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Oil Palm *Phytochrome-Interacting Factor4* (*PIF4*) Gene is Conserved and Highly Expressed During Somatic Embryogenesis

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ABSTRACT

Oil palm is used in food, fuel and cosmetic industries. Tissue culture is the best way to propagate oil palm; unfortunately the somatic embryogenesis during tissue culture takes long time. The molecular mechanism of somatic embryogenesis in oil palm remains unknown. Recent research reported that auxin plays an important role in early and post-embryogenic plant. PHYTOCHROME-INTERACTING FACTOR4 (PIF4) regulates levels of auxin and the expression of key auxin biosynthesis genes. Our research aims to characterize oil palm *PIF4* gene. Thus, we cloned *EgPIF4*, analyzed the domain using bioinformatic and examined the expression of *EgPIF4* during somatic embryogenesis at different tissue including callus and somatic embryo stages; globular, torpedo, cotyledon, and plantlet stage using real-time PCR method. The result showed that *EgPIF4* gene comprised 1,737 bp with 9 exons, which encode 578 amino acid residuals. It contains a conserved domain called basic helix-loop-helix domain. *EgPIF4* has high level of expression at somatic embryogenetic stage specifically globular and torpedo stage suggested that *EgPIF4* plays an important role during somatic embryogenesis. The future characterization of *EgPIF4* function in oil palm will help to understand somatic embryogenesis process and facilitate the improvement of the oil palm tissue culture.

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) belongs to the Arecaceae family. It is the most important oil crop giving the highest yield per hectare among all oil crops in the world. Palm oil is the source for producing in several industries such as food, fuel and cosmetic. The plantation area in many countries including Indonesia, Malaysia, and Thailand is expanding because of the rapid increase in demand for the oil palm product (Aratrakorn *et al.* 2006). Tissue culture process are used to propagate high yielding oil palm to meet the demand. Oil palm tissue culture is composed of callus induction, somatic embryogenesis, maturation stage (shoot and root induction) and finally the regeneration of viable plantlets (Thuzar *et al.* 2011). Normally the production of oil palm by tissue culture technique takes at least one and a half years to obtain small plantlets (Konan *et al.* 2010). The longest stage is somatic embryogenesis stage which takes 7-8 months (Thuzar *et al.* 2011). Oil palm tissue culture has high economic importance but the biological mechanisms of changes associated with embryogenesis especially at molecular level still unknown.

The most widely used plant growth regulator to control callus induction or somatic embryogenesis induction is 2,4-dichlorophenoxyacetic acid (2,4-D) which is an auxin analog. It is well known that auxin is a key regulator controlling plant cell division and differentiation (Nic-Can and Loyola-Vargas 2016). Auxin plays an important role in early and post-embryogenic plant development (Cueva-Agila *et al.* 2016; Elhiti and Stasolla 2016). From recent researches, the levels of auxin were controlled by PHYTOCHROME-INTERACTING FACTOR4 (PIF4) transcription factor. PIF4 transcription factor regulates levels of auxin and the expression of key auxin biosynthesis gene at high temperature (Franklin *et al.* 2011). PIF4 and PIF5

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directly controlled auxin biosynthesis and signaling components to ultimately regulate stem growth of plants (Hornitschek *et al.* 2012). Furthermore, at an early stage of somatic embryogenesis, (*Gossypium hirsutum*) TEOSINTE BRANCHED1-CYCLOIDEA-PCF transcription factor15, GhTCP15, was phosphorylated. Phosphorylated GhTCP15 has an enhanced binding to promoter of *GhPIF4* to regulate the transcription of *GhPIF4*, so that regulating auxin biosynthesis. During later stage of somatic embryogenesis, GhTCP15 was reduced the phosphorylation activity, thereby decrease auxin biosynthesis, and promote somatic embryo formation (Min *et al.* 2015).

However, *PIF4* gene in oil palm has not been characterized. To understand how *PIF4* in oil palm controlling somatic embryogenesis during tissue process, we cloned *EgPIF4* gene and examined its expression in different stage of somatic embryogenesis using quantitative real-time PCR. We also investigated the relationship between oil palm and other plant species based on the sequence variation of *PIF4* gene. The information from this study will be a preliminary data to show that *EgPIF4* may have an important role during somatic embryogenesis stage in oil palm. The investigation and characterization of genes involved in the somatic embryogenesis during tissue culture process will facilitate the understanding of the mechanism and provide the useful information for oil palm improvement and hopefully lead to shortening the length of the oil palm tissue culture process.

2. Materials and Methods

2.1. Plant Materials

Young leaves of an oil palm Tenera clone from Golden Tenera plantation, Krabi, Thailand were used as an explant for tissue culture based on Thuzar *et al.* (2011). Oil palm tissues at different developmental stages including callus, somatic embryo at globular, torpedo, cotyledon, and plantlet were used in this study (Figure 1). These samples were collected and kept at -80°C for RNA extraction.

2.2. RNA Extraction and cDNA Synthesis

Total RNAs were isolated from oil palm tissue samples including callus, globular, torpedo, cotyledon, and plantlet using Spin Plant RNA (STRATEC Molecular, Germany). Quantity and quality

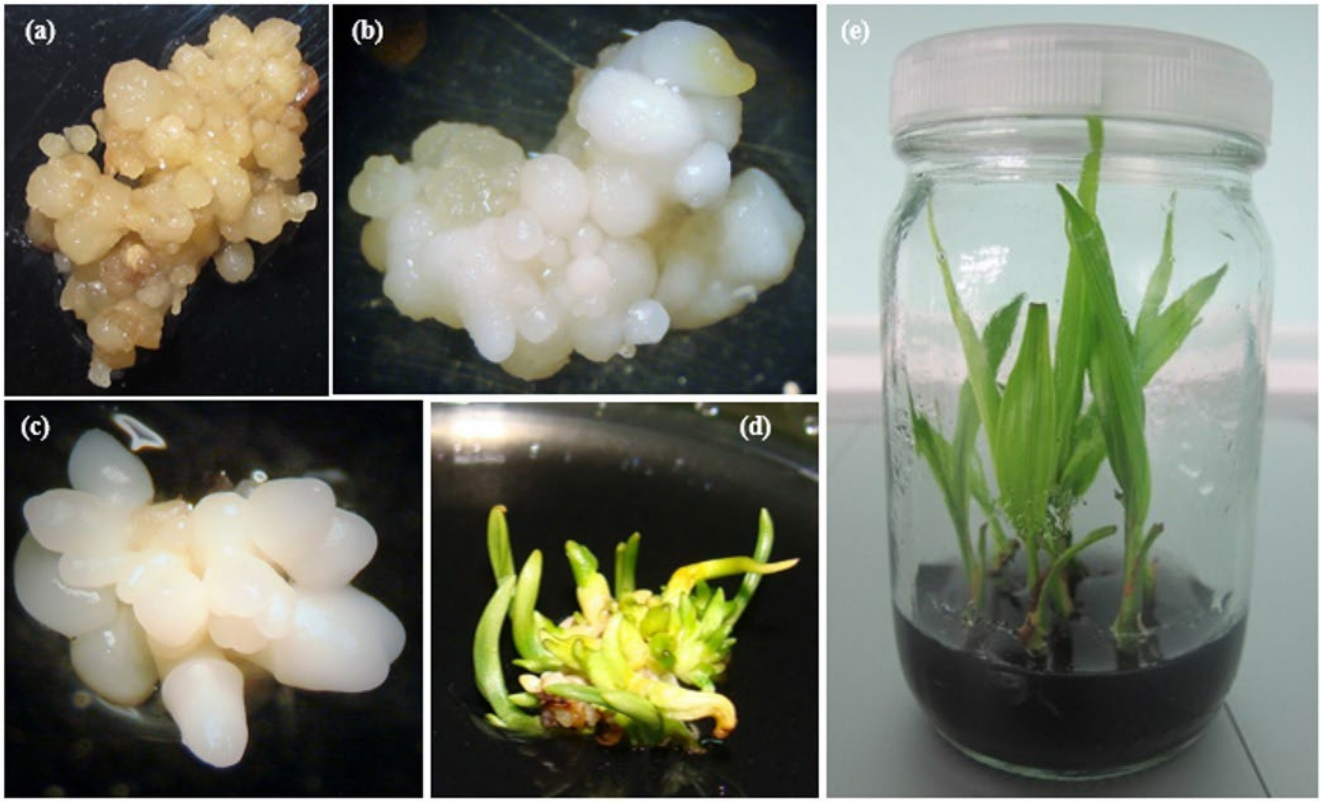


Figure 1. Tissue of oil palm during tissue culture; (a) callus, (b) globular stage, (c) torpedo stage, (d) cotyledonary stage, and (e) plantlet

of RNAs were assessed using Nanodrop (Thermo Scientific, USA) and agarose gel electrophoresis. The genome DNA was removed by RNAase-free DNAase, then Verso cDNA kit (Thermo Scientific, USA) was used for cDNA synthesis according to the manufacturer's instruction.

2.3. Cloning of *EgPIF4*

Predicted sequence of oil palm *PIF4* gene was downloaded from GenBank database (XM_010906351.1). Primer used for gene cloning were *EgPIF4_F* (5'-ATGAATCACTATGTTCCTCATTG-3') and *EgPIF4_R* (5'-TTATTGAGTTGAGGGGTACTGG-3'). The PCR was performed as follows: denaturing at 95°C for 5 minutes, followed by 35 cycles of amplification (95°C for 30 s, 56°C for 30 s, 72°C for 45 s) and extension at 72°C for 5 minutes (Vivantis, USA). The PCR product fragment was cut out from the gel, extracted and eluted using GF-1 AmbiClean Kit (Gel and PCR) (Vivantis, USA). Finally, the purified DNA was cloned into the pGEM-T easy vector (Promega, USA.). The recombinant vector was transformed to *E. coli* DH5α by heat shock method. Recombinant vector from the selected clones were sent out for sequencing at Macrogen, South Korea. The nucleotide sequences obtained from this experiment were annotated by using a BLAST program from the National Center for Biotechnology Information (NCBI) with non-redundant databases (Altschul *et al.* 1997).

2.4. Bioinformatics Analysis of *EgPIF4* Protein Motif

The physicochemical properties of the protein encoded by *EgPIF4* were predicted with the online Expasy Protparam tool (http://web.expasy.org/protparam/). The amino acid sequence of PIF4 were downloaded from GenBank database. The multiple sequence alignment of PIF4 amino acid sequences from different plant species were aligned using mafft online sequence alignment (https://mafft.cbrc.jp/alignment/server/). Information of the amino acid sequence of *PIF4* gene from 18 plant species used for bioinformatic analysis are shown in Table 1.

Table 1. Information of the amino acid sequence of *PIF4* gene from 18 plant species used for bioinformatic analysis

Common name	Sciencename	Accession no.
Arabidopsis	*Arabidopsis thaliana*	NP_001323428.1
green algae	*Coccomyxa subellipsoidea*	XP_005644417
cucumber	*Cucumis sativus*	XP_011653988
oil palm	*Elaeis guineensis*	XM_010906351.1
rose gum	*Eucalyptus grandis*	KCW89798
blue lupin	*Lupinus angustifolius*	XP_009757613.1
tobacco	*Nicotiana sylvestris*	XP_009757613.1
rice	*Oryza sativa*	XP_015631787.1
date palm	*Phoenix dactylifera*	XP_008811817.2
desert poplar	*Populus euphratica*	XP_011026200.1
peach	*Prunus persica*	XP_020416275.1
sesame	*Sesamum indicum*	XP_011100372.1
tomato	*Solanum pennellii*	XP_027774395.1
sorghum	*Sorghum bicolor*	XP_021302236.1
cacao	*Theobroma cacao*	XP_017971849.1
mungbean	*Vigna radiata*	XP_014522272.1
grape	*Vitis vinifera*	XP_010657098.1
corn	*Zea mays*	NP_001348421.1

2.5. Analysis of Gene Expression by Quantitative Real-time PCR (qRT-PCR)

cDNAs from five somatic embryo stages (callus, globular, torpedo, cotyledon, and plantlet) were used for quantitative amplification using *EgPIF4* gene specific primers. The primers used for *EgPIF4* gene expression were *PIF4_F* (5'-AACACAATGCCACCTCCTAA-3') and *PIF4_R* (5'-GCCACCTGATGATGAAGTAAC-3'). Real-time qPCR was performed on a LightCycler 480 (Roche, Switzerland) in 96 well plates in 10 µl reaction volume containing 2 µl of 1/40 time dilution cDNA, 1.5 µl of 2 µM for each primer and 5 µl SYBR® Green Master mix (Roche, Switzerland). Real-time PCR was initiated by denaturation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 70°C for 1 minute. Expression was normalized with the amplification of oil palm elongation factor gene; *EgEF1-α_F* (5'-ACATTGTCGTCATTGGTCAT-3') and *EgEF1-α_R* (5'-GGGTAAAGGCAAGCAAAGCA-3') (*EgEF1-α*, accession number: NM_001303577). Amplification of RNA matrices were also conducted to validate the absence of DNA in each sample. The gene expression experiment was performed in triplicate using two independent biological samples.

3. Results

3.1. Conservation of a Basic Helix-loop-helix Domain in Plant Species

Somatic embryo tissue of oil palm was used for RNA extraction including callus, globular, torpedo, cotyledon, and plantlet stage (Figure 1). *EgPIF4* gene was cloned using cDNA from globular tissue during somatic embryogenesis process. The nucleotide sequence was blast against the oil palm genome database. The results showed that *EgPIF4* gene located on chromosome 1 in oil palm genome. The coding sequence of *EgPIF4* gene comprised 1,737 bp with 9 exons (Figure 2a). *EgPIF4* protein comprises of 578 amino acid residuals,

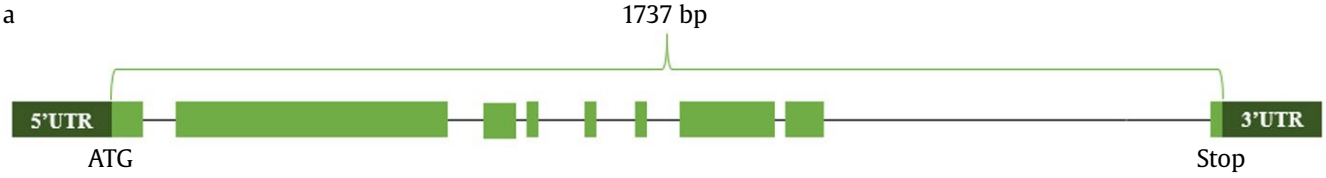
which contains a basic helix-loop-helix domain (bHLH domain) in the amino acid position 346-413 (Figure 2b). Analysis of physicochemical properties revealed a protein molecular weight of 63.71 kDa and an isoelectric point of 6.48.

Since the amino acid sequence of PIF4 proteins from different plant species has no conserved domain at the start and the middle of the sequence, an alignment of a conserved amino acid sequence of EgPIF4 protein and PIF4 proteins from different plant species is shown in Figure 3. The amino acid sequence alignment revealed that PIF4 protein contains a conserved structural domain, namely bHLH domain. The result showed that the deduced amino acid sequence of EgPIF4 was highly similar to that of other PIF4 proteins.

3.2. *EgPIF4* Expressed During Somatic Embryogenesis in Oil Palm Tissue Culture

To investigate the *EgPIF4* expression levels at different developmental stages during oil palm tissue culture, a qRT-PCR experiment was performed. From the result, transcript level of *EgPIF4* when

a



b

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CDS  atgaatcactatgttcctcattggaacttggaagatgagacaacatctttcggaggacttccagcgacaaatcagaaaaaaactatctgg
aa    M  N  H  Y  V  P  H  W  N  L  E  D  E  T  T  S  F  G  G  L  P  A  T  N  Q  K  K  T  I  W    30
CDS  ccggacaatgagcttgtagagctcttgtggcaagatgggcgtgtaatcatgcacagtcagactcatcggaaatcccctcccaccgtcggt
aa    P  D  N  E  L  V  E  L  L  W  Q  D  G  R  V  I  M  H  S  Q  T  H  R  K  S  P  P  T  V  G    60
CDS  gaatttaaacaagctcagaaggctgagccagcatcaaaatgcaggggggctctcgggaattcgagcaatttgagtcaagaagatgaaact
aa    E  F  K  Q  A  Q  K  A  E  P  A  S  K  C  R  G  A  L  G  N  S  S  N  L  S  Q  E  D  E  T    90
CDS  gcctcatggttccaacactctctcgatgattcgctagaaaaagagttctcagaatttcttttgcgaaacgccaaatgtagatgccattggt
aa    A  S  W  F  Q  H  S  L  D  D  S  L  E  K  E  F  S  E  F  F  C  E  T  P  N  V  D  A  I  G   120
CDS  agcagcaagatgactaaagatgtcactgaagagggagagagatccacaaactttggtgcgaatgaggaaacgaatgtgtttgctgcttct
aa    S  S  K  M  T  K  D  V  T  E  E  G  E  R  S  T  N  F  G  A  N  E  E  T  N  V  F  A  A  S   150
CDS  gatcccaagcaatccaggatttgtttcaaggaaaacacaatgccacctcctaaatcccacattattgcttctacccagcaggcttcttgt
aa    D  P  K  Q  S  R  I  C  F  K  E  N  T  M  P  P  P  K  S  H  I  I  A  S  T  Q  Q  A  S  C   180
CDS  ttggcaaatagcaatctcgtaaatttttcacacttcacaaggccacggccggtgatggctgatgtgggatcattaaatgggcagctgggg
aa    L  A  N  S  N  L  V  N  F  S  H  F  T  R  P  R  P  V  M  A  D  V  G  S  L  N  G  Q  L  G   210
CDS  aaggaatcagggaagagaattcgggcagggggcggtagagtccacctcgacgatgaccattgcatcaagtatctgtgagagcaaccagatt
aa    K  E  S  G  K  R  I  R  A  G  A  V  E  S  T  S  T  M  T  I  A  S  S  I  C  E  S  N  Q  I   240
CDS  caagctcaggccgatccaagccatactataagcagtgatgctgcaggagttgttgtaagaggtctgaaggaggacgcccagatgacatca
aa    Q  A  Q  A  D  P  S  H  T  I  S  S  D  A  A  G  V  V  V  R  G  L  K  E  D  A  Q  M  T  S   270
CDS  ctatctgagagaacacgaacgaacacgtatgaagctactgttacttcatcatcaggtggctctggttgtagctttggaagaacaggacag
aa    L  S  E  R  T  R  T  N  T  Y  E  A  T  V  T  S  S  S  G  G  S  G  C  S  F  G  R  T  G  Q   300
CDS  cagagtgccatcaatcagagccacaaaagaaagggaagagatgcagaggaatatgaaagccagagcgaggaggctgactatgagtatgtc
aa    Q  S  A  I  N  Q  S  H  K  R  K  G  R  D  A  E  E  Y  E  S  Q  S  E  E  A  D  Y  E  Y  V   330
CDS  gaggcaaacaagctagcgcagcggtctgcattgccacgaagaagccgttctgctgaagtccacaatctctccgagaggagaagaagggac
aa    E  A  N  K  L  A  Q  R  S  A  L  P  R  R  S  R  S  A  E  V  H  N  L  S  E  R  R  R  R  D   360
CDS  agaataaatgaaaagatgaaggcactacaagagctcatacctcattgcaacaagctactttattctcggaacatcggacatcgtcatgat
aa    R  I  N  E  K  M  K  A  L  Q  E  L  I  P  H  C  N  K  L  L  Y  S  R  N  I  G  H  R  H  D   390
CDS  gtaatcaagtttgtaataacagacaaagcatcgatgctagatgaggcaattgagtacttgaagtcccttcaactgcaagttcagataatg
aa    V  I  K  F  V  I  T  D  K  A  S  M  L  D  E  A  I  E  Y  L  K  S  L  Q  L  Q  V  Q  I  M   420
CDS  tggatgggaagtggcatggcaccaatgatgtttcctgctgtccaacaatgcatgtcacgcatcggtatgggagtgggtcatgcttctatg
aa    W  M  G  S  G  M  A  P  M  M  F  P  A  V  Q  Q  C  M  S  R  I  G  M  G  V  G  H  A  S  M   450
CDS  ccctctatgcatggtccagtccaattgccaagagtcccatttctcaatcagtctatagcctcggcttcgacggcacaccagacaccgctc
aa    P  S  M  H  G  P  V  Q  L  P  R  V  P  F  L  N  Q  S  I  A  S  A  S  T  A  H  Q  T  P  L   480
CDS  tgcctatcaacggccttgaatgcattgaactttccaaatcagatgcagaatatccatcttcctggttcgtatgccccataccttggcttc
aa    C  L  S  T  A  L  N  A  L  N  F  P  N  Q  M  Q  N  I  H  L  P  G  S  Y  A  P  Y  L  G  F   510
CDS  catcacatgcaagcatcctctcaggaaatgaattgttgtacctatggatctcacatggtgcagcagaatccgacaacagcagtgccgagc
aa    H  H  M  Q  A  S  S  Q  E  M  N  C  C  T  Y  G  S  H  M  V  Q  Q  N  P  T  T  A  V  P  S   540
CDS  agcagcatctttcctactgctggagaagttcccgctgagaataatcaaaataacaaatctggatcttgcaaatggttcacatttagtggg
aa    S  S  I  F  P  T  A  G  E  V  P  A  E  N  N  Q  N  N  K  S  G  S  C  K  W  F  T  F  S  G   570
CDS  tccctccagtacccctcaactcaataa
aa    S  L  Q  Y  P  S  T  Q  -    579
```

Figure 2. Characteristic of *EgPIF4* gene. (a) Gene structure of *EgPIF4* gene contain 9 exons of 1,737 bp and (b) coding sequence and amino acid sequence of *EgPIF4* gene. Red color highlight indicates the bHLH domain. Dash (-) refers to stop codon

compared with the expression level of *EgEF1-α*, was not detected in in callus but detected in all somatic embryo tissue (globular, torpedo, and cotyledon), with the highest relative expression observed in globular stage. The relative expression of *EgPIF4* was then gradually decreased after globular stage (Figure 4). This result suggested that *EgPIF4* may play an important role at somatic embryogenesis process during tissue culture.

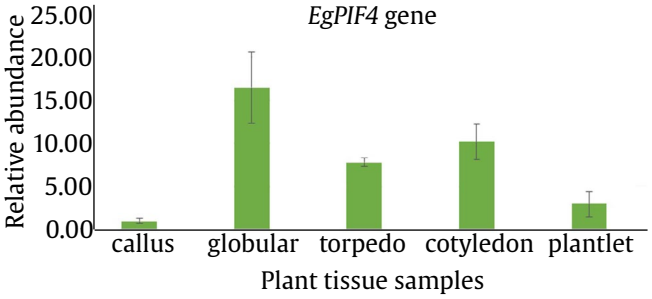

Figure 4. Quantitative real-time PCR analysis of *EgPIF4* transcription levels in oil palm tissue samples including somatic embryo tissues: callus, globular, torpedo, cotyledon, and plantlet. Oil palm elongation factor gene, *EgEF1-α*, was used as internal control

4. Discussion

Since clonal propagation is the best way to propagate high yield oil palm, tissue culture technique was used.

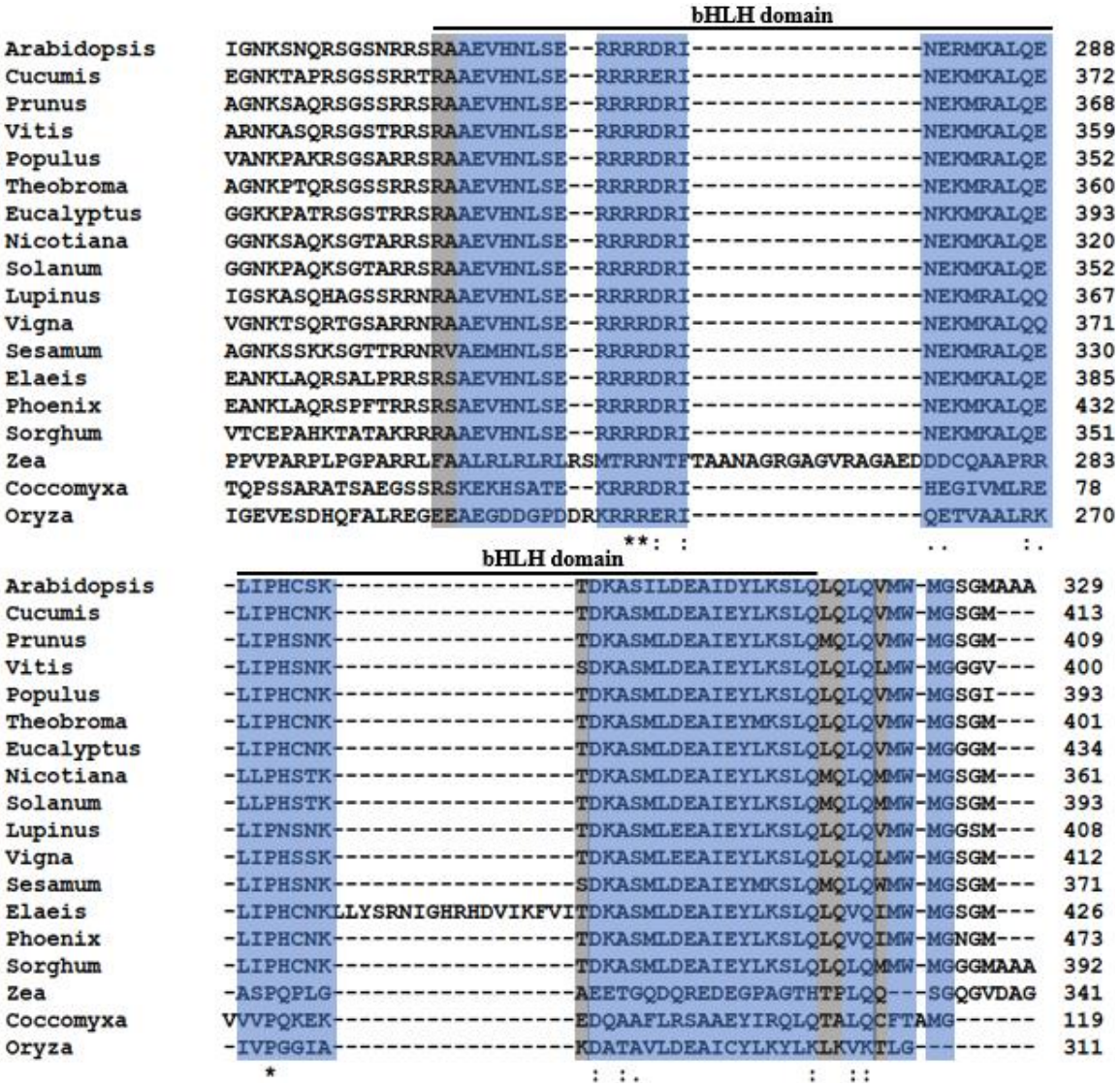

Figure 3. Sequences alignment between the deduced amino acid of EgPIF4 and other homologous genes in plants. Blue background represents amino acid identity is over 80%, and gray background represents amino acid identity is over 50%, The bHLH domains is marked with an overline

Unfortunately, the somatic embryogenesis during tissue culture process in oil palm take longer time that other plants. Genes involved in this process were studied. PIF4 is a transcription factor that was reported to have a role in controlling the level of auxin which is an important hormone during somatic embryogenesis process. The molecular mechanisms of PIFs have been studied extensively in *Arabidopsis*. Auxin is an important plant hormone essential for many aspects of plant growth and development and also has an essential role during somatic embryogenesis. It plays an important role in early and post-embryogenic plant development (Cueva-Agila *et al.* 2016; Elhiti and Stasolla 2016). The basic helix-loop-helix (bHLH) transcription factor phytochrome-interacting factor 4 (PIF4) has a key role in controlling the levels of auxin. PIF4 was reported to be a regulator that control levels of auxin and the expression of key auxin biosynthesis gene at high temperature (Franklin *et al.* 2011). However, not many studies focus on the role of PIF4 during somatic embryogenesis and *PIF4* gene in oil palm has not been characterized. The results from our studies showed that *EgPIF4* is located on chromosome 1 and contains 1,737 bp open reading frame encoding a polypeptide comprised of 578 amino acid residuals.

The results from a database search revealed that PIF4 contains a region with strong homology to the bHLH superfamily of transcription factors (Evan and Littlewood 1998; Atchley *et al.* 1999). From our results, sequence comparison of the bHLH domain from different plant species revealed that the residues that define the bHLH domain are conserved in the PIF4 amino acid sequence. This suggested that EgPIF4 transcription factor may play an important role during embryo development as in other plants.

Recent researches reported the expression of *PIF4* gene in various tissue. *PIF4* was found to be significantly induced at the early stage of leaf senescence and decreased at the late stage of leaf senescence in *Arabidopsis* (Song *et al.* 2014). RT-qPCR analysis showed that *PIF4* gene in maize is expressed in roots, stems, coleoptiles, and leaves at the six-leaf stage (Shi *et al.* 2018). *PIF4* gene in oil palm has not been studied and characterized before. From our studies, we showed that *EgPIF4* gene in oil palm, specifically expressed in somatic embryo tissues with the highest expression at globular and cotyledon stages which are the somatic embryogenesis stage. Furthermore, *EgPIF4* showed no expression at callus stage. These results supported the idea that *PIF4* gene is the important regulator for the embryogenesis in plant species including oil palm.

The results from this study were a preliminary data to show that EgPIF4 may plays an important role during somatic embryogenesis. EgPIF4 protein will be used for characterizing the function and the role during somatic embryogenesis in the future.

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Conflict of Interest

The authors report no conflict of interest.

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