γ-tocopherol may also be important to human health and that it possesses unique features that distinguish it from α-tocopherol, such as anti-inflammatory and anticancer activity. However, the bioavailability and bioactivity of γ-tocopherol are lower than those of α-tocopherol (Jiang and Ames 2003; Jiang *et al.*, 2009). Thus, α-tocopherol is expected to be more potent antioxidant than either β- or γ-tocopherol. The relative activity or potency of β-, γ- and δ-tocopherols to α-tocopherol is estimated to be 0.5, 0.1 and 0.03, respectively, in human (Kamal-Eldin and Appelqvist, 1996). The major sources of vitamin E for human or animal consumption are from plants, but the concentration of vitamin E varies depending on plant species. Of various plant species, oil crop plants generally contain high levels of tocopherols in their seeds. However, most common oilseeds contain relatively higher levels of δ-tocopherol, a precursor of α-tocopherol than α-tocopherol itself (Shintani

and DellaPenna, 1998; Grusak, 1999). Therefore, humans or animals would have more efficient intake if γ-tocopherol could be metabolically converted to α-tocopherol (Shintani and DellaPenna, 1998). Plant transgenic technology has been quite successful in introducing and subsequently changing the tocopherol composition by overexpressing the *γ-TMT* gene to convert the precursor γ-tocopherol to α-tocopherol (Shintani and DellaPenna 1998; Kim *et al.,* 2000; Cho *et al*., 2005). Shintani and DellaPenna (1998) reported that the seeds of transgenic *Arabidopsis* overexpressing *γ-TMT* driven by the carrot DC3 seed specific promoter showed an 80 fold increase in α-tocopherol levels as compared with the wildtype control. It was also reported that many useful crops with high α-tocopherol content could be developed by introducing this gene. Cho *et al*. (2005) and Kim *et al.* (2000) developed transgenic lettuce (*Latuca sativa* L.) with high level of α-tocopherol by overexpressing a cDNA encoding the *γ-TMT* gene from *Arabidopsis thaliana*. Recently it was shown that transgenic soybean (Tavva *et al.,* 2007) and transgenic *Brassica juncea* (Yusuf and Sarin, 2007) with increased α-tocopherol content were developed by expressing a perilla *γ-TMT* gene. *α-TMT* genes encoding γ-tocopherol methyl-transferase have been previously cloned from different plant species such as *Arabidopsis thaliana* (Shintani and DellaPenna, 1998) and *Perilla frustescens* (Kim *et al.,* 2002). Therefore, it should now be possible to convert γ- tocopherol to α-tocopherol by overexpression of a *γ-TMT* gene under the control of a seed-specific promoter in the seeds of engineered crops.

Fatty acids are the main components of plant membrane lipid and seed storage lipid, and the major source of energy in plant. The large variety of fatty acids in nature plays a vital physiological role and possesses high food and industrial value (Topfer *et al.*, 1995; Ohlrogge and Browns 1995). More studies have shown that monounsaturated fatty acids (such as oleic acid) are superior to polyunsaturated fatty acids in physiological activity and oxidative stability (Pattee *et al.*, 2002). Nutritionally, edible oil with high oleic acid content can lower total serum cholesterol by lowering the level of the undesirable low-density lipoprotein cholesterol and not reducing the desirable high-density lipoprotein cholesterol (Lapointe *et al.,* 2006; Colomer and Menendez 2006). The production of soybeans containing low linolenic acid levels in the oil fraction is desirable for the demands of modernmarkets. Through mutagenesis, a set of soybean lines has been developed with lowered seed linolenic acid levels, and these lines have mutations at the *fan* locus (Wilcox and Cavins 1987; Rennie and Tanner, 1991; Rahman *et al.,* 1996). Other lines have been described in which lower linolenic acid phenotypes were generated, and the inheritance was characterized as multigenic (Fehr *et al.,* 1992; Rahman *et al.,* 1997; Rahman *et al.,* 1998; Takagi *et al.,* 1999; Ross *et al.,* 2000). Omega-3 fatty acid desaturase enzymes introduce the third double bond into linoleic acid precursors to produce linolenic acid precursors. Nuclear-encoded, chloroplast-targeted omega three fatty acid desaturases may also contribute to seed linolenic acid levels (Yadav *et al.,* 1993). Omega-3 fatty acid desaturases are members of an enzyme family characterized by the presence of a diiron cofactor which interacts with three regions of conserved histidine motifs in the protein (Shanklin *et al.,* 1994). Omega-3 fatty acid desaturases catalyze a third double bond into linoleic acid precursors to produce linolenic acid. Both chloroplast-targeted and microsomal omega-3 fatty acid desaturases have been identified in plants, but the microsomal enzymes have been shown to be the major contributors to seed linolenic acid levels (Yadav *et al.,* 1993).

In this study, the full-length cDNA of *FAD2* and *γ-TMT* were obtained from maize, *Perilla frustescens.* Here we show that it was undertaken to analyze characterization of maize *FAD2* and *γ-TMT* gene. The protein was also expressed in *E. coli* and observed. Furthermore, we developed novel plasmids contain *FAD2* and *γ-TMT* genes were driven by the constitutive CaMV 35S promoter that more useful form of the plant transformation.

## MATERIALS AND METHODS

### RNA isolation, reverse transcriptase reactions PCR

For *FAD2* gene amplification, total RNA isolated from high oil maize (*Zea mays*) inbred line 4K261 was used for 1st strand cDNA synthesis reaction by using PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. RNA samples (0.5-1g) were vacuum dried and used for RT (reverse transcriptase) reactions. 10 µL of PCR mixture containing 5 µL of Premix Taq Version 2.0 (TaKaRa), 1.0 µL of dNTP (10 mmol/L), 20 pmol of forward primer P1ZmFAD2, 20 pmol of reverse primer P2ZmFAD2 (Table 1) and 5 ng of template DNA were used. The experimental conditions of RT-PCR were 95ºC for 5 min, then 30 cycles of 95ºC 30 s, 60ºC 30 s, 72ºC 1.5 min, followed by a final extension at 72ºC for another 10 min.

For *γ-TMT* gene amplification, total RNA isolated from leaves of *Perilla frustescens* was used for 1st strand cDNA synthesis reaction by using PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. RNA samples (0.5-1g) were vacuum dried and used for RT (reverse transcriptase) reactions. 10 µL of PCR mixture containing 5 µL of Premix Taq Version 2.0 (TaKaRa), 1.0 µL of dNTP (10 mmol/L), 20 pmol of forward primer P1PfTMT, 20 pmol of reverse primer P2PfTMT (Table 1) and 5 ng of template DNA were used. The experimental conditions of RT-PCR were 95ºC for 5 min, then 30 cycles of 95ºC 30 s, 55ºC 30 s, 72ºC 1.5 min, followed by a final extension at 72ºC for another 10 min.

**Table 1.** Primers used in this study

| Primers | Sequences (5'-3') |
|---|---|
| P1PfTMT | CCATGGCGGAGATGGAGACGGAGATGGAG |
| P2PfTMT | CTCGAGAGATGCAGGTTTTCGGCATGTA |
| P3PfTMT[a] | CCCGGGTGGTCAGTCCCTTATGGCGGAGATGGAGACGGA |
| P4PfTMT[b] | GAGCTCTTAAGATGCAGGTTTTCGGCATGTAATG |
| P7PfTMT[c] | GAGCCCAACAGCTTGCTGATGCTCAAGGATT |
| P8PfTMT[d] | GTCGACTTAAGATGCAGGTTTTCGGCATGTAATG |
| P1ZmFAD2 | TATTATATGGGTGCCGGCGGCAGGATGACCGAG |
| P2ZmFAD2 | GCGCGCTAGAACTTCTTGTTGTACCAGAAGACG |
| P3ZmFAD2[a] | CCCGGGTGGTCAGTCCCTTATGGGTGCCGGCGGCAGG |
| P4ZmFAD2[b] | GAGCTCTTACTAGAACTTCTTGTTGTACCAGAAGACG |
| P5ZmFAD2[c] | GGATCCGATGGGTGCCGGCGGCAGG |
| P6ZmFAD2[d] | GTCGACTTACTAGAACTTCTTGTTGTACCAGAAGACG |
| P7ZmFAD2[e] | TTACGAATTCCCATGGAGTCAAAGATTCAAATAGAGG |
| P8ZmFAD2[e] | GAATTCAAGCTTGGACAATCAGTAAATTGAACGGAGA |

[a] The *Sma*I restriction site is underlined; [b] The *Sac*I restriction site is underlined
[c] The *Bam*HI restriction site is underlined; [d] The *Sal*I restriction site is underlined
[e] The *Eco*RI restriction site is underlined

### Transformation plasmid into DH5a, BL21 strain of *E. coli*

Amplification products were fractionated on 1% agarose gel from which the selected band was purified. The amplified DNA was inserted into the pMD19-T vector (TaKaRa) and transformed into *E. coli* DH5α. One hundred microlitres of bacteria stock solution was mixed with 100ng plasmid, chilled on ice for 30 min and put in 42ºC water bath for 2 min. The mixture was put immediately on ice again for about 2 min, incubated in LB broth in shaking incubator (160 rpm) for 60 min at 37ºC and streaked onto different LB agar plates, each containing ampicillin (for DH5a) or kanamycin (for BL21) at a concentration of 100 mg/ml. The plates

were incubated overnight at 37ºC and analysed. The positive plaques were identified by PCR and then sequenced by Jieli Biotech (Shanghai, China). The negative control would be untransformed *E. coli* strain. The bacteria plate was sub-cultured once every week to make sure that the bacteria were fresh before every plasmid extraction process.

**Culture of *E. coli* in LB broth**

A colony of the transformed *E. coli* was picked and mixed into antibiotic containing LB broth. (in g/L: tryptone 10.0, yeast extract 5.0, NaCl 5.0; pH 7.2– 7.5) at 37ºC on orbital shaker (180 rpm) for 12–16 h. In the case of the recombinant strains, LB medium was supplemented with ampicillin (100 mg/mL) for DH5a strain or kanamycin (100 mg/mL) for BL21 strain. Plates containing LB medium supplemented with agar (16 g/L) were incubated at 37ºC for 12–16 h. The broth was then incubated in shaking incubator (150 rpm) at 37ºC overnight prior to be used for plasmid DNA extraction.

**Expression *FAD2* and *γ-TMT* gene in *E. coli* BL21**

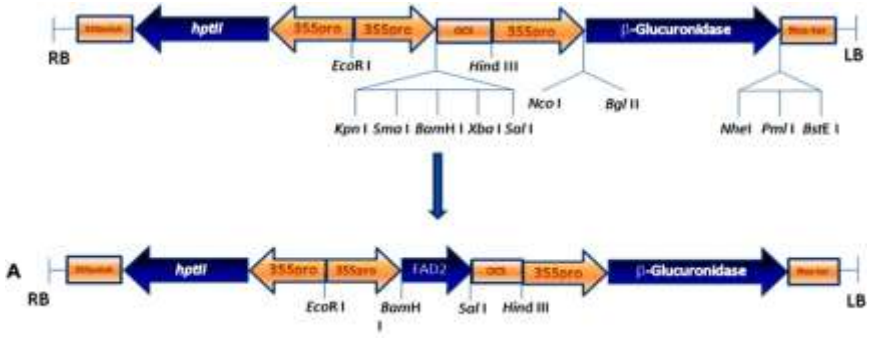
The full-length cDNA of *FAD2* and *γ-TMT* were amplified by PCR with forward primer P5ZmFAD2/P7PfTMT and the reverse primer P6ZmFAD2/P8PfTMT (Table 1). The fragments were digested by the *Bam*HI and *Sal*I enzymes and then ligated into the pET28a vector, generating the plasmid pET-TMT and pET-FAD2. The transformants harboring plasmid pET-TMT and pET-FAD2 were cultured at 37ºC in LB medium until $OD_{600nm}$ reached 0.5-0.7. IPTG was added to final concentration of 0.5 mmol/L and the cultivation was continued for another 4 hours at 37ºC. The cell was harvested by centrifugation. The protein concentration was measured by Bio-Rad Protein Assay. Total bacterial protein was run 12% SDS-PAGE.

**Vector construction**

The full-length cDNA of *FAD2* was amplified by PCR using the forward primer: P3ZmFAD2 and the reverse primer: P4ZmFAD2 (Table 1). The *FAD2* fragment was digested by the *Bam*HI and *Kpn*I enzymes, inserted between the cauli-flower mosaic virus (CaMV) 35S promoter and *A. tumefaciens* octopine synthase (OCS) terminator sequences (Barker *et al.*, 1983) and then ligated into the pCAMBIA1301S vector driven by cauli-flower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator (Nos polyA). The resulting plasmids were designated as pCAMBIA1301S-FAD2 (Fig. 2A)

The full-length cDNA of *γ-TMT* was amplified by PCR using the forward primer: P3PfTMT and the reverse primer: P4PfTMT (Table 1). The binary vector pBI121 (Jefferson *et al.*, 1987) contains an nptII gene as a selective marker and a *GUS* gene under the control of a cauliflower mosaic virus (CaMV) 35S promoter. Plasmid pBI-TMT (Fig. 2B) was produced by removing the GUS sequence from pBI121 by digestion with *Sma*I and *Sac*I and *γ-TMT* fragment was cloned into the binary vector pBI121, generating the plasmid pBI121-TMT.

We continued to modify the pCAMBIA1301S-FAD2 vector. The first, 35S promoter-TMT-Nos was amplified by PCR from as pBI121-TMT plasmid using the forward primer P7ZmFAD2 (Table 1) corresponding to the 5’ coding region of Cauliflower mosaic virus 35S promoter and reverse primer P8ZmFAD2 (Table 1) corresponding to the 5’ coding region nopaline synthase terminator (Nos-ter) of the *γ-TMT* gene.



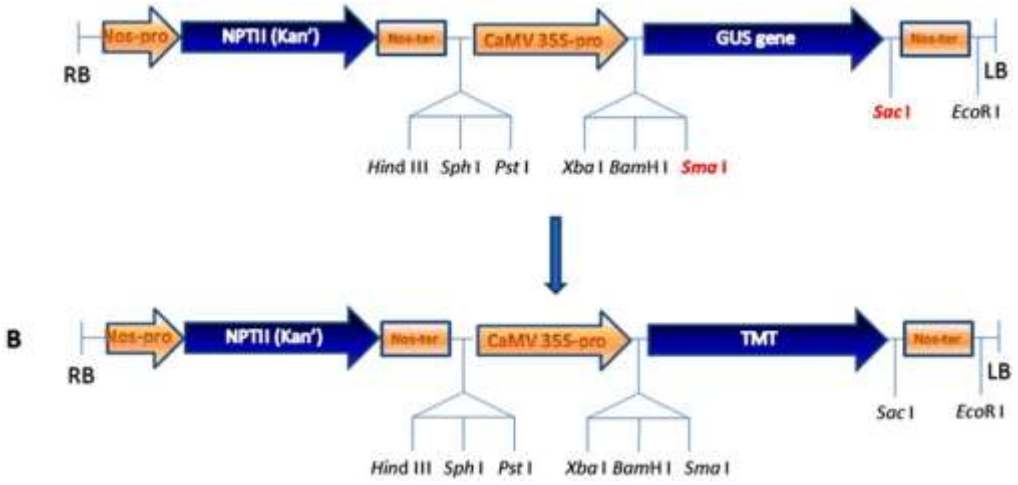



**Fig. 2:** Schematic diagram of the binary vectors **(A)** The binary vectors pCAMBIA1301-FAD2 (13,913 bp) were constructed by ligation of *FAD2* sequencing at the *Bam*HI and *Sal*I sites of binary vector pCAMBIA1301 vector. The binary vector pCAMBIA1301-FAD2 contained the *FAD2* gene, β-Glucuronidase and *hptII* gene. The *FAD2* gene was driven by the constitutive CaMV 35S promoter. OCS octopine synthase terminator, *35SpolyA* cauliflower mosaic virus 35S terminator, Nos-ter nopaline synthase terminator, RB right border, LB left border. **(B)** The binary vectors pBI121-TMT (7,425bp) were constructed by ligation of *γ-TMT* sequencing at the *Sma*I and *Sac*I sites of binary vector pBI121. The binary vector pBI121-TMT contained the *γ-TMT* gene and *nptII* gene. The *γ-TMT* gene was driven by the constitutive CaMV 35S promoter. Nos-pro nopaline synthase promoter, Nos-ter nopaline synthase terminator, RB right border, LB left border.

## RESULTS AND DISCUSSION

### Characterization of maize *FAD2* gene

RT-PCR products only by primers P1ZmFAD2/P2ZmFAD2 were obtained from immature embryos. Then PCR products (Fig. 3) of the same length were amplified from the maize genome. The sequence of cloned *FAD2* cDNA of maize has about 89–99% homology to unidentified mRNA sequences from *Zea mays* and *Oryza sativa* with BLASTN searches. Several pairs of primers were designed according to the cDNA sequences of 5'-end and 3'-end translated regions. Analysis of the sequences showed that all of them were 1236 bp and the sequences were consistent, indicative of no intron in the *FAD2* gene. These differences may be the results either of amplification artifacts resulting from the inherent inaccuracy of DNA polymerase, or of some sequencing error. The open reading frame of 1236 bp fragment encodes a predicted peptide of 411 amino acid residues and has three highly conserved histidine motifs. Alignment of the deduced amino acid sequences of plants *FAD2* in Fig. 3 shows an identity of about 70% between the predicted amino acid sequences derived from maize *FAD2* gene and *Arobidopsis*, *Glycine max*, *Brassica napus*, and *Gossypium hirsutum FAD2* genes. *FAD2* shares only approximately 33% identity to *FAD7* and *FAD8* in maize (data not show) (Berberich *et al*., 1998).

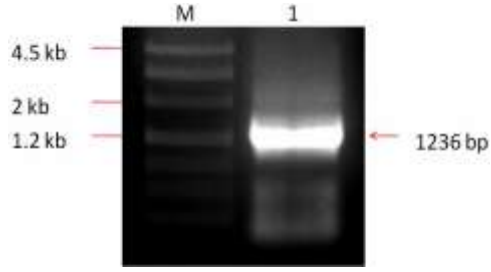



**Fig. 3:** PCR amplification of *FAD2* gene from maize genomic DNA. M: marker IV; 1: PCR product (1236bp)

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Arabidopsis     MGAGGRMFVPTSSKKSETD------TTKRVPCEKPPFSVGDLKKAIPPHCFKRSIPRSFS 54
Brassica        MGAGGRMQVSPPSKKSETD------TIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFS 54
Gossypium       MGAGGRMSVPPSQRKQESG------SMKRVPISKPPFTLSEIKKAIPPHCFQRSLIRSFS 54
Glycine         MGAGGRTDVPPANRKSEVD------PLKRVPFEKPQFSLSQIKKAIPPHCFQRSVLRSFS 54
Zea             MGAGGRMTEKEREKQEQLARATGGAAMQRSPVEKPPFTLGQIKKAIPPHCFERSVLKSFS 60
                ******      .::.:        . :* * ..* *::.::*********:**: :***

Arabidopsis     YLISDIIIASCFYYVATNYFSLLPQPLSYLAWPLYWACQGCVLTGIWVIAHECGHHAFSD 114
Brassica        YLIWDIIIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSD 114
Gossypium       YLVYDFILVSIFYYVATTYFHNLPQPLSFVAWPIYWTLQGSVLTGVWVIAHECGHHAFSD 114
Glycine         YVVYDLTIAFCLYYVATHYFHLLPGPLSFRGMAIYWAVQGCILTGVWVIAHECGHHAFSD 114
Zea             YVVHDLVIAAALLYFALAIIPALPSPLGYAAWPLYWIAQGCVCTGVWVIAHECGHHAFSD 120
                *:: *: :.  : *.*   :  ** **.: . .:**  **.: **:**************

Arabidopsis     YQWLDDTVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKQKSAIKWYGKYLNN 174
Brassica        YQWLDDTVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKSDIKWYGKYLNN 174
Gossypium       YQWIDDTVGLILHSSLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKRSSIRWWAKYLNN 174
Glycine         YQLLDDIVGLILHSALLVPYFSWKYSHRRHHSNTGSLERDEVFVPKQKSCIKWYSKYLNN 174
Zea             YSLLDDVVGLVLHSSLMVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKEALPWYTPYVYN 180
                *. :** ***::** *:*******************************::. : *:  *: *

Arabidopsis     -PLGRIMMLTVQFVLGWPLYLAFNVSGRPYDG-FACHFFPNAPIYNDRERLQIYLSDAGI 232
Brassica        -PLGRTVMLTVQFTLGWPLYLAFNVSGRPYDGGFACHFHPNAPIYNDRERLQIYISDAGI 233
Gossypium       -PPGRFVTVTIQLTLGWPLYLAFNVAGRPYEG-LACHYNPYGPIYNDRERLQIYISDVGV 232
Glycine         -PPGRVLTLAVTLTLGWPLYLALNVSGRPYDR-FACHYDPYGPIYSDRERLQIYISDAGV 232
Zea             NPVGRLVHIVVQLTLGWPLYLATNASGRPYPR-FACHFDPYGPIYNDRERAQIFVSDAGV 239
                 * ** : :.: :.******** *.:****   :***: * .***.**** **::**.*:

Arabidopsis     LAVCFGLYRYAAAQGMASMICLYGVPLLIVNAFLVLITYLQHTHPSLPHYDSSEWDWLRG 292
Brassica        LAVCYGLFRYAAAQGVASMVCFYGVPLLIVNGLLVLITYLQHTHPSLPHYDSSEWDWLRG 293
Gossypium       LAVTYGLYRLVLAKGLAWVICVYGVPLLIVNAFLVMITYLQHTHPALPHYDSSEWDWLRG 292
Glycine         LAVVYGLFRLAMAKGLAWVVCVYGVPLLVVNGFLVLITFLQHTHPALPHYTSSEWDWLRG 292
Zea             VAVAFGLYKLAAAFGVWWVVRVYAVPLLIVNAWLVLITYLQHTHPSLPHYDSSEWDWLRG 299
                :** :**:: . * *:  :: .*.****:**. **:**:******:**** *********

Arabidopsis     ALATVDRDYGILNKVFHNITDTHVAHHLFSTMPHYNAMEATKAIKPILGDYYQFDGTPWY 352
Brassica        ALATVDRDYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDGTPVV 353
Gossypium       ALATVDRDYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYSFDGTPVY 352
Glycine         ALATVDRDYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYRFDETPFV 352
Zea             ALATMDRDYGILNRVFHNITDTHVAHHLFSTMPHYHAMEATKAIRPILGDYYHFDPTPVA 359
                ****:********:*********************:********:****:** ** **
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**Fig. 4:** Alignment of *FAD2* protein sequences from Zea mays and four other organisms using ClustalW2 software using ClustalW2 software. The deduced amino acid sequences compared are from: *Arabidopsis FAD2* gene (L26296), *Brassica napus FAD2* cDNA (AF243045), *Gossypium hirsutum* (AF331163), *Glycine max FAD2* cDNA (L43921) and *Zea mays FAD2* gene (this paper)

**Characterization of *Perilla frustescens γ-TMT* gene**

The sequence of cloned *γ-TMT* cDNA of *Perilla frustescens* has about 89–99% homology to unidentified mRNA sequences from *Perilla frustescens* with BLASTN searches. Several pairs of primers were designed according to the cDNA sequences of 5'-end and 3'-end translated regions. RT-PCR products only by primers P1PfTMT/ P2PfTMT were obtained from leaf tissue. Then PCR products (Fig. 5) of the same length were amplified from the maize genome. Analysis of the sequences showed that all of them were 894 bp and the sequences were consistent, indicative of no intron in the *γ-TMT* gene. These differences may be the results either of amplification artifacts resulting from the inherent inaccuracy of DNA polymerase, or of some sequencing error. The open reading frame of 894 bp fragment encodes a predicted peptide of 298 amino acid residues and has three highly conserved histidine motifs. Alignment of the deduced amino acid sequences of plants *γ-TMT* gene in Fig. 6 shows an identity of about 72% between *Perilla frustescens γ-TMT* gene and *Arabidopsis*, 70% between *Perilla frustescens* γ-TMT and *Brassica oleracea γ-TMT*. The Glycine max *γ-TMT* protein shares a high degree of amino acid sequence similarity with *Perilla frustescens γ-TMT* (74%)

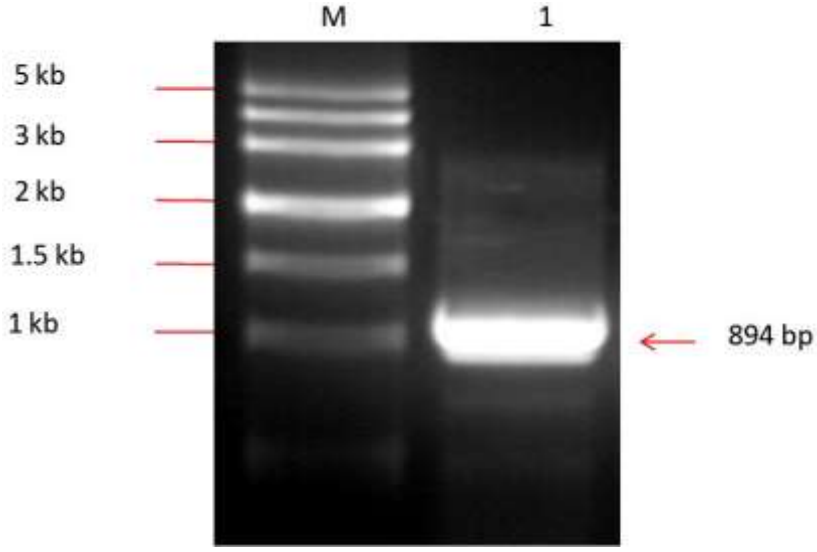


**Fig. 5:** PCR amplification of *γ-TMT* gene from *Perilla frustescens* genomic DNA. M: marker III; 1: PCR product (894 bp)

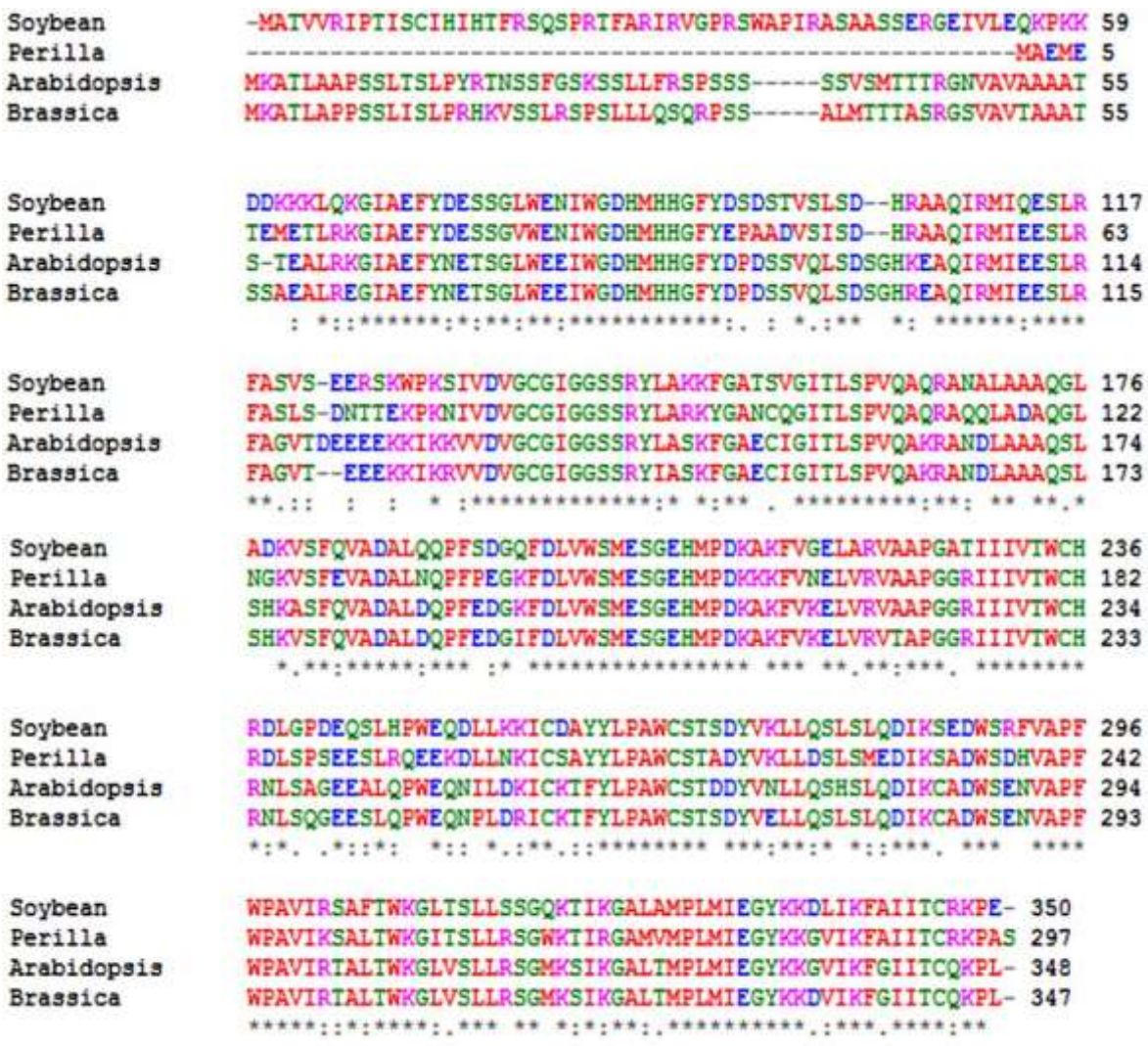


**Fig. 6:** Alignment of *γ-TMT* protein sequences from *Perilla frustescence*, *Brassica oleracea* and two other organisms using ClustalW2 software. The deduced amino acid sequences compared are from: *Arabidopsis thaliana γ-TMT* gene (AF104220), *Glycine max γ-TMT* (AY960126), *γ-TMT Brassica oleracea* (AF381248) and *γ-TMT Perilla frustescens* (this paper)

**Expression of *FAD2* and *γ-TMT* gene in *E. coli***

The deduced amino acid sequence of *FAD2* and *γ-TMT* indicates the presence of a putative plastidial transit peptide in the preprotein. The plastidial transit peptide induces the preprotein transporting into the plastids. The plastidial processing of the preprotein in removal of the targeting peptide is necessary to yeild a mature enzyme. This N-terminal signal sequence could affect the conformation of *γ-TMT* protein when expressed in *E. coli* and render the protein inactive. Therefore, a 894 bp sequence of *γ-TMT* was modified by PCR using a pair of primers P7PfTMT and P8PfTMT to produce a truncated protein (34 kD) devoid of a majority of the putative N-terminal plastidial signal sequence. Beside that, a 1236 bp sequence of *FAD2* was also modified by PCR using a pair of primers P5ZmFAD2 and P6ZmFAD2 to produce a truncated protein (46 kD) devoid of a majority of the putative N-terminal plastidial signal sequence.

In order study the possible function of *γ-TMT* and *FAD2*, the prokaryotic expression vectors pET-TMT and pET-FAD2 were constructed and transformed into *E. coli* BL21 (DE3). After induction at 37ºC for 4h with IPTG, a specified protein band of vector pET-TMT and pET-FAD2 were observation in 12% SDS-PAGE, which had the same molecular weight of the recombination protein with a 6xHis tag sequence of pET28a (Fig. 7A, lane 1 and Fig. 7B, lane 2), while negative control did not procedure this band (Fig. 7A, lane 2 and Fig. 7B, lane 1).

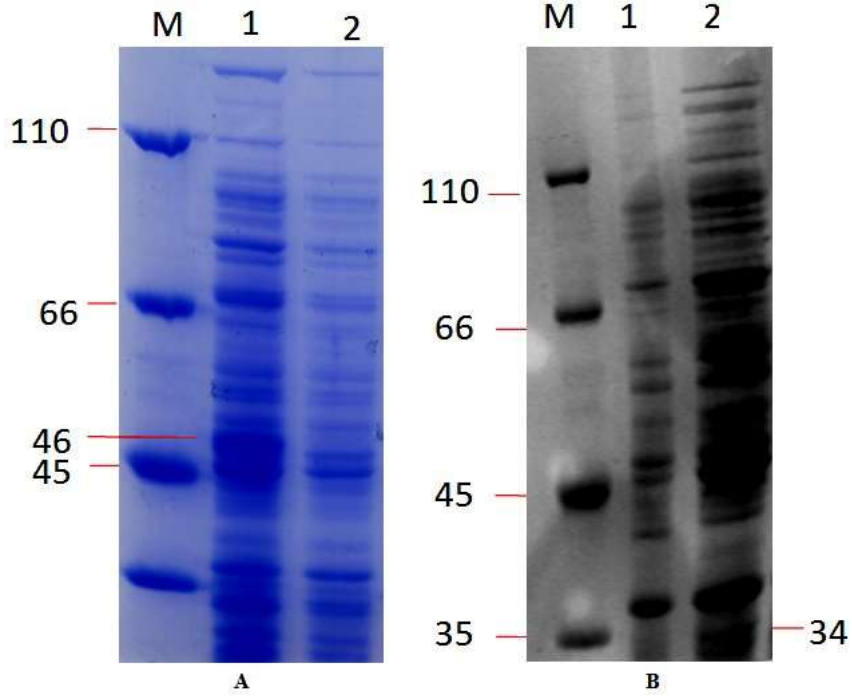


**Fig. 7:** Expression of the recombinant *FAD2* and *γ-TMT* in *E. coli* induced by IPTG (12% SDS-PAGE). (A) M, molecular marker, the sizes of marker are shown on the left; 1, total protein extracted from *E. coli* BL21 (DE3)/pET-FAD2; 2, total protein extracted from *E. coli* BL21 (DE3)/pET28a. (B) M, molecular marker, the sizes of marker are shown on the left; 1, total protein extracted from *E. coli* BL21 (DE3)/pET28a; 2, total protein extracted from *E. coli* BL21 (DE3)/pET-TMT

**Construction of plasmid vector pCAMBIA1301S-FAD2 and pCAMBIA1301S-TMT**

To provide researchers with convenient tools for various applications in plant gene expression, we constructed expression cassettes containing the *FAD2* and *γ-TMT* gene. The first binary express construct *FAD2* was generated by cloning *FAD2* fragment into the *Bam*HI and *Sal*I sites of the backbone vector pCAMBIA1301S to generate the plasmid pCAMBIA1301S-FAD2, respectively. The plasmid pCAMBIA1301S-FAD2 was generated by inserting *FAD2* fragment into a multiple cloning site (MCS) between cauli-flower mosaic virus (CaMV) 35S promoter and octopine synthase terminator (OCS), whose maps are shown in Fig 2a. This construct contain the *GUS* reporter gene under the control of CaMV 35S promoter, and the *Hpt* selection gene under the control of CaMV 35S promoter (Fig. 2a). We constructed a first cassette pCAMBIA1301S-FAD2, with the *FAD2* gene cloning from maize. Delta-12 fatty acid desaturase of *Arabidopsis* is encoded by a single *FAD2* gene in all kinds of tissues while there are two different cDNA sequences in soybean, *FAD2*-1 and *FAD2*-2, encoding microsomal delta-12 fatty acid desaturase. The *FAD2*-1 gene is strongly expressed in developing seeds only, while *FAD2*-2 gene is constitutively expressed in both vegetative tissues and developing seeds (Heppard *et al.*, 1996). In *Olea europaea*, the desaturase encoded by *FAD2*-1 is possibly responsible for the desaturation of reserve lipids in young seed, while the desaturase encoded by *FAD2*-2 may be involved mainly in storage lipid desaturation in mesocarp and maturing seeds (Hernandez *et al.,* 2005). In cotton, this enzyme is encoded by multiple genes expressed in different tissues (Liu *et al.*, 1999a, b; Pirtle *et al.*, 2001). Similar to Arabidopsis *FAD2*, soybean *FAD2-2*, and cotton *FAD2-2, FAD2-3*, and *FAD2-4*, maize *FAD2* gene is expressed in vegetative tissues and developing seeds, but it distinguishes itself as being strongly expressed in developing seeds. Lipid is an important component of the plant cell membrane. It will be interesting if other *FAD2* genes homologous to the cloned *FAD2* gene are also expressed in plant transformation.

The second binary express construct *γ-TMT* was generated by removing the *GUS* sequence from pBI121 by digestion with *Sma*I and *Sac*I and the *γ-TMT* fragment was cloned into the binary vector pBI121, respectively (Fig. 2B). The plasmid pBI121-TMT contains an *nptII* gene as a selective marker and a *γ-TMT* gene under the control of a cauliflower mosaic virus (CaMV) 35S promoter. We created a second cassette pBI121-TMT, containing a *Perilla frustescens γ-TMT* gene for the improvement of tocopherol composition in some of crop. γ-tocopherol methyltransferase, the final enzyme in the tocopherol pathway, uses S-adenosylmethionine (SAM) to generate α- and β-tocopherols from γ- and δ-tocopherols, respectively (d'Harlingue and Camara 1985; Hofius and Sonnewald 2003; Koch *et al.,* 2003). Consequently, seed specific overexpression of the *γ-TMT* gene could be a way to convert the large food pool of γ-tocopherol into α-tocopherol in soybean seed. Increases in α-tocopherol content in soybean seed have previously been demonstrated using the *γ-TMT* gene from the model plant *Arabidopsis thaliana* (At-VTE4) (Kim et al. 2005; Van Eenennaam *et al.,* 2003), and it has also been used to increase the α-tocopherol content in lettuce leaves (Cho *et al.,* 2005).

We therefore created two plasmid vectors, containing the *FAD2* gene and the *γ-TMT* gene following CaMV 35S promoter for improved expression omega-3 and vitamin E content in seeds of plant species. To obtain more information about the characteristics of the cloned maize *FAD2* gene and *Perilla frustescens γ-TMT* gene, we will perform the transformation in seeds of plant species and molecular characterization of the transgenes.

## CONCLUSION

*Zea maize FAD2* and *Perilla frustescens γ-TMT* gene, have been cloned successfully. The expression of the recombinant *FAD2* and *γ-TMT* gene in *E. coli* BL21(DE3) were finally obtained by SDS-PAGE. We developed two

plasmid vectors containing *FAD2* gene and *γ-TMT* gene following CaMV 35S promoter which is mostly used for driving trangene expressions in both dicot and monocot *plant transformation* for improved expression of omega-3 and vitamin E content in seeds of plant species.

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## TÓM TẮT

*Axit béo là thành phần chính của lipid màng tế bào trong cây và hạt, và là nguồn năng lượng chính của cây trồng. Tocopherols là vi chất dinh dưỡng cần thiết cho người và động vật, tham gia nhiều phản ứng tạo sản phẩm có lợi cho cây trồng. Kết quả phân tích chuỗi cDNA của gen FAD2 được ly trích từ phôi non hạt bắp và gen γ-TMT được ly trích từ lá non cây tía tô qua phương pháp RT-PCR. Kết quả phân tích trình tự gen cho thấy gen FAD2 chứa khung đọc mở dài 1.236 bp mã hóa chuỗi polypeptide 46 kD và gen γ-TMT chứa khung đọc mở dài 894 bp mã hóa chuỗi polypeptide 34 kD. Trong nghiên cứu này, chúng tôi đã thiết kế được hai plasmid pBI121-TMT và pCAMBIA1301S-FAD2 dùng cho chuyển nạp gen với mục đích gia tăng hàm lượng omega-3 và vitamin E trong cây trồng. Gen FAD2 và γ-TMT trong mỗi plasmid được điều khiển bởi promoter CaMV 35S-được sử dụng rộng rãi trong chuyển nạp gen cho cây một lá mầm và hai lá mầm.*

***Từ khóa:*** *FAD2 gene; Perilla frustescens; γ-TMT*