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Molecular approach for enhancing phosphate uptake in oil palm

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Abstract

Oil palm is grown in acidic tropical soils with low bioavailability of inorganic phosphate (orthophosphate; Pi), the form that can be assimilated by plant. An understanding on the molecular mechanism for acquiring Pi by oil palm roots under Pi deficient condition may help to enhance oil palm productivity and reduce excessive input of fertilizer. A high-affinity and energy-mediated co-transport system is believed to be the major system responsible for uptake of Pi from soil into the root cells against a steep concentration gradient. This paper reports on the characterization of an oil palm gene encoding a high affinity phosphate transporter designated as *EgPHT1*. The deduced protein sequence of the *EgPHT1* cDNA specifically expressed under Pi starvation condition indicates that it is a plasma membrane localized protein containing 12 trans-membrane spanning domains. Among the regulatory motifs identified in the promoter sequence includes several root-specific, phosphate starvation inducible, water stress-responsive and hormone signaling elements. Functional study using β-glucuronidase reporter gene showed that *EgPHT1* promoter can drive root-specific and phosphate starvation-inducible expression in transiently transformed tissues of the monocotyledonous oil palm as well as in stably transformed dicotyledonous model plant, *Arabidopsis thaliana.* The potential application of the information generated on improving Pi uptake by oil palm will be discussed.

Keywords: oil palm, high affinity phosphate transporter, promoter analysis, transgenic *Arabidopsis*

INTRODUCTION

Improvement of fertiliser uptake is important for oil palm sustainable development as this addresses both the economic and environmental aspects through reducing the cost of production and environmental pollution, respectively (Kassam et al., 2009). Phosphate as a macronutrient plays a critical role in crop development and production but its efficient use is hampered in acidic tropical soil where oil palm is widely grown. This issue is being addressed in other crop species by attempting to identify the key transporters, traffic facilitators and transcription factors and their contributions towards the molecular mechanism in enhancing nutrient uptake and use efficiencies (Wu et al., 2013).

The existence of a plant adaptive response that is triggered in the root under low Pi (inorganic phosphate) availability has been the subject of interest in various crop species. There are several reports indicating the importance of molecular regulation at the transcriptional level (Misson et al., 2005; Bustos et al., 2010). In vascular plants, genes encoding Pi transporters play a crucial role in Pi transport systems. Pi transporters belong to four families based on functional and structural classifications namely, PHT1, PHT2, PHT3 and PHT4. The high-affinity Pi transporters (PHT1) were confirmed by kinetic studies and they are involved in acquisition of Pi by the roots from low external concentrations in the soil. Members of the PHT1 family have been identified in different plant species. They are mainly located in root epidermal cells and root hairs. Their presence in other tissues including leaves,

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stems, seeds and flowers suggest their involvement not only in Pi uptake by roots but also in internal root-to-shoot distribution.

This paper reports on the isolation and characterisation of the first high affinity phosphate transporter from oil palm that is expressed under phosphate-starvation condition. It displays the typical structure of the PHT1 protein with twelve plasma membrane spanning domains. The promoter sequence was subsequently isolated and was found to contain several root-specific and phosphate-starvation inducible motifs.

MATERIALS AND METHODS

Plant materials

Oil palm genotype D X P was obtained from Federal Land Development Authority (FELDA), Malaysia.

Hydroponics treatments on D x P oil palm seedling

Pi treatments in the presence of Pi or under Pi-deficient conditions have been performed to determine the effects on the oil palm Pi transporters gene expression. For the phosphate treatment, hydroponics culture system was applied. Firstly, the seedlings were grown in container using Cooper solution. All of the oil palm seedlings were grown in adequate Pi in hydroponics culture. After the plant was stable in the hydroponics system, the seedling was grown in two treatments which was Cooper solution with Pi application and while for the Pi starvation, plants were supplied with Cooper solution without addition of Pi. The plants were then harvested on day five after Pi deprivation for preparation of RNA from roots.

Partial cDNA isolation using degenerate primers

Total RNAs were extracted using the method of Azzreena et al. (2016). Purification of mRNA from 100 µg total RNA was done using Absolutely mRNA Purification Kit (Stratagene). First-strand cDNA was synthesized using a Superscript III Reverse Transcriptase (Invitrogen Ltd, Paisley, UK). Based on known high-affinity phosphate transporters sequences, forward and reverse degenerate primers were designed based on highly conserved regions identified by multiple sequence alignments using Consensus-degenerate hybrid oligonucleotide primers (CODEHOP) program (Staheli et al., 2011). The hybrid structure (5' consensus and 3' degenerate) of CODEHOP primers allow the PCR amplification to be specific during the cycles from the original source DNA and selective during the late cycles from the PCR synthesis products. The multiple alignment sequence was obtained from a group of related protein sequence of phosphate transporters isolated from *Arabidopsis thaliana*; *Oryza sativa*; *Nicotianatabacum*, *Solanum lycopersicum*, *Solanum tuberosum*, *Lupinusalbus*, *Catharanthusroseus*, *Triticumaestivum*, *Medigotruncatula* and *Sesbaniarostrata* using the Clustal W program. PCR reaction were performed on a Thermal Cycler programmed to give a temperature profile of 2 min at 94^0C followed by 30 cycles of 30 s at 94^0C; 2 min at 55^0C; 2 min at 72^0C and final 10 min extension at 72^0C. PCR products were analysed on a 1% (w/v) TAE-agarose. The amplified PCR products were cloned into a TOPO TA cloning vector and sent for sequencing.

5' and 3' RACE PCR

RACE PCR (5' and 3') was performed using SMART RACE cDNA Amplification, Advantage 2 PCR Kit (Clontech) and gene-specific primers designed for target gene according to the suggested PCR parameter by the manual. The amplified product was extracted from agarose gel, cloned and sequenced.

Isolation of the promoter sequence and identification of regulatory motifs

The promoter sequence was isolated using the Universal GenomeWalker Kit according to the manufacturer's instructions (BD Biosciences Clontech, USA) using oil palm genomic DNA. To identify cis-acting regulatory elements, the DNA sequences upstream of translation start codon (ATG) were analysed using the online PLACE and PlantCARE databases.

RESULTS AND DISCUSSION

Isolation of high-affinity oil palm phosphate transporter cDNA clone

Plant high-affinity Pi transporters have a pH optimum of between 5.0 and 6.0. Pi is most readily taken up by plants as orthophosphate ion, which is predominantly found in media buffered below pH 6.0 (Nagy et al., 2006). Oil palm seedlings grown in the presence of Pi or under Pi-deficient conditions were studied to determine the effects of Pi deprivation on the oil palm Pi transporters gene expression.

cDNA was synthesized from normal and Pi-deprived root tissues and used as templates in PCR reaction using the degenerate primers. This resulted in amplification of partial cDNA of about 670bp in length from the Pi-deprived roots only (Figure 1A). This result demonstrated that the oil palm Pi transporter gene is only expressed in the Pi-starved oil palm seedlings suggesting that the oil palm phosphate transporter expression is tightly linked to the level of Pi in the solution.

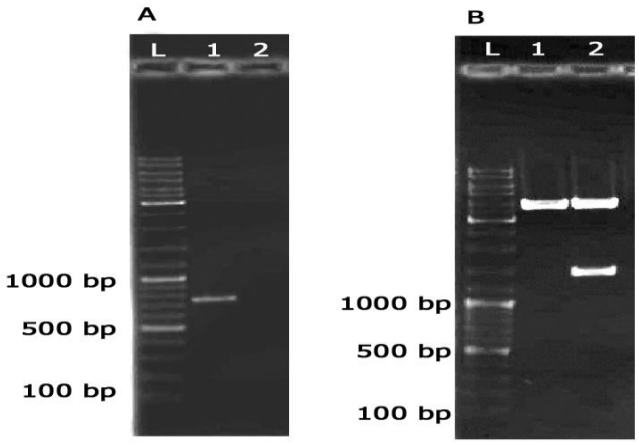

Figure 1. A: PCR product obtained using forward and reverse degenerate primers and oil palm cDNA from roots as template. Lane L: Gene Ruler DNA Ladder Mix Marker (Fermentas); Lane 1: Amplified product using the degenerate primer pair from Pi deprived root tissues; Lane 2: No amplification from root tissues with Pi application. B: Agarose gel electrophoresis analysis of purified recombinant plasmid containing 1.4 kb oil palm Pi transporter cDNA before digestion (Lane 1) and after digestion with Eco RI (Lane 2) to confirm the presence of insert.

RACE approach was carried out to isolate the full-length cDNA of oil palm Pi transporter. Based on the partial sequence, two pair of primers (a gene-specific primer pair and nested gene –specific primers) were designed to generate the 5'-end and 3'-end sequence of oil palm Pi transporter. The 5'-RACE reaction produced a DNA fragment of 0.65 kb and the 3'-RACE reaction generated a DNA fragment of 0.83 kb.

Sequence information obtained from 5' and 3' RACE was used in end to end PCR using cDNA from Pi-deprived roots as a template. This resulted in the amplification of approximately 1.4 kb PCR product. The PCR product was cloned into TOPO TA vector. Figure 1B showed the result of the restriction analysis of the cloned PCR product. The product was sequenced to completion. Sequencing results demonstrated that the cDNA sequence was 1412 bp in length.

Homology comparison and sequence analysis

A successfully isolated full-length cDNA sequence from oil palm was analysed at the nucleotide and protein levels for homology through BLAST Search Engines using BLASTX algorithm. Identities of the clones were determined based on the sequence homology search. The sequence was identified as homologous to the high-affinity Pi transporters where the BLASTX result showed strong amino acid sequence homology with the Pi transporter genes

from other plant species in the database such as *Catharanthusroseus, Oryzasativa, Hordeum vulgare, Zea mays* and *Arabidopsis thaliana* with greater than 77% amino acid sequence identity. The identified gene was named according to the Commission on Plant Gene Nomenclature as *ELAgu; Pht1*. For simplification, it will be referred to as *EgPHT1*.

In silico analysis of *EgPHT1* cDNA sequence

High-affinity Pi transporters are actually proton/phosphate ($H^+/H_2PO_4^-$) symporters. *EgPHT1* encodes a Pi transporter gene in oil palm. According to the computational analysis, this protein belongs to the Major Facilitator Superfamily (MFS) based on protein configuration. MFS is classified into five clusters including drug-resistance proteins, sugar facilitators, facilitators for Krebs cycle intermediates, phosphate ester-phosphate antiporters and a distinct group of oligosaccharide-H^+ symporters. The MFS transporters help in transporting small solutes in response to chemiosmotic ionic gradients across plant plasma membranes. Plant Pi transporter was originally identified by sequence similarity to the PHO84, a high-affinity Pi transporter from *Saccharomyces cerevisiae* (Smith et al., 2000).

The full length cDNA and deduced amino acid sequence of oil palm phosphate transporter gene, EgPHT1 (1963 bp) contained an open reading frame (ORF) of 1533bp, encoding a putative protein of 511 amino acids. The amino acids sequence encodes by EgPHT1 have been analyzed using Protparam program. This program shows that the deduced molecular weight of the predicted protein was approximately 55.6kDa with a predicted isoelectric point (pI) of 8.6. Hydrophobicity profiles analysis for EgPHT1 amino acids sequence was performed using Protscale program (Kyle and Doolitle, 1982). Using this program, positively index value shows hydrophobic characteristic while negatively index value shows hydrophilic characteristic. Transmembrane protein topology prediction, TMHMM is used to predict location and transmembrane helices weather it is located inside or outside cell based on a hidden Markov model (Figure 2). Through the hydrophobicity profiles, the transmembrane region can be predicted where the N-terminal and C-terminal are found faced towards inside of the membrane. There are 12 candidate membrane-spanning segments found in the EgPHT1 amino acids sequence and a central hydrophilic loop between TM6 and TM7 domains. This is a common feature shared by other membrane-associated cotransporters. This result demonstrated that EgPHT1 sequence is similar in overall structure to known high-affinity Pi transporters. The conservation indicates a similar conservation of function and role for these proteins in phosphate uptake.

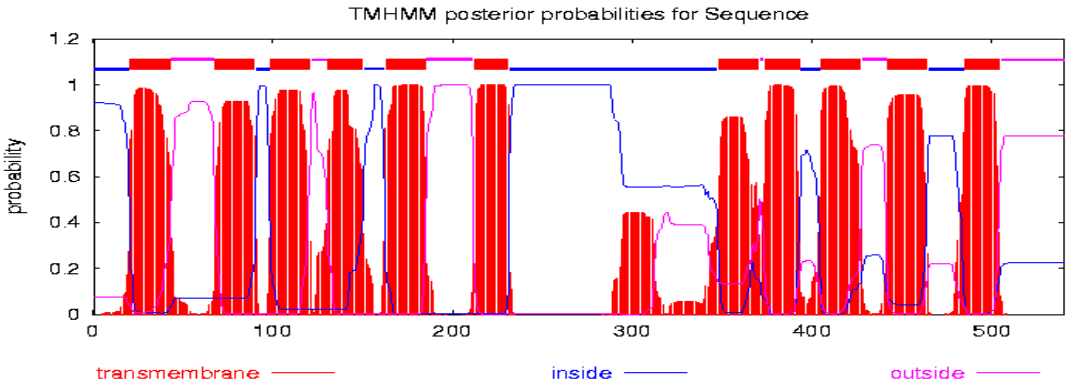

Figure 2. Transmembrane domains of EgPHT1 from oil palm.

To predict the putative location for EgPHT1 programs such as WoLF PSORT and ProtComp Version 6 have been used. Both program agreed that the EgPHT1 sequence as an integral membrane protein. However, to prove exact location of the EgPHT1 sequence, present of certain predicted motifs or *in vivo* experiment needs to be carried out.

Isolation of the promoter sequence and identification of putative regulatory motifs

Via the genome walking approach, a genomic region of 1400 bp upstream of the ATG was successfully isolated. It was found to contain several regulatory motifs for inducible expression under Pi deficient condition in the roots including several root-specific, Pi starvation inducible and water stress-responsiveelements (Table 1). The promoter was further characterized using β-glucuronidase reporter gene. The resultsshowed that *EgPHT1*promoter can drive root-specific and phosphate starvation-inducible expression in transiently transformed tissues of the monocotyledonous oil palm as well as in stably transformed dicotyledonous model plant, *Arabidopsis thaliana.*

Table 1. Examples of important putative *cis*-regulatory elements found in the promoter sequence of *EgPHT1* gene of oil palm.

Response elements type	Regulatory element name	*Cis*-acting regulatory sequence	Position from translation start site
Pi-starvation response	P1BS	GNATATNC	-1150
	W-box	TTGAC(C/T)	-704, -1168, -1250
Root- specific	ROOTMOTIFTAPOX1	ATATT	-120, -748, -756, -847, -848, -1148, -1289, -1290
	RAV1AAT Box	CAACA	-188, -200
Water- stress response	MYBCORE	CNGTTR	-384, -1130, -1348
	MYCATERD1	CATGTG	-204, -642
	ACGTATERD1	ACGT	-515, -1171, -1395

CONCLUSIONS

The following conclusions can be drawn from the study:

- The oil palm high-affinity phosphate transporter cDNA hasbeen successfully isolated from Pi-starved oil palm roots and its promoter sequence containing several root specific and Pi-starvation inducible motifs was obtained.
- This gene could be further characterized to evaluate its potential for future application in genetic improvement to produce high-yielding oil palm in Pi-deficient soils through genetic engineering and molecular breeding.

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