SHORT COMMUNICATION

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POTASSIUM NUTRITION IN THE OIL PALM: A MOLECULAR PERSPECTIVE

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ABSTRACT

Potassium (K^+) is a major nutrient essential for plant growth and development. Acquisition of this vital element and maintenance of K^+ homeostasis are complex processes, facilitated by an array of membrane transporters including carriers and channels. Key mediators of K^+ uptake are the K^+ Transporters (KT) /K^+ Uptake Permease (KUP)/High Affinity K^+ (HAK) family of transporters. The oil palm (Elaeis guineensis)*, is an agriculturally and economically important crop, but the molecular mechanisms of nutrient acquisition in this plant are poorly understood. Here we report the first molecular characterisation of potassium transporters from KT/KUP/HAK superfamily in the oil palm, named EgKUP3, EgKUP8 and EgKUP11.*

Keywords: potassium, KT/KUP/HAK, oil palm, molecular characterisation.

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INTRODUCTION

Elaeis guineensis or the oil palm is by far the most productive oil bearing crop (Kushairi *et al*., 2019; Bourgis *et al*., 2011; Yan, 2017), produces almost 70 million tonnes of palm oil in 2017 or 30% out of the total world edible oil production, claiming the top spot amongst 16 other vegetable oils as the most consumed oil worldwide. The global demand for oils and fats has seen a staggering increase of 167% in less than three decades from 83.5 million tonnes in 1991-92 to 223 million tonnes in 2017 (Mielke, 2017) and is expected to continue rising proportionally with the growing world population. Malaysia is the second largest producer of palm oil after Indonesia, and both countries currently account for 85% of the total palm oil production in the world (Yan, 2017). In order to meet the growing demand for oils and fats, expansion of the current 5.8 million hectares of plantation area in Malaysia is not a viable option, thus optimising the existing land to produce maximum yield is the only alternative for sustainable palm oil production. One of the key areas to improve productivity is mineral nutrition.

Potassium (K^+) is one of the most important mineral nutrients which play diverse roles in plants daily survival including enzymatic functions, opening and closing of stomata in the leaf (to allow water vapour and waste gases to escape and at the same time to provide plants with the ability to withstand drought conditions), maintaining the turgor pressure of the cell (meaning it keeps plants from wilting) as well as regulation of cell size (Maathuis *et al*., 1996; Schachtman and Schroeder, 1994; Very and Sentenac, 2003). In the oil palm, K^+ is a crucial element in fertilisation, which directly affects bunch weight and bunch number (Lamade *et al*., 2014). For this reason, palms exposed to K^+ deficiency have been reported to produce significantly less oil (Diomande *et al*., 2004). K^+ is also involved in the transport of sugar assimilates or 'food' from photosynthetic tissues to other parts of the plant (Lamade *et al*., 2014) such as inflorescence and developing fruits. K^+ deficiency has been associated with the occurrence of vascular wilt disease, cercospora leaf spot, *Ganoderma* basal stem rot, and the physiological disorders which cause bunch and plant failure (Rankine and Fairhust, 1999).

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In many plantations, the availability of K^+ often is very low (Maathuis, 2009), and to compensate the shortage of K^+ and other nutrients in soil, large amount of fertilisers are applied. Approximately 0.52 t ha^{-1} yr^{-1} is required for K fertilisers alone with an associated cost of approximately USD 185 t^{-1}. The use of fertilisers can contribute up to 50% of the total production cost especially in time of weakened Malaysian ringgit (MYR). Besides that, with the growing concern of the deteriorating environment, there has been a drive towards mitigating the use of chemical fertilisers. Hence, palms with enhanced traits are the future planting materials for a more sustainable oil palm industry.

Development of crop less dependent on fertilisers and optimisation of fertilisation has been impeded by current lack of knowledge in the molecular aspects of agriculturally and economically important plants. Nevertheless, advances in molecular genetics and genomics in recent years such as the publication of the *Elaeis guineensis* genome in 2013 has provided us with endless opportunities for genomic-driven crop improvement. Moving towards this goal we looked at a few gene candidates responsible for K^+ uptake, specifically from the superfamily of K^+ Transporters/K^+ Uptake Permease/High Affinity K^+ (KT/KUP/HAK) transporters. These groups of transporters are found in bacteria, fungi and plants. In plants, the transporters can be grouped into four distinct clusters (I-IV). Members of cluster I play a key role in K^+acquisition particularly when K^+ availability is low (Banuelos, 2002; Rodriguez-Navarro, 2006) and are likely to function as H^+-K^+ co-transporter (Rodríguez-Navarro, 2000). Members of cluster II are involved in diverse physiological functions such as complementing the role of K^+-channels for low-affinity K^+-transport, response to salinity and regulation of cell size (Grabov, 2007), while transporters in cluster III and IV are still not well characterised.

The objective of this study was to identify and characterise KT/KUP/HAK potassium transporters from the oil palm (EgKUP). This was achieved by obtaining: (i) full-length sequences of *EgKUP* using a combination of homology screening from several plant databases and Rapid Amplification of cDNA Ends (RACE), (ii) phylogenetic analyses of the obtained sequences, (iii) genes expression analysis, and (iv) functional characterisation in the heterologous system, *Escherichia coli.*

MATERIALS AND METHODS

Plant Growth, Total Ribonucleic Acid (RNA) Extraction and cDNA Synthesis

The *Elaeis guineensis* plantlets of three-month old were grown in modified-MS liquid media under 12 hr light (2000 lux) and 12 hr dark cycles, and 60% humidity prior to the experiment. Approximately 100 mg of root tissues were disrupted using a tissue lyser. RNA was immediately extracted from the disrupted tissues using the RNeasy extraction kit (Qiagen) according to the manufacturer's protocol. The RNA obtained was then used as templates for cDNA synthesis using the Omniscript Reverse Transcription kit (Qiagen) according to the manufacturer's protocol.

Construction of Full-length cDNA of the Candidate Genes

Few gene sequences of the KT/KUP/HAK family of potassium transporters from other plant species such as *Oryza sativa, Arabidopsis thaliana* and *Phoenix dactylifera* were used as a reference to generate gene specific oligonucleotide primers for *EgKUP*. The construction of full-length *EgKUP* cDNA was then performed using SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol.

Phylogenetic Analysis of EgKUP

The full-length EgKUP sequences were queried against *Arabidopsis thaliana* databases (www.arabidopsis.org), identified and named based on the closest homologues from *A. thaliana*. Sequences of other known KT/KUP/HAK transporters from different species were downloaded from their respective database websites including rice (http://crep.ncpgr.cn/crep-cgi/home.pl), maize (http://www.maizesequence.org/index.html), tomato (http://chibba.agtec.uga.edu/duplication/), wheat and barley (from NCBI database). Alignment of all the sequences was conducted using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the subsequent phylogenetic tree was built using MEGA5 software, and the Neighbour-Joining method (Tamura *et al.*, 2011). Bootstrap testing was performed with 1000 resampling.

Gene Expression Analysis

The plantlets were transferred into modified MS media containing three different concentrations of KNO_3 namely 0.2, 10 and 20 mM KNO_3 for periods of up to 21 days. The pH of the media was adjusted to 5.7 using $Ca(OH)_2$ prior to use. For each K^+ concentration, roots tissues were harvested at 7, 14 and 21 days of growth from experimental and control media. For each test condition 100 mg of ground root/leaf tissues were used for RNA extraction. The RNA extraction was conducted as described. The cDNA synthesis as previously described was then performed, followed by quantitative real-time polymerase chain reaction (PCR) using the Quantitect SYBR Green RT-PCR kit in 0.2 ml thin-

walled PCR tubes (Qiagen) in a final volume of 10 µl. The PCR signals obtained for the target transcripts were normalised against the signal obtained for GAPDH (oligonucleotide primer sequences) are given in *Table 1* and the expression levels were then compared to the control group using the method described by Livak and Schmitten (2001).

Growth Test Assay

The full-length of *EgKUP3*, *EgKUP8* and *EgKUP11* were individually cloned into plasmid pBAD24 (oligonucleotide primer sequences) are given in *Table 1* and then transformed into the *Escherichia coli* strain TKW4205, deficient in all K^+ uptake systems. For bacterial growth tests, at low K^+, serial dilution drops of strains grown in LB supplemented with 30 mM K^+ were inoculated on a solid medium containing 5 mM PO_4H_3, 0.4 mM $MgSO_4$, 6 µM $FeSO_4$, 1 mM citric acid, 1 mg $litre^{-1}$ thiamine, 0.2% glycerol, 8 mM asparagine, 20 µM Ca_2Cl, and oligo-elements brought to pH 5.5 or pH 7.5 with HCl or arginine respectively and supplemented with 13 mM arabinose and either 50-, 5- or 1 mM K^+ concentrations. This medium is based on that described by Senn *et al*. (2001). The petri dishes were then incubated at 37°C for 48 hr. The assay was performed to evaluate whether the defect of *E. coli* TKW4205 can be substantially corrected by the introduction of a plasmid harbouring *EgKUP* genes.

RESULTS AND DISCUSSION

Identification of the Oil Palm KT/KUP/HAK Transporters

In order to understand the molecular basis of K^+ uptake and homeostasis, it is essential to obtain the genes encoding K^+ transporters from *E. guineensis*. The combination of homology screening from several plant databases such as rice, Arabidopsis, date palm and oil palm itself, total RNA extraction, RACE and PCR techniques had successfully led to the identification of three full-length genes from the KT/KUP/HAK family named *EgKUP3*, *EgKUP8* and *EgKUP11*. The full-length sequences were subjected to phylogenetic analysis together with their counterparts from other species for conformity. Results from the analysis confirmed that all EgKUP belong to the KT/KUP/HAK family of membrane transporters. EgKUP3 and EgKUP8 are grouped under cluster II, while EgKUP11 is in cluster III (*Figure 1*).

Regulation of EgKUP at Gene and Protein Levels

To further characterise *EgKUP*, we conducted quantitative real time-PCR (qRT-PCR) to evaluate the gene expression level following K^+-starvation of the oil palm plantlets. Level of expression for EgKUP3 and EgKUP11 remained unaffected by K^+-starvation treatment, whereas for EgKUP8, the expression was markedly increased by seven- and five-fold respectively at 14- and 21-day of treatment (*Figure 2*). This result triggers the question on whether some of the cluster II transporters are also involved in high-affinity transport. To investigate whether the changes of *EgKUP8* observed at the gene level is translated at the protein level, we conducted functional complementation assay by cloning the full-length of *EgKUP8* into *Escherichia coli* knockout strain defective in K^+ uptake systems. The cells harbouring *EgKUP8* were able to complement the cells' defect at 50 mM and 5 mM but not at the lowest K^+-concentration, 1 mM, when tested at pH 7.5. At low pH (pH 5.5), only positive control cells were able to survive. Based on previous studies, high-affinity transporters have certain distinct

TABLE 1. OLIGONUCLEOTIDE PRIMER SEQUENCE for (A) QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR) EXPERIMENT AND (B) CONSTRUCTION OF pBAD24 PLASMIDS CONTAINING *EgKUP*

	Primer identification	Sense primer (5′-3′)	Antisense primer (5′-3′)
A	EgKUP3_qrt	GCCAAGTTCATTCAGATGG	CCTCACTACCAGCCTTGAGC
	EgKUP8_qrt	GCATCGTCCGCTATGGATAC	CACCATGCCATTGGGTTC
	EgKUP11_qrt	TGTATGGCAATTGGAGATGG	GACCACAGCAACAAGTACAACC
	GADPH_qrt	GAGAGAGCGTGCTACTCATCTT	CGGAAGTGCTTCTGAGATCC
B	EgKUP3-pBAD	GGGCCC**GAATTC**ATGGATCA GGAGAGAGGGATGCA	GGGCCC**CCATGG**CTA CATAGTAGATCATA CCCACTTCAA
	EgKUP8-pBAD	GGGCCC**CCATGG**ATGGATCT CGAGGGCGGT	GGGCCC**AAGCTT**TCA GACATGGTAAATCATCCCC
	EgKUP11-pBAD	GGGCCC**TCTAGA**ATGGCATC GGAGACGG	GGGCCC**AAGCTT**CTATACAT AGAATATCTGTCCCACATTCA

Note: Fonts in bold indicate restriction enzyme sequences.

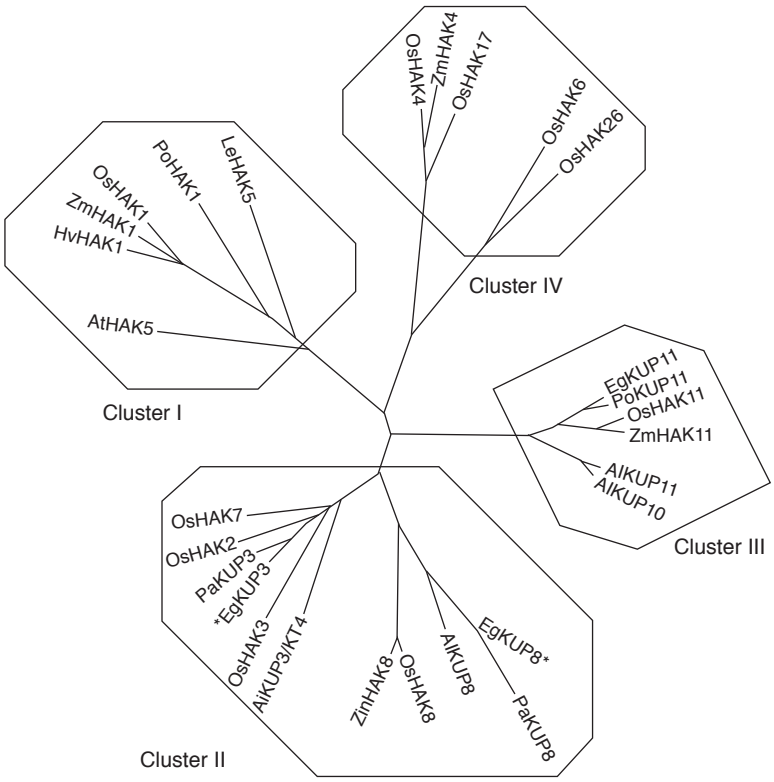

Figure 1. Phylogenetic relationship between representatives of KT/KUP/HAK family in several plant species including Oryza sativa *(Os),* Zea mays *(Zm),* Phoenix dactylifera *(Pd),* lycopersicon esculentum *(Le) and* Hordeum vulgare *(Hv). EgKUP are indicated by asterisks.*

characteristics including (i) upregulation of gene level under K^+ deprivation (under 1 mM K^+), and (ii) complement growth of K^+ deficient cells under low K^+ (1 mM and lower) and low pH (pH 5.5).

The fact that EgKUP8 does not fulfil the second criteria may indicate that this transporter is not energised by inwardly directed H^+ gradients or in other words it may not function as high-affinity H^+-K^+ co-transporter, at least when recombinantly expressed in *E. coli*. A very similar expression profile was reported for another cluster II transporter, AtKUP3, with expression was strongly upregulated by K^+ starvation (40 μM) in Arabidopsis seedlings, but failed to mediate high-affinity transport when expressed heterologously in *E. coli* (Kim *et al*., 1998). The result, however, should not be treated as conclusive before further investigation takes place. Perhaps, EgKUP8 needs some 'adjustment' to function at lower K^+ concentration and low pH when expressed in prokaryotic system. This system-dependent behaviour has been demonstrated for the AtHAK5 of *A. thaliana*. *In planta* the AtHAK5 mediates high-affinity transport, however only after the introduction of a point mutation, F130S, that the transporter was able to function as high-affinity transporter (at 100 μM K^+) in *E. coli* (Alemán *et al*., 2014). Nevertheless, the results from expression and functional complementation assays may indicate the importance of EgKUP8 in K^+-sufficient environment with low-affinity mode of transport.

On the other hand, the expression of *EgKUP3* and *EgKUP11* was not affected by changes in environmental K^+ conditions. At protein level, EgKUP3 and EgKUP11 demonstrated K^+ transport function only at 50 mM external K^+ concentration when tested at pH7.5. These observations may suggest that primary function of EgKUP3 and EgKUP11 may not be in K^+ transport.

CONCLUSION

This study provides insights into the oil palm potassium uptake at molecular level. Three membrane transporter candidates from the KT/KUP/HAK family, EgKUP3, 8 and 11, have successfully been identified and cloned. All of the transporters demonstrated K^+ transport function based on the functional complementation assay. Taking together results from the gene expression study, it is clear that none of the EgKUP transporters reported here fulfill the criteria of being a high-affinity transporter. However, the observation that *EgKUP8* is transcriptionally activated in plants at K^+-deficiency may indicate its importance for K^+ uptake in K^+-sufficient environment, because such activation may compensate for low efficiency of these transporters in sub-mM concentrations. The study serves as a foundation for further molecular physiology research in understanding the precise

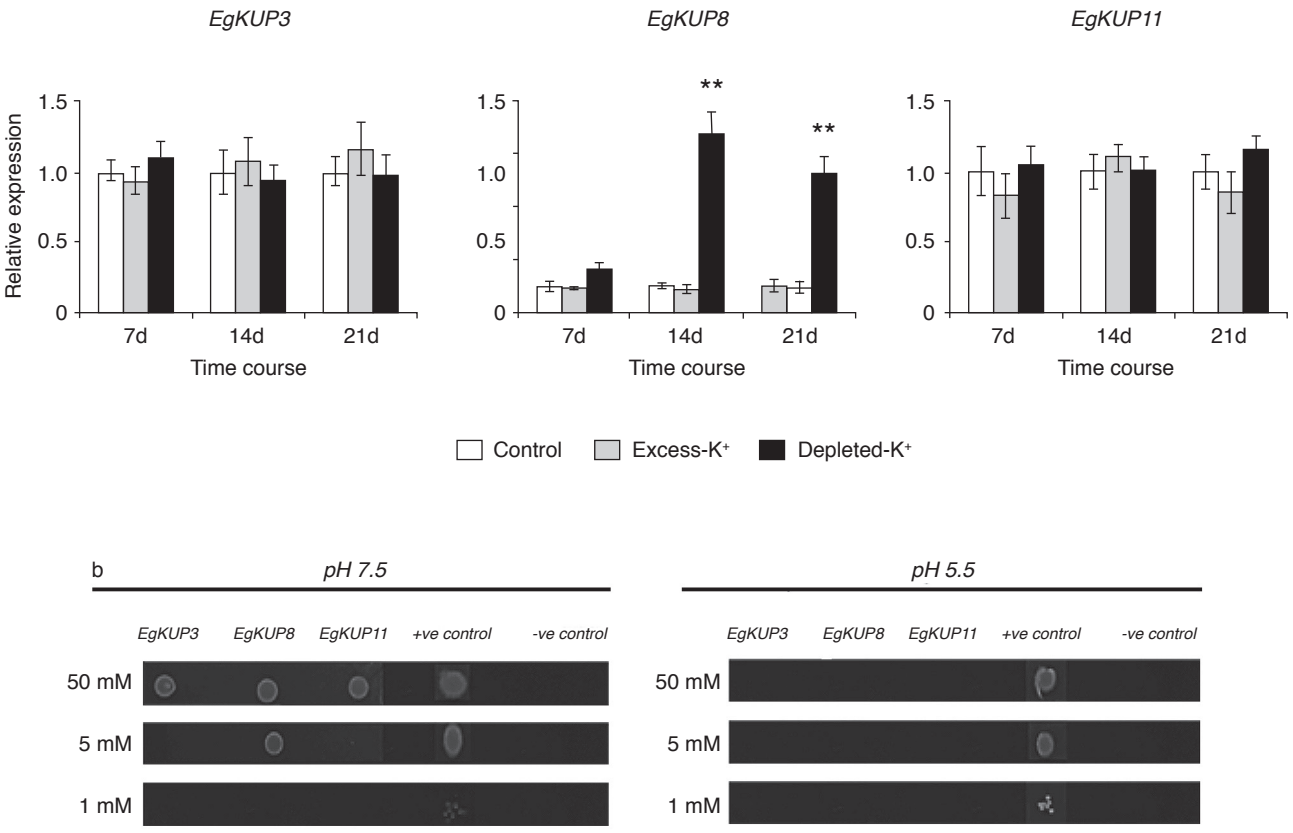

*Figure 2. Regulation of EgKUP3, 8, and 11 at gene and protein level. (a) Gene expression of EgKUP in three-month old oil palm plantlets following incubation at different K⁺ concentrations. The graphs show the expression of EgKUP3, 8 and 11 in root tissues following excess K⁺ treatment (20 mM K⁺) and depleted K+ treatment (0.2 mM K⁺) compared to control group (10 mM K⁺) at 7-, 14- and 21-day after treatment. Data points are the average of three experiments carried out in triplicate. Statistical analysis was performed using a student's t-test comparing high and low concentration K⁺ samples with the control; ** represents p<0.01. (b) EgKUP mediated bacterial growth at three different external K⁺ concentrations; 50 mM, 5 mM and 1 mM of K+, tested at pH 7.5 and pH 5.5. The negative control was TKW4205 cells containing empty vector while the positive control was BL21 cells also containing empty vector.*

mechanism of nutrient acquisition in oil palm. The growing knowledge in this area will greatly assist us in designing planting materials with improved nutrient efficiency.

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