BIODIVERSITAS
Volume 21, Number 6, June 2020
Pages: 2462-2466

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210618

Short Communication: Polymorphism of *Fumarate Hydratase* 1 (FUM1) gene associated with nitrogen uptake in oil palm (*Elaeis guineensis*)

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Manuscript received: 12 February 2020. Revision accepted: 12 May 2020.

Abstract. *Maryanto SD, Tanjung ZA, Sudania WM, Kusnandar AS, Roberdi, Pujianto, Utomo C, Liwang T. 2020. Short Communication: Polymorphism of* Fumarate Hydratase *1 (FUM1) gene associated with nitrogen uptake in oil palm (*Elaeis guineensis*). Biodiversitas 21: 2462-2466.* Nitrogen (N) is an essential element for oil palm vegetative growth and fruit development. Fumarase is known to participate in the tricarboxylic acid (TCA) cycle in the mitochondrial matrix. The *FUM* gene is required for fumarate accumulation in leaves and necessary to enhance growth under low nitrogen condition. SNPs were obtained in oil palm *FUM1* gene based on *in silico* analysis using local database. The SNP is further verified with Sanger sequencing and qPCR analysis. The genomic DNA of oil palm with high and low efficient to N based on phenotype characters was sequenced using Sanger method. The *EgFUM1* gene was located in chromosome 14 and had two SNP positions located in 9.0711 cM and 9.0714 cM. Furthermore, four months oil palm seedlings from three progenies were treated with 30% (N-low) and 100% (control) dosages. Transcription level of *EgFUM1* gene was measured using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) method. The value of fold changes was significantly up-regulated (6.14-fold) on Progeny 1 (high efficient characters); 1.05-fold on Progeny 2 (medium efficient characters); and 1.08-fold on Progeny 3 (low moderate efficient characters) in the leaf. According the result, there was correlation between SNP and transcription level of *EgFUM1* gene. Therefore SNP markers of *EgFUM1* gene could be used in oil palm selection with potential N uptake efficiency.

Keywords: *EgFUM1*, molecular marker, N uptake, polymorphism

INTRODUCTION

Global use of nitrogen in 2007 amounted to 110 million metric tons. It is projected to increase between 125 and 236 million metric tons by the year 2050. The ability of plants to capture nitrogen from the soil is dependent on a number of variables including crop, soil type, and the environment. In many cases up to 50-75% of nitrogen applied to agricultural lands is not used by the crop plants (Good and Beatty 2011). Nitrogen (N) is an essential element for rapid vegetative growth and fruit development of the oil palm (Tinker 2008). N is applied to agricultural systems in large quantities. Deficiency of this nutrient leads to yield losses and triggers complex molecular and physiological responses (Mohidin et al. 2015). The improvement of nutrient absorption efficiency in crops can be achieved by two strategies; (i) adopting more efficient crop management practices in agronomic field such as nutrient rate, timing of application and placement, and (ii) breeding more nutrient efficient cultivars (crop improvement with genetic basis) (Baligar and Fageria 2015). The understanding in molecular and physiology basis of complex mechanism in nutrient usage is needed. Thus, crop improvement with genetic basis can be established (Hawkesford 2011).

Nitrogen absorption efficiency involved complex molecular and physiological mechanisms. Thus, an appropriate method to identify genes involved in N absorption efficiency is needed. The research for N uptake efficiency with genetic basis has been conducted since 2013 using *in silico* analysis approach. The *fumarate hydratase 1* of *Elaeis guineensis* (*EgFUM1*) is one of specific genes associated with N uptake. This gene was obtained from selection of thirty candidate genes with known functions derived from oil palm local database. The expression level among genes was varied in low fertilizer dosage (30 percent fertilizer dosage compares to normal), several genes were up-regulated while others were down-regulated. *EgFUM1* gene had expression correlated with N uptake efficiency based on RT-qPCR method.

Fumarase (FUM, fumarase hydratase; EC.4.2.1.2) catalyzes the reversible hydration of fumarate to L-malate (Zubimendi et al. 2018). Fumarase is known to participate in the tricarboxylic acid (TCA) cycle in the mitochondrial matrix in eukaryotes (Akram 2014), urea cycle, and in amino acids metabolism. Fumarase is localized both in mitochondria and cytosol in all eukaryotes (Yogev et al. 2010). There are two fumarase genes which are *FUM1* and *FUM2*. *FUM1* encodes a protein with mitochondrial targeting information. The *FUM1* gene is expressed as a

single translation product, which is distributed between the cytosol and the mitochondria (Heazlewood and Millar 2005). The mitochondrial *FUM1* is an essential gene, while cytosolic *FUM2* is not required for plant growth. *FUM2* is required for the massive accumulation of fumarate in *Arabidopsis* leaves (Pracharoenwattana et al. 2010). In *Arabidopsis thaliana*, *FUM1* could act in concert with *FUM2* for the accumulation of fumarate of the TCA cycle (Yogev et al. 2011).

DNA based marker could be develops from single base difference between the same DNA fragments. It could cause by differences in the base in the same locus as well as the occurrence of INDEL (insertion and deletion) as a result of the diversity of DNA sequence in plant genomes which is called Single Nucleotide Polymorphism (SNP). SNP is a single base pair position in genomic DNA at which different sequence alternatives or alleles exist in normal individuals within population. Recently, there is a considerable interest in the development of SNP based marker system. SNP is the most common form of sequence variation between individuals within a species (Pootakham et al. 2013).

SNPs have been widely used for advance quantitative, functional and evolutionary genomics (Mammadov et al. 2012; Sutanto et al. 2013). SNPs are ideal molecular markers due to their higher abundance (Roorkiwal et al. 2013). SNP markers have been applied to select nitrogen use efficiency in rice (*Oryza sativa*) (Duan and Zhang 2015). Transcriptome study could be used to validate the SNP markers. Wang et al. (2000) reported that the correlation between gene expression level and variation of DNA sequences as a response to nitrogen treatment. This correlation will be helpful for developing DNA based marker which is cheaper and easy in compared to RNA based markers. Furthermore, polymorphism was obtained on cytosolic fumarase (FUM2) gene in *Arabidopsis thaliana*. This gene has function in carbon assimilation and nitrogen utilization. Polymorphism was reported as insertion or deletion (InDel) polymorphism located on two stretches of 2.1 kb and 3.8 kb. The effect of polymorphism in *Arabidopsis thaliana* was reduced FUM2 mRNA expression, reduced fumarase activity, reduced fumarate or malate ratio in leaves, malate and fumarate levels, and with dry weight at 15 days after sowing (DAS) (Riewe et al. 2016) . There has been no reported polymorphism on FUM gene in oil palm, so far. Therefore, the study aimed to obtain SNP of *fumarate hydratase 1* of *E. guineensis* (*EgFUM1*) that might associated with efficient N uptake in oil palm.

MATERIALS AND METHODS

Materials

The planting materials for leaf sample unit (LSU), SNP and gene expression analysis were Tenera (DxP) oil palms. These Tenera were produced from crossing between three different Dura and single Pisifera. The Dura palms were selected based on their N uptake efficiency (high, moderate and low) by measured of N-contents in leaves (previous research-not published). Thus Tenera oil palms in this study consisted of Progeny 1 which is high N uptake efficiency group, Progeny 2 is moderate N uptake efficiency group and progeny 3 is low N uptake efficiency group.

Methods

Main nursery trial

Randomized complete block design with two factors namely: progeny and levels of N fertilizer, with three replications was used as the experimental design. There was consisted of 3 progenies x 2 fertilizer dosage x 3 replication x 3 repetition = 54 palm. Real-time qPCR analysis comprised of progeny 1, progeny 2, and progeny 3. Four months old oil palm seedlings from 3 progenies were treated with two selected levels of N, i.e. 30% and 100% dosages. Two selected dosages are based on effect of N fertilizer treatment to the growth of oil palm progeny in the main nursery (Fadhila 2015). Some progenies were have good growth performance where treated with 30% of N compared to recommendation dosage (100%). The leaf samples from 26 selected palms, 13 of each from progeny 1 and progeny 3, were used for SNP analysis. Furthermore, leaf and root tissues from three progenies were collected for RT-qPCR at seven months old palm.

LSU Analysis

Leaflets are sampled from leaf at seven months old palm. Leaf samples were dried in a forced draft oven at 70-80^0 C. Leaf samples of54 individual palms were brought to laboratory to estimate their N content using Kjeldahl method (Gholizadeh et al. 2009; Rahmawati and Santoso 2017). Furthermore, progenies were divided into two groups (high and low) based on critical value (2.50%) of N-content (Fairhurst et al. 2015).

RNA, DNA isolation and construction of single-strand cDNA

Leaf and root samples from three progenies were extracted using RNeasy® Plant Mini Kit (Cat. no 74904, Qiagen, Hilden, Germany), while total DNA from 26 plants were extracted using GenEluteTM Plant Genomic DNA Miniprep Kit (Cat. no. G2N350, Sigma-Aldrich, Missouri, USA). Quantity and quality of RNA and DNA were measured by absorbance measurement at 260, 280 and 230 nm wavelength using NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, Massachusetts, USA). DNA integrity was visualized using 1% agarose gel (Bioron, Ludwigshafen, Germany) electrophoresis in TAE buffer 1x (0.04 M Tris, 0.001 M EDTA-Na_2 $2H_2O$, 0.02 M acetic acid pH 8.5). Single strand cDNA was constructed from total RNA using QuantiTect® Reverse Transcription Kit (Cat. no. 205313, Qiagen, Hilden, Germany).

Gene expression analysis

Gene expression analysis was conducted using RT-qPCR method (Applied Biosystems Fast 7500/7500 machine, USA). The reagent was conducted using Quantifast SYBR Green PCR Kit (Catalog Number 204054, Qiagen, Hilden, Germany). RT-qPCR was applied for fumarate hydratase 1 (EgFUM1) gene. In silico study associated to N uptake from local database was carried.

Primer real-time qPCR was design using Primer 3 online software (https://www.ncbi.nlm.nih.gov). There was designed to amplify all SNP position targeted in the EgFUM1gene (Table 1). Gene expressions associated with nitrogen uptake on root and leaf were analyzed using relative quantification (Feckler et al. 2017). Cq value and quantity value of the sample were obtained automatically from the qPCR analysis software v2.0.6 7500 while Relative Quantitation (RQ) value was calculated using formula based on Guide from Applied Biosystems (Applied Biosystems 2008).

Sequencing and data analysis

The purified DNA was sequenced using Sanger method at 1st Base Sequencing (Singapore). Primer was used to polymerase chain reaction and Sanger-sequencing showed on Table 2. SNP targeting was analyzed with *Geneious version 9.0.5* software (Biomatters Ltd) while statistical analysis by two by two table analysis with *Simple Interactive Statistical Analysis* (SISA) online software (http://www.quantitativeskills.com).

Table 1. *EgFUM1* gene primer was used to real-time qPCR

Primer Name	Primer Sequence (5'-3')	Size (bp)
q-FUMF	TAACCCAGTTCCAACGGCAG	175
q-FUMR	GGTAAACACAATTTCCGCTTCTCT	

*Note: F: Forward primer; R: Reverse primer

Table 2. *EgFUM1* gene primer was used to sequencing

Primer Name	Primer Sequence (5'-3')	Size (bp)
snpFUMF	AGCAACAGGATCACATAAGACA	904
snpFUMR	CGGGTACCATTTTGTGTTCGG	

*Note: F: Forward primer; R: Reverse primer

RESULTS AND DISCUSSION

Phenotypic characters

According to N-content analysis, the 54 individual palm divided into two groups (high and low). The leaf nitrogen content in high group were up to 2.50%, ranged from 2.60% to 2.89% (left), while in low group were below 2.50%, ranged from 1.99-2.34% (right) (Figure 1). Thirteen palms of the highest (2.69-2.89%) N-content and thirteen palms of the lowest (1.99-2.21) N-content were selected to determine of polymorphism of *EgFUM1* in oil palm.

Single nucleotide polymorphism

Twenty six oil palm samples consisted of 13 palms with extreme high and low N uptake efficiency was analyzed. The *EgFUM1* gene have 10,796 base pairs totally genome region; 1,497 base pairs coding sequence; 17 exon and 16 intron. The amplicon length of *EgFUM1* gene was 904 base pairs that located of significantly SNP targeted. Two SNPs motifs were found among samples (Table 3). Statistical analysis of SNPs was shown in Table 4, SNPs obtained on 9.0711 cM and 9.0714 cM position was strong positive correlation. It might be both 9.0711 cM and 9.0714 SNP positions has correlation with phenotypic characters. The allele variants in 9.0711 cM were AA (prolific), AG (neutral), and GG (non-prolific) genotype, while in 9.0714 cM position were TT (prolific), TA (neutral), and AA (non-prolific) genotype. The AA and TT genotype was related high efficiency; AG and AT genotype was related with moderate efficiency, while GG and AA genotype was related with low N efficiency uptake in both SNP positions, respectively.

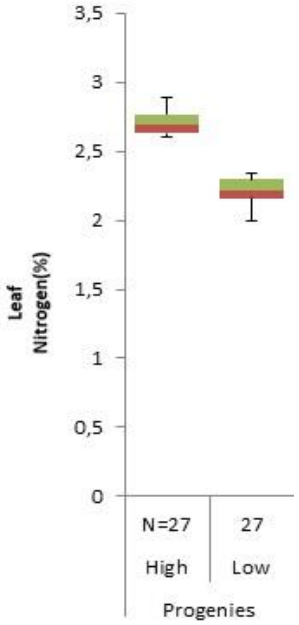

Figure 1. Nitrogen content in leaf

Table 4. Statistical analysis of SNPs EgFUM1 gene

SNP (cM)	Flanking	Allele	Genotype	Efficient (N+)	Not efficient (N-)	P-value	Odds ratio (OR)	95% Confidence interval (CI)	Finding
9.0711	TG(A>G)GT	A, G	AA	12	6	6,5	14	1.385 >14> 141.486	Significant, strong positive correlation
			Non AA	1	7	6,5	0,07	0.007 >0.071> 0.722	
9.0714	GT(A>T)TT	A, T	TT	12	1	18,6	144	8.043 >144> 2578.099	Significant, strong positive correlation
			Non TT	1	12	18,6	0.007	0 >0.007> 0.124	

Table 3. SNPs of *EgFUM1* gene in oil palm

Sample	Progeny	Phenotype	Position	
			9.0711 cM	9.0714 cM
S1	P1	High efficient	A	T
S2	P1	High efficient	A	T
S3	P1	High efficient	A	T
S4	P1	High efficient	A	T
S5	P1	High efficient	A	T
S6	P1	High efficient	A	T
S7	P1	High efficient	A	T
S8	P1	High efficient	A	T
S9	P1	High efficient	A	T
S10	P1	High efficient	A	T
S11	P1	High efficient	A	T
S12	P1	High efficient	G	W
S13	P1	High efficient	A	T
S14	P3	Low efficient	R	W
S15	P3	Low efficient	R	W
S16	P3	Low efficient	R	W
S17	P3	Low efficient	A	T
S18	P3	Low efficient	A	W
S19	P3	Low efficient	R	W
S20	P3	Low efficient	A	W
S21	P3	Low efficient	R	W
S22	P3	Low efficient	R	W
S23	P3	Low efficient	A	W
S24	P3	Low efficient	R	W
S25	P3	Low efficient	A	W
S26	P3	Low efficient	A	W

*Note: A: adenine; G: guanine; C: cytosine; T: thymine; R: adenine or guanine; W: adenine or thymine; S: sample code

Gene expression

SNPs were validated by measured of *EgFUM1* gene transcription level using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) method. Expression level of *EgFUM1* gene in the leaf and root were shown in Figure 2. The transcription level of *EgFUM1* in leaf of progeny1was up-regulated 6.14-fold, while in other progenies, the transcription level only 1.05-fold (P2); and 1.08-fold (P3) consecutively. The expression level of *EgFUM1*in root were 1.50, 1.83, and 1.84-fold for P1, P2 and P3, respectively (Figure 2).

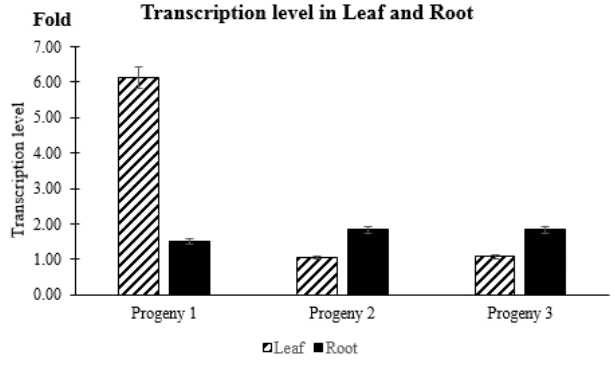

Figure 2. The expression level in leaf and root of oil palm

Discussion

Molecular marker can be used to identify both useful genotypes for inclusion in breeding and interesting progeny or variety for further study (Daryono and Maryanto. 2018). Recently, there has been considerable interest in the development of single nucleotide polymorphism (SNP) based marker system. Single Nucleotide Polymorphism (SNP) is a type of the molecular marker which can be used as a selection tool and crop genetic diversity analysis (Borlay et al. 2017).

The *EgFUM1* gene polymorphism is associated with N uptake. The FUM gene participates in the tricarboxylic acid (TCA) cycle. It is found within mitochondria and cytosolic in plant. In *Arabidopsis thaliana* possesses two FUM genes consisted of FUM1 and FUM2 (Eprintsev et al. 2017). The FUM1 gene was encoded for the mitochondrial isoform while the FUM2 for the cytosolic fumarase (Pracharoenwattana et al. 2010). In the TCA cycle, the FUM1 is an essential gene while the FUM2 is for the major fumarase activity measured in leaves (Zell et al. 2010). Those genes were mainly required for the massive fumarate accumulation during the day (12 h) in plants grown under high nitrogen (N). In fact, the FUM2 acts in the direction of fumarate synthesis (L-malate dehydratase-MD-activity) during the N-rich autotrophic phase. However, the stored fumarate was mobilized for replenishing TCA intermediates and for respiration at night (Araújo et al. 2011; Chia et al. 2000). The FUM2 would run in the opposite direction (fumarate hydratase-FH-activity) which was supported the heterotrophic growth stage. It has been demonstrated that L-malate dehydrogenase (MDH) protein abundance and the FUM1 activity was increased during the day (12 h) in *Arabidopsis* (Lee et al. 2010). The FUM1 could act in line with the FUM2 for the accumulation of fumarate through a reductive branch of the TCA cycle (Sweetlove et al. 2010).

Accumulation of fumarate in leaves would increase of expression level the *EgFUM1*. The function of *EgFUM1* is to facilitate the production of energy which is required for rapid nitrogen assimilation and growth. However, in this study, the transcription level of *EgFUM1* was elevated on low N (30% N-fertilizer dosage) condition. This low N condition was determined on the leaf tissue of progeny 1, which transcription level was up-regulated 6.14-fold. In other progenies, the transcription level only 1.05-fold (P2); and 1.08-fold (P3) consecutively. Progeny 1 with higher transcription level might correlate with SNPs in the *EgFUM1* gene. P1 has genotype AA in 9.0711 cM and TT in 9.0714 cM as dominant homozygote that changed regulation of *EgFUM1* gene. This gene should be shown a low expression or down-regulated on the low nitrogen condition but substitution A become T nucleotides could be elevated the expression gene regulation, while substitution A become G nucleotides in 9.0711 cM position could be decreased of the expression gene. Furthermore, the *EgFUM1* in root expression was up-regulated 1.50-fold (P1); 1.83-fold (P2); and 1.84-fold (P3) consecutively (Figure 2). The transcription level was equal on the three progenies of root samples. It means that not found nitrogen assimilation activities in the root. The key enzymes of

nitrogen assimilation activities only occurs in leaf. There was also related with photosynthesis activities that occurs in leaf (Chia et al. 2000; Tschoep et al. 2009). Furthermore, there was a correlation with accumulation of fumarate taken from leaf organs. Gene expression level has correlation with the variation of DNA sequences as a response to nitrogen treatment in oil palm. The *EgFUM1* in leaf expression was up-regulated could be improved the plant growth. Subsequently there was increasing of energy produced caused by the higher efficient of nitrogen assimilation in the cell.

ACKNOWLEDGEMENTS

We would like to thank Victor Aprilyanto, Reno Tryono, and Marcelinus Rocky Hatorangan for helpful suggestions regarding this manuscript. We also thank to Ranny Hanifah, Fikih Imam Rahman, and Ainun Najib as technician in laboratory and field.

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