# *EgNRT2.3* and *EgNAR2* expression are controlled by nitrogen deprivation and encode proteins that function as a two-component nitrate uptake system in oil palm.

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Keywords: NRT2, NAR2, nitrogen deprivation, two-component nitrate uptake system, Oil palm

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## Abstract

Oil palm (*Elaeis guineensis* Jacq.) is an important crop for oil and biodiesel production. Oil palm plantations require extensive fertilizer additions to achieve a high yield. Fertilizer application decisions and management for oil palm farming rely on leaf tissue and soil nutrient analyses with little information available to describe the key players for nutrient uptake. A molecular understanding of how nutrients, especially nitrogen (N), are taken up in oil palm is very important to improve fertilizer use and formulation practice in oil palm plantations. In this work, two nitrate uptake genes in oil palm, *EgNRT2.3* and *EgNAR2*, were cloned and characterized. Spatial expression analysis showed high expression of these two genes was mainly found in un-lignified young roots. Interestingly, *EgNRT2.3* and *EgNAR2*

were up-regulated by N deprivation, but their expression pattern depended on the form of N source. Promoter analysis of these two genes confirmed the presence of regulatory elements that support these expression patterns. The *Xenopus* oocyte assay showed that EgNRT2.3 and EgNAR2 had to act together to take up nitrate. The results suggest that EgNRT2.3 and EgNAR2 act as a two-component nitrate uptake system in oil palm.

Keywords: NRT2, NAR2, nitrogen deprivation, two-component nitrate uptake system, Oil palm

## Introduction

African oil palm (*Elaeis guineensis* Jacq.) is an economically important crop in Thailand and many Southeastern Asia countries (Meijaard et al., 2020). Oil palm needs a huge amount of fertilizer for growth and development, especially during the early seedling stage. Nitrogen (N) is one of the essential macronutrients for plant growth and development and is typically available in soil in form of ammonium and nitrate. In temperate climates, nitrate is the major form of soil N for uptake by land plants and it is also a signal to trigger nitrate uptake and assimilation (Andrews et al., 2013; Crawford and Glass, 1998). Nitrate uptake in plants consists of two systems, a low-affinity transport system (LATS) and a high-affinity transport system (HATS). LATS includes the nitrate/peptide transporter family (NPF) which sense and transports nitrate in the millimolar (mM) range while HATS usually includes a nitrate transporter2 (NRT2) and a small companion protein called nitrate assimilation related protein2 (NAR2 or NRT3) which operate at micromolar (μM) concentrations of nitrate (Tong et al. 2005; Lezhneva et al., 2014; Feng et al., 2011a).

There are seven members of the NRT2 family in *Arabidopsis* and all have been functionally characterized. AtNRT2.1 is the main family member for nitrate uptake from the

soil but it is less active during N deficiency (Li et al., 2006). AtNRT2.2 also plays a role in nitrate uptake acting together with AtNRT2.1 in high-affinity uptake (Li et al., 2006). AtNRT2.4 and AtNRT2.5 have a role in nitrate influx, particularly in response to nitrate starvation (Kiba et al., 2012; Lezhneva et al., 2014). Additionally, AtNRT2.5 and AtNRT2.6 are important in rhizobacterial symbiosis (Kechid et al., 2013), while AtNRT2.7 governs the nitrate reservoir in seeds (Chopin et al., 2007). The rice genome encodes four NRT2 family members, *OsNRT2.1, OsNRT2.2, OsNRT2.3a,b,* and *OsNRT2.4* and each has a responsibility for nitrate uptake and transport, but each has different characteristics. In rice, OsNRT2.1 and OsNRT2.2 are the major players in nitrate uptake from the soil (Feng et al., 2011a). OsNRT2.3 has two spliced forms which are OsNRT2.3a, taking a role in long-distance nitrate transport, and OsNRT2.3b, involved in pH homeostasis in the cytosol (Fan et al., 2016). OsNRT2.4 has a role in maintaining nitrate transfer between root and shoot (Feng et al., 2011a).

Most NRT2 members need a partner protein called NAR2 for nitrate uptake (Feng et al., 2011a; Kotur et al. 2012), In *Arabidopsis*, there are two *NAR2* genes, *AtNAR2.1* and *AtNAR2.2*, but only AtNAR2.1 acts as a co-operation unit with most members of (Kotur et al., 2012). Similarly, rice also has two *NAR2* genes, *OsNAR2.1* and *OsNAR2.2*. Only OsNAR2.1 cooperates with OsNRT2.1, OsNRT2.2, and OsNRT2.3a for driving high-affinity nitrate uptake (Chen et al., 2017, Chen et al., 2020, Feng et al., 2011b, Naz et al., 2019).

Nutrients are taken up mainly by roots and the root structure of a mature oil palm (Fig. 1A) comprises adventitious primary roots that emerge from the basal cone and grow either downwards from the base of the palm or radically in a horizontal direction (Jourdan and Rey, 1997). The primary roots have a heavily lignified hypodermis and branch out from the secondary and tertiary roots that have a similar structure as the primary roots except for the

un-lignified tips of the growing primary, secondary and tertiary roots. The smallest type of roots but greatest in numbers are the quaternary roots which are only a few centimeters long and almost entirely un-lignified (Jourdan and Rey, 1997) (Fig. 1C). Root development in the juvenile stage (0 - 1 year) of oil palm (Fig. 1B) is mainly composed of primary roots with lignified hypodermis and un-lignified secondary roots (Fig. 1D). There are no root hairs in oil palm (Phosri et al., 2010), therefore un-lignified young root types might play a critical role in nutrient uptake (Rivera-Mendes et al., 2016). However, there is no direct evidence regarding the spatial expression of nutrient transporters in each type of oil palm root.

Oil palm plantations require extensive fertilizer management, especially for added N to achieve high yields. Oil palm growers usually apply N fertilizer as urea which is quickly converted by soil microbes to ammonium and nitrate. Nowadays, fertilizer application decisions in oil palm are solely managed by leaf tissue and soil nutrient analyses. Fundamental knowledge of key players in nutrient uptake especially at the molecular level to help improve fertilizer efficiency in oil palm is not fully investigated. Currently, N transporters have been identified in oil palm but none have been characterized. Moreover, the functions of NRT2/NAR2 as a two-component nitrate uptake system are also still unknown in oil palm. Thus, it is crucial to investigate the expression patterns, responses to fertilizer application, and the possible molecular functions of NRT2 and NAR2 in oil palm. In this work, two full-length nitrate uptake-related genes, *EgNRT2.3* and *EgNAR2* were identified, cloned, and characterized. The expression of these genes under different N application regimes was studied. In addition, promoter sequences of these two genes were cloned and investigated for the presence of regulatory elements. The uptake function of these proteins was studied using the *Xenopus* oocyte assay system. The study of nitrate transporters in oil palm is beneficial not only for a fundamental understanding of N uptake mechanisms but also for improving N use efficiency in this major economically important crop.

## Materials and methods

### Plant material and growth conditions

Oil palm (*Elaeis guineensis* Jacq.), variety Suratthani1 (Dura C 2120:184 D x Pisifera IRH 629:319) provided by Suratthani Oil Palm Research Center, Thailand was used in this study for gene expression analysis and cloning of full-length *EgNRT2.3* and *EgNAR2*. Oil palm juvenile plants (4-5 months old) were grown in a pot (15 cm x 12 cm). The soil comprised 1 part of clay soil, 2 parts of organic matter, and ¼ part of sand. The pH of the soil is about 5.5 – 6.0 which is optimal for oil palm. (Mutert, 1999; Department of Agriculture, 2009). Four types of fertilizer were used in this study; fertilizer with an equal percentage of N-P-K at 15:15:15 (N was in both nitrate and ammonium forms) was used as a commercial controlled fertilizer (Total-N). Two modified N fertilizers, nitrate fertilizer (1.73 g $Ca(NO_3)_2$, 0.5 g $KH_2PO_4$, 0.33 g KCl) and ammonium fertilizer (in form of urea) (0.57 g $CO(NH_2)_2$, 0.5 g $KH_2PO_4$, 0.33 g KCl), were formulated with a ratio of N-P-K at 1.8:1.8:2.6 (see Mutert, 1999). No-N fertilizer treatment was also used with P and K at the same ratio but without any N-source. Juvenile plants (1 plant/pot) were treated with fertilizer or starved of N-supply for 42 days, all fertilizer treatments were applied every two weeks 3 times after starting the experiment with three replications. After 42 days, oil palm tissues including roots and leaves were harvested. Roots from juvenile plants were divided into primary and secondary types. Roots from mature plants were harvested and pooled into two groups which were mature root type (primary and secondary roots) and young root type (tertiary and quaternary roots). Both root types were stored at -80 °C for DNA and RNA analyses. Soil parameters including pH, organic matter, N, P, and K were also collected and analyzed at the time of harvesting (Supplemental Table A16).

**Total RNA isolation and First-strand cDNA synthesis**

RNA was extracted from oil palm root tissues using Invitrogen's Concert ™ Plant RNA Reagent (Invitrogen, USA). The total RNA quality and quantity were checked with NanoDrop™ One Microvolume UV-Vis Spectrophotometers (Thermo Fisher Scientific, USA). All isolated RNA samples were used as a template for cDNA synthesis by using The SuperScript ® III First-Strand Synthesis System (Thermo Fisher Scientific, USA).

**Quantitative real-time PCR (qRT-PCR)**

85 nanograms of cDNA were used as a template and SYBR® Green Realtime PCR Master Mix (QPK-201) (Toyobo, Japan), was used to quantify gene expression. The primers used for this experiment were listed in primer number 17 - 20 in Supplementary Table A13. The mixture reactions were monitored by Applied Biosystems™ 7500 Real-Time PCR (Thermo Fisher Scientific, USA). The PCR conditions included initial denaturation at 95 °C for 10 min, followed by 40 cycles of 2 steps PCR as denaturation at 95 °C for 15 sec, annealing, and extension at 60 °C for 1 min. *EgeIF1* listed in primer number 25,26 in Supplementary Table A13 was used as an internal control.

**Chlorophyll content and C & N content analysis**

Leaf fresh weight and chlorophyll content was measured at the end of the experiments. Fully expanded leaf samples were used to determine total chlorophyll content according to Sumanta et al., 2004. Analysis of the C and N content was conducted using CHN elemental analysis (Perkin-Elmer elemental analyzer, model 2400). Plant tissues were dried at 65 °C for 72 h to achieve a constant weight. Ground samples containing 1.0-3.0 mg were weighed in small tin capsules and submitted to combustion at 925 °C for about 2 min in the

147 combustion box of a Perkin-Elmer CHN elemental analyzer, model 2400. Final concentrations
148 of C, H, and N in each sample were stoichiometrically calculated, and shown as the percentage
149 of elements in the total mass of ground samples.
150
151 **Statistical analysis**
152 The qRT-PCR data included both technical triplicates and triplicates of biological
153 samples. The data was analyzed by using IBM® SPSS® Statistics22 software by using
154 analysis of variance (ANOVA) and tested for significance with the Duncan method at
155 $P < 0.05$.
156
157 **5'/3'RACE PCR, full-length DNA cloning, and DNA sequencing**
158 Cloning of full-length *EgNRT2.3* and *EgNAR2* were carried out using SMARTer®
159 RACE 5'/3' Kit (TaKaRa Bio, Japan) with gene-specific primers (Supplementary Table
160 A13). 5' and 3' RACE reactions were performed by following manufacturer protocol and the
161 condition of PCR as described (Supplementary Table A8, A9). To obtain a full-length DNA
162 sequence of *EgNRT2.3* and *EgNAR2*, a high-fidelity DNA polymerase; Phusion™ (Thermo
163 Fisher Scientific, USA) DNA polymerase enzyme was used. The PCR conditions were
164 described in Supplementary Table A10. All PCR products were ligated with pGEM® - T Easy
165 vector (Promega, USA) with A-tailing ligation. All samples were submitted to MACROGEN
166 INC. (Korea) for DNA sequencing.
167
168 ***EgNRT2.3* and *EgNAR2* promoter cloning**
169 The putative promoter domains of *EgNRT2.3* and *EgNAR2* were isolated by
170 amplification of 3,000 bases and 1,000 bases upstream from the start codon of *EgNRT2.3* and

*EgNAR2* respectively. The PCR reaction was performed using a high-fidelity DNA polymerase; Phusion™ (Thermo Fisher Scientific, USA enzyme. The PCR primers were listed in Supplementary Table A13 and PCR conditions were described in Supplementary Table A11. PCR product was ligated to pGEM®-T Easy vector (Promega, USA) with A-tailing ligation and sent to MACROGEN INC. (Korea) for DNA sequencing.

**Oligonucleotide primers design**

All oligonucleotide primers were constructed by using the Primer3Plus program (https://primer3plus.com/) and the Oligo Analyzer program (https://sg.idtdna.com/calc/analyzer). All primers used are listed in Supplementary Table A13.

***In silico* analysis**

The retrieved DNA sequence was analysed in the blastn NCBI software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the amino acid alignment was determined by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Genedoc programs (ver. 2.7). A phylogenetic relationship was drawn using the MEGA7 program (Kumar et al., 2016). The membrane topology was constructed by using Protter platform ver. 1.0 (http://wlab.ethz.ch/protter/start/) (Omasits et al., 2013). The STRING ver11.0 database (https://string-db.org/) was used to predict the protein-protein interaction of EgNRT2.3 and EgNAR2. The promoter analysis was accomplished using PlantCARE (https://bio.tools/plantcare) and PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/) databases.

**cRNA preparation and *Xenopus* oocyte assay**

pGEM®-T Easy *EgNRT2.3* and pGEM® - T Easy *EgNAR2* were amplified and then subcloned to pT7TS vector (Tong et al., 2005) by using an In-Fusion® HD Cloning Kit (TaKaRa Bio, USA). The procedures were followed by the manufacturer's recommendation and PCR conditions are shown in Supplementary Table A12. One microgram of the subcloning PT7TS vectors was linearized with *XbaI* (Thermo Scientific™, UK) according to the manufacturer-recommended and used with mMESSAGE mMACHINE® T7 Transcription Kit (Ambion, USA) for cRNA synthesis. *In vitro* transcription was followed using the company's instructions. Preparation of *Xenopus* oocytes and injection of cRNA was performed as described previously (Tong et al., 2005). The cRNA-injected oocytes were incubated with 10 mM $^{15}N$ - $NaNO_3$ to monitor nitrate uptake (Tong et al., 2005). The δ15N was measured by a Finnigan Delta plus XP isotope ratio mass spectrometer (IRMS, Thermo Fisher Scientific).

## Results

### Identification and *in silico* analysis of full-length *EgNRT2.3* and *EgNAR2*

In this study, two nitrate uptake-related genes, *EgNRT2.3* and *EgNAR2* were identified and cloned from oil palm. *EgNRT2.3* (LOC105046035) and *EgNAR2* (LOC105051272) genes have whole coding sequences of 1533 bp and 612 bp, encoding 510 and 203 amino acid residues and were predicted to locate on chromosome 5 and chromosome 9 respectively (Singh et al., 2013). Membrane topology prediction results showed that EgNRT2.3 has 12 transmembrane spanning domains while EgNAR2 contains only 1 domain (Supplementary Figs. B1A, B1B). Sequence analysis of *EgNRT2.3* revealed the conserved protein sequences of the major facilitator superfamily (MFS) domain which are (AGGWGN(X)MLG) and PFV(XX)R(X)LGLI(X)GMT(XX)GG (Forde, 2000; Trueman et

al. 1996; Pao et al. 1998) (Supplementary Fig. B2) while *EgNAR2* gene contains a conserved NAR family motif which is K(2)K(2)LCY(2)S(3)RxWR(3) (Tong et al., 2005) (Supplementary Figs. B1B, B3).

In addition, both *EgNRT2.3* and *EgNAR2* amino acid sequences showed high identity with rice genes *OsNRT2.3a* and *OsNAR2.1* at 75.2% and 62.6 % respectively. (Supplementary Figs. B4, B5). The phylogenetic relationships of NRT2 and NAR2 proteins revealed that EgNRT2.3 and EgNAR2 are closely related to OsNRT2.3a and OsNAR2.1 in rice respectively (see Supplementary Figs. B1C, B1D).

**Spatial expression study of *EgNRT2.3* and *EgNAR2* in oil palm**

To study the function of *EgNRT2.3* and *EgNAR2* in oil palm, a spatial expression study was performed. Different tissue samples from mature oil palms including mature roots (pooled $1^o$ + $2^o$ roots), young roots (pooled $3^o$ + $4^o$ roots), young leaves, and mature leaves (Fig. 1D) were collected to observe the expression of *EgNRT2.3* and *EgNAR2*. The results showed that high expression levels of *EgNRT2.3* and *EgNAR2* were primarily in un-lignified young roots with very low expression in mature roots (Fig. 2A, 2B). Low expression of both genes was also found in leaf tissues except for the expression of *EgNAR2* in young leaves which was comparable to young roots (Fig. 2B). Altogether, younger root and leaf parts closer to the meristems were shown to be the main tissue sites of *EgNRT2.3* and *EgNAR2* expression.

**Expression of *EgNRT2.3* and *EgNAR2* was upregulated during N-deprivation**

It was found that juvenile oil palms treated with N-fertilizer had a greener appearance (Fig. 3A) with significantly more leaf fresh weight, total chlorophyll and N content but with a

much lower C/N ratio when compared to non-N-fertilized plant. (Fig. 3B, 3C, 3D and 3E). To study the expression patterns of *EgNRT2.3* and *EgNAR2* in these oil palms, qualitative expression analyses of both genes were examined in different tissues in juvenile oil palms treated with or without N-fertilizer. The results showed significant up-regulation of *EgNRT2.3* expression in young roots (R2) of non-N-fertilized plants (Fig. 4A). A similar situation was also observed in old roots (R1) but with a less degree of up-regulation of *EgNRT2.3* expression. Likewise, up-regulation of *EgNAR2* expression was also found in R1 and R2 roots of non-N-fertilized plants (Fig. 4B). Altogether, these results suggested the up-regulation pattern of both genes during N-deprivation in oil palm.

**Expression responses of *EgNRT2.3* and *EgNAR2* to different N sources**

To further analyze the N-source that might play a role in the N-deprivation response of *EgNRT2.3* and *EgNAR2*, varied N-source experiments were conducted by applying juvenile oil palms with nitrate, ammonium, and total-N fertilizers and compared with non-N-fertilized plants as a control. It was shown that N contents in leaf tissues were different depending on the availability and form of N sources when the P and K supply in each treatment were not significantly different (Supplemental Table A17). The *EgNRT2.3* transcript showed significant up-regulation in non-N-fertilized plants compared to other treatments (Fig. 5A). Meanwhile, *EgNAR2* expression also showed a similar expression pattern to *EgNRT2.3* except in the nitrate treatment when *EgNAR2* expression was significantly higher than with ammonium, total-N treatments, and plants that had received no N-fertilizer (Fig. 5B).

**Identification and *in silico* analysis of possibly N, ammonium, and N deprivation responsive elements of *EgNRT2.3* and *EgNAR2* promoters**

To study transcriptional regulation of *EgNRT2.3* and *EgNAR2* in oil palm, the promoter sequences of both genes have been investigated. We successfully cloned and sequenced 3000 bases upstream sequence of *EgNRT2.3* and 1000 bases upstream sequence of *EgNAR2* to explore any nitrate or nitrogen responsive domains (Supplementary Figs. B6, B7). The sequences of *EgNRT2.3* and *EgNAR2* promoters were analyzed using PlantCARE and PlantPAN 3.0 databases. The results indicated that both *EgNRT2.3* and *EgNAR2* promoter sequences consist of many members of Arabidopsis NIGT1/HRS1/HHO family elements which play an important role in N deprivation response (Kiba et al., 2018) including HHO2, HHO3, and HRS1 as shown in table A14. Furthermore, *EgNRT2.3* and *EgNAR2* promoter sequences also contain many N responsive, N metabolism, auxin-responsive, and light-responsive promoter elements. The list of putative nitrate responsive elements was shown in Table A15. Some examples include, myb-like transcription factor (TF) family members which corresponded to suppression of N starvation (Kiba et al., 2018), Arabidopsis response regulator11 (ARR11) which positively responds to nitrate during root development (Gifford et al., 2008), bHLH DNA-binding protein which corresponds to nitrate trigger for root formation (Gaudinier et al., 2018) and a Dof TF which modulates nitrate and C metabolism (Tsujimoto-Inui et al., 2008). Moreover, both *EgNRT2.3* and *EgNAR2* promoters also contain some sequences that may involve nitrate responses, such as (5'-GATA-3'); (Bi et al., 2005), (5'-A(C/G) TCA-3'); (Hwang et al., 1997), and (5'-GACtCTTN10AAG-3'); (Konishi and Yanagisawa, 2010) (Supplementary Fig. B8).

In addition, some sequences involved with the regulation of ammonium uptake and transport were also identified. For instance, GATA motif (5'-GAT(A/T)A-3') (Howitt and Udvardi, 2000), elements for Dof family (5'-AAAG-3', 5'-CTTT-3') (Wu et al., 2017) and bHLH transcription factor (5'-CACGTG-3') (Chiasson et al., 2014) were found in both *EgNRT2.3* and *EgNAR2* promoters. (Supplementary Fig. B9).

292

293 **Nitrate uptake function of EgNRT2.3 and EgNAR2 in *Xenopus* oocyte system**

294 To investigate nitrate uptake function and test for a two-component nitrate transport
295 system heterologous expression of *EgNRT2.3* and *EgNAR2* was done using the *Xenopus*
296 oocyte system. Co-injection of *OsNRT2.3a* and *OsNAR2.1* was assayed at pH5.5 and pH7.5
297 as a positive control. The results indicated that a significant increase in nitrate uptake was
298 only found when *EgNRT2.3* and *EgNAR2* were co-injected (Fig. 6). However, no difference
299 was found when the cRNA of each gene was injected alone compared to the water-injected
300 oocytes. This result suggested that EgNRT2.3 and EgNAR2 operate together as a two-
301 component nitrate transport system. In addition, co-injection of *EgNRT2.3* and *EgNAR2* at
302 pH 5.5 showed larger $^{15}$N-nitrate accumulation than at pH 7.5 (Fig. 6) which indicated the
303 possibility that *EgNRT2.3* could be a nitrate-proton symporter.

304

305 **Discussion**

306 ***EgNRT2.3* and *EgNAR2* sequence analyses suggested a function as a two-component**
307 **nitrate uptake system**

308 *EgNRT2 and EgNAR2* genes were cloned from oil palm and characterized to
309 investigate expression, regulations, and possible functions with the hypothesis that EgNRT2
310 and EgNAR2 work together in a two-component system to take up nitrate in oil palm.
311 Currently, the NCBI database contains 3 putative *EgNRT2* family members from oil palm
312 which are XM_010924517.2, XM_010924814.2, and XM_010928825.2, and categorized into
313 2 different subgroups while only one NAR2 family member from oil palm
314 (XM_010931613.2) is found. In this study, we cloned *EgNRT2.3* (XM_010924517.2) and
315 *EgNAR2* (XM_010931613.2). Phylogenetic analyses showed the closest family members of

316 *Eg*NRT2.3 and *Eg*NAR2 were from rice (Supplementary Figs. B1C, B1D) which is
317 reasonable since both plants are monocots. A highly conserved NNP domain has been found
318 in *Eg*NRT2.3 and a topology domain prediction also exhibited 12 transmembrane domains
319 which is a typical character of a high-affinity transporter NRT2. *Eg*NRT2.3 also has a long
320 C-terminus which is generally found in higher plant NRT2s and may be important for
321 co-operation with NAR2 in nitrate uptake function (Tong et al., 2005). These results
322 indicated that EgNRT2.3 has all the sequence features of the NRT2 family, which has a high-
323 affinity nitrate transport function and takes up nitrate at low concentrations. Meanwhile,
324 *EgNAR2* contains a conserved NAR family motif and membrane topology prediction of one
325 transmembrane domain in EgNAR2 which is similar to CrNAR2.1 (Zhou et al., 2000).
326 Besides, the *Eg*NAR2 amino acid sequence shows the conserved residues R100 and D109 of
327 OsNAR2.1 (Supplementary Fig. B4) which are essential for OsNRT2.3a/OsNAR2.1
328 interaction at the plasma membrane (Liu et al., 2014b). In addition, protein-protein
329 interaction analysis using the STRING database also confirmed a potential interaction
330 between *Eg*NRT2.3 and *Eg*NAR2 (Supplementary Fig. B10). Therefore, these results suggest
331 the interplay between *Eg*NRT2.3 and *Eg*NAR2 for nitrate transport in oil palm.
332 It is also interesting that the bioinformatics analysis of oil palm genome revealed that there
333 are only two members of NRT2 family, *EgNRT2.3* and *EgNRT2.4* (Supplementary Table A2)
334 when many plants including rice also contain NRT2.1 and NRT2.2 (Feng et al., 2011a, Li et
335 al., 2006, Wang et al., 2018) family members. This characteristic might be unique to the palm
336 order (Aracales order) in family Arecaceae since there are only *PdNRT2.3* and *PdNRT2.4* in
337 date palm (*Phoenix dactylifera*) (NCBI database).
338
339 In addition, studies with NRT2.4 in Arabidopsis and rice showed that it does not require the
340 co-operation of NAR2 for nitrate transport (Kiba et al., 2012, Wei et al., 2018). However, we

341 could not identify the predicted EgNRT2.4 in our work which might depend on the oil palm
342 genetic background. Therefore, this information led us to focus only on EgNRT2.3 and
343 EgNAR2 and hypothesized that EgNRT2.3 and EgNAR2.1 function as two-component
344 nitrate transport, thereby providing the only NRT2-type nitrate uptake system in oil palm.
345
346 **Growth analyses of juvenile oil palm showed responses to N fertilizer application**
347 N is a very important element for plant growth and it is crucial for chlorophyll synthesis (Liu
348 et al., 2014a). Our results revealed that the N-fertilized juvenile oil palms appeared greener
349 leafy (Fig. 3A) and showed the increase of leaf fresh weight, total chlorophyll content, and
350 total N content when compared to N-depleted plants (Fig. 3B, 3C, and 3D). These results
351 from oil palm confirmed the importance of N for oil palm growth during the experiment. In
352 addition, a higher C/N ratio found in N-deprived juvenile oil palm (Fig. 3D) suggested the
353 C/N balance might control the expression of *EgNRT2.3* and *EgNAR2*. Typically, the C/N
354 ratio is an indicator of plant N status because it reflects the balancing between C and N
355 metabolites and internal nutrient status (Zheng, 2009). An increased C/N ratio number has
356 been found in N deprivation (Krapp and Traong, 2006). Therefore, N deprivation in oil palm
357 might affect the expression of genes involved in N uptake and N metabolism in oil palm.
358 Another interesting point is that oil palm only very slowly exhibited an increased C/N ratio
359 with a duration of 42 days. This prolonged feedback was found for potassium in *Pinus*
360 *resinosa,* it displayed a very slow response to potassium fertilizer after 25 years of
361 application (Miller et al., 1979), so slower-growing woody plants like oil palm or pine might
362 show prolonged nutrient dynamic responses after fertilizer addition when compared with
363 herbaceous plants like Arabidopsis.
364

**Expression of *EgNRT2* and *EgNAR2* genes found mainly in young roots and affected by N deprivation**

In this study *EgNRT2.3* and *EgNAR2* expression in oil palm was reported for the first time and it was shown that *EgNRT2.3* and *EgNAR2* was expressed abundantly in young roots but was less in older root types. These results seem reasonable since older roots usually have lignified hypodermis whereas younger and un-lignified root cells are more active likely to play a role in nutrient absorption (Rivera-Mendes et al., 2016). In fact, oil palm does not have root hairs. Therefore, the majority of nutrient uptake might take place at the un-lignified young root region by allowing the absorption of water and nutrients from the rhizosphere (Jourdan and Rey, 1997). This finding was similar to *OsNRT2.3a* and *OsNAR2.1* expression patterns in rice, in which both genes localize at the root tip zone (Feng et al.,2011b). Previously, the feeder roots (young roots with less or no lignified cell walls) were suggested to actively absorb water in oil palm (Rivera-Mendes et al., 2016; Intara et al., 2018). However, this is the first report showing differential nutrient transporter expression in each order of roots in oil palm. This novel result suggested the potential use of feeder root density as a phenotypic marker for high N use efficiency (NUE) in oil palm breeding programs in a similar fashion to this selection trait in grapevine (Cuneo et al., 2018).

**Expression of *EgNRT2.3* and *EgNAR2* genes in juvenile oil palm responded differently to nitrate and ammonium**

Our results showed that both *EgNRT2.3* and *EgNAR2* were up-regulated significantly in plants when N starved (Fig. 4A, 4B). Expression of some NRT2 and NAR2 family members are induced by N deprivation (Lejay et al., 1999; Kiba et al., 2012; Lezhneva et al., 2014, In addition, our results also showed the decrease of *EgNRT2.3* and *EgNAR2* transcripts

after ammonium exposure. This down-regulation had been found with *OsNRT2.1*, *OsNRT2.2*, *OsNRT2.3a,* and *OsNRT2.3b* under exposure to ammonium treatment (Feng et al., 2011b). Besides, *AtNRT2.5* in Arabidopsis was also characterized as a component of HATS (Lezhneva et al., 2014), therefore EgNRT2.3 together with EgNAR2 might function in HATS in response to prolonged N deprivation treatment. However, more extensive kinetic studies on both proteins are required to address this possibility.

Interestingly, the expression of *EgNAR2* responded to nitrate supply in Fig. 5B and showed a similar pattern to the N-depleted treatment. This scenario was interesting since N in form of nitrate did not inhibit *EgNAR2* expression but N in form of ammonium could do so. The possible reason might depend on the N pools within soils of both treatments that contained different availability of nitrate and ammonium. From phylogenetic analysis, there are few members of NRT2 and AMTs with only one member of urea transporter (DUR3) in oil palm (Supplementary Fig. B12). Generally, urea is quickly converted to ammonium by urease activity from microorganisms in the soil (Pinton, et al., 2016, Wang et al., 2008, Watson et al., 1994) (Supplementary Fig.B11). Subsequently, ammonium is rapidly oxidized to nitrite and nitrate by microbially-mediated nitrification (Norton and Ouyang, 2019). Conversely, in anaerobic soil nitrate fertilizer may be reduced to nitrite and ammonium by dissimilatory nitrate reduction to ammonium (DNRA) processes by DNRA-capable bacteria and fungi (Philippot, 2005). However, it was reported that DNRA did not provide a significant contribution to ammonium pools (Inselsbacher et al., 2010). Similarly, it was found that DNRA accounted for only 3% of N mineralization in a tropical forest system (Silver et al., 2005). This evidence indicates that low ammonium pools in nitrate-treated soil might cause up-regulation of *EgNAR2.* Therefore, the possibility that regulation of *EgNAR2* during the N-starvation period was controlled mainly by ammonium availability while *EgNRT2.3* may involve both nitrate and ammonium status suggest that rhizosphere N

cycling may be important for oil palm. However, further experiments are needed to explore this scenario in oil palm plantations including rhizosphere microbiome analysis to identify changes in N cycling organisms. There may be an opportunity to improve oil palm NUE by inoculation of seedling roots.

**The bioinformatic analysis of *EgNRT2.3* and *EgNAR2* promoter identify N, ammonium, and N deprivation responsive elements**

The analysis of *EgNRT2.3* and *EgNAR2* promoter domains suggested the possible regulation of both genes by similar factors including nitrate regulation, N metabolism, and C metabolism. The significant sequences are several NIGT1/HRS1 HHO family elements (Kiba et al., 2018) in *EgNRT2.3* and *EgNAR2* promoters which supported the correspond to N deprivation in this study. Previous studies showed that both nitrate and C supply can regulate the expression of *NRT2* or *NAR2* (Feng et al., 2011b; Krouk et al., 2010). However, *EgNRT2.3* and *EgNAR2* expression need further experiments to confirm the impact of C supply on *EgNRT2.3* and *EgNAR2* expression. It is also interesting that the *EgNAR2* promoter domain location is very similar to that of *OsNAR2.1* (Feng et al., 2011b). Feng et al., 2011b demonstrated that the sequence, 5'-GACtCTTN10AAG-3', was crucial for the regulation of *OsNAR2.1* by nitrate. Furthermore, it was found that *OsNAR2.1* expression was affected by N and C supplies. In our results, a high C/N ratio in N-depleted juvenile oil palm was also found which suggested the possible correlation of the C/N ratio and *EgNAR2* expression.

Regarding *EgNRT2.3* and *EgNAR2* expression in Fig. 5, *in silico* analysis of promoter domains of *EgNRT2.3* and *EgNAR2* also showed various important DNA motifs that are important in the modulation of ammonium transport (Chiasson et al., 2014), ammonium

438 uptake (Santos et al., 2012 Wu et al., 2017) and regulation of ammonium metabolism (Howitt
439 and Udvardi, 2000) in other species. The GATA factor recognizes the 5'-GAT(A/T) A-3'
440 sequence to turn on the modulation of N responses (Howitt and Udvardi, 2000). The
441 sequencing of *EgNRT2.3* and *EgNAR2* promoters also revealed 7 and 3 copies of the
442 recognition binding site of GATA motif respectively. Thus, it can be implied that *EgNRT2.3*
443 and *EgNAR2* might also be regulated by ammonium. In addition, it is interesting to point out
444 that a GATA motif is also important to control two nitrate reductase genes, *NR1* and *NR2*
445 (Jensen et al., 1996) so the GATA motif might be important for the interplay between nitrate
446 and ammonium regulation. Down-regulation of *NRT2* genes was found under exposure to
447 ammonium treatment with *OsNRT2.1*, *OsNRT2.2*, *OsNRT2.3a,* and *OsNRT2.3b* but less
448 sensitivity in *OsNAR2.1* expression (Feng et al., 2011b).

449

450 **Co-expression of *Eg*NRT2.3 and *Eg*NAR2 in *Xenopu*s oocyte demonstrated a two-**
451 **component system to uptake nitrate**

452 Most NRT2 family members co-operate with NAR2 to transport nitrate transport
453 function (Kotur et al., 2012; Yan et al., 2011). The result in Fig 6 that confirmed the co-
454 function of EgNRT2.3/EgNAR2 was consistent with many previous studies in other plants
455 like *C. reinhardtii* (Zhou et al., 2000) *Arabidopsis* (Orsel et al., 2006), *O. sativa* (Yan et al.,
456 2011), *H. vulgare* (Tong et al., 2005). Nitrate accumulation was found at pH 5.5 than pH 7.5
457 indicating co-transport of nitrate with protons like in many previous studies (Tong et al.,
458 2005; Zhou et al., 2000). Further studies with co-expression of *Eg*NRT2.3 and *Eg*NAR2 in
459 model plants and oil palm should be done to confirm the co-function of these two proteins to
460 uptake nitrate as well as comprehensive kinetic studies to determine the affinity to nitrate of
461 both proteins.

## Conclusion

In this study, nitrate transporters in oil palm *EgNRT2.3* (LOC105046035) and *EgNAR2* (LOC105051272) were cloned and characterized using computational analysis, expression patterns, and nitrate uptake studies in oocytes. *EgNRT2.3* was predicted by *in silico* analysis to be a member of HATS and *EgNAR2* as a nitrate assimilation-related protein. The spatial expression analysis of oil palm roots indicated high expression of *EgNRT2.3* and *EgNAR2* in un-lignified young roots. The expression of *EgNRT2.3* and *EgNAR2* responses to different N sources showed an up-regulation in N-depleted treatment when compared with N-treated plants. This data indicated the N-deprivation response of *EgNRT2.3* and *EgNAR2* was possibly regulated by nitrate and ammonium availabilities or perhaps the C/N status of the oil palm. The *Xenopus* oocyte assay demonstrated that EgNRT2.3 and EgNAR2 could function as a two-component nitrate uptake system.

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## CRediT authorship contribution statement

Kwanhathai Sinsirimongkol performed most experiments and wrote the manuscript, Atcharaporn Buasong and Yada Teppabut provided some qRT – PCR data, Nutthida Pholmanee provided *EgNRT2* promoter data, Yi Chen contributed the *Xenopus laevis* oocyte experiments, wrote and reviewed the manuscript, Anthony J. Miller and Napassorn Punyasuk designed the experiments, supervised the project, wrote and reviewed the manuscript.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## List of Figures

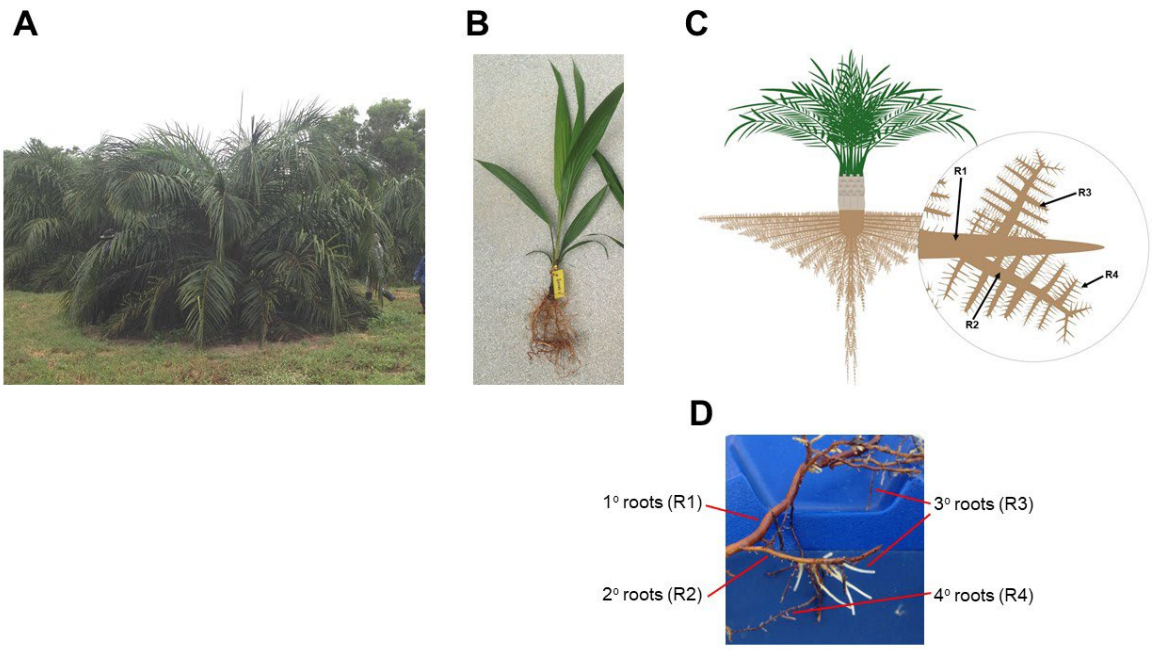



**Fig. 1 Oil palm morphology and root architecture showing different root types**

A = Mature oil palm, B = Juvenile oil palm, C = Mature oil palm illustration indicates different root levels, R1 = Primary root, R2 = Secondary root, R3 = Tertiary root, R4 = Quaternary root, D = Juvenile oil palm root sample shows different root types, 1° root = Primary root (R1), 2° root = Secondary root (R2), 3° root = Tertiary root (R3), 4° root = Quaternary root (R4)

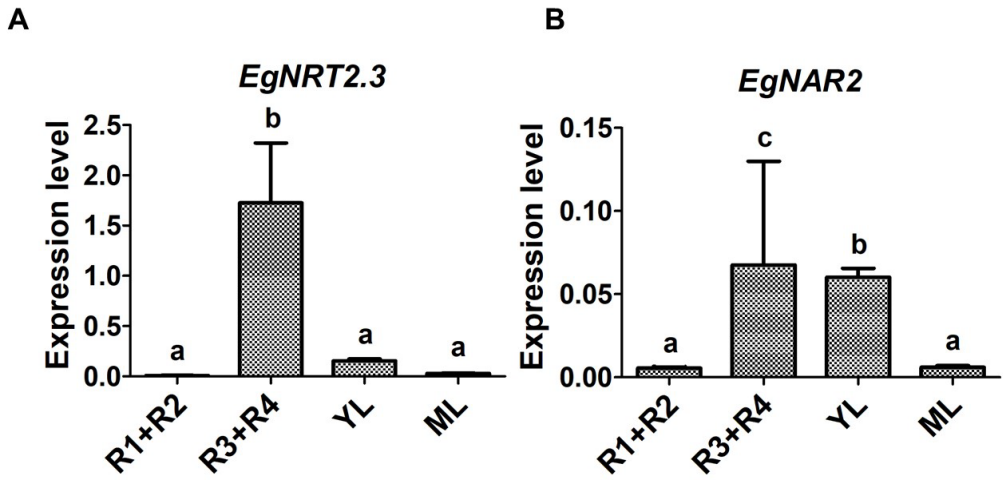
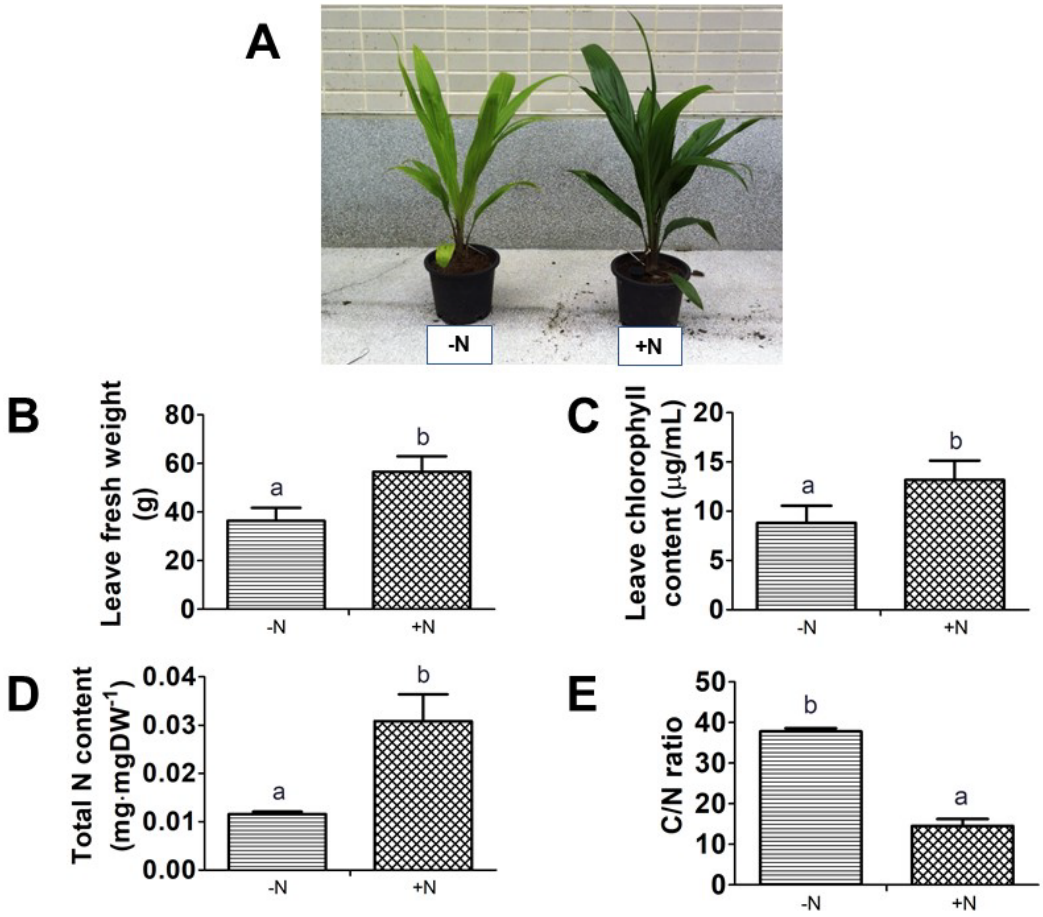



**Fig. 2 The expression levels of *EgNRT2.3* and *EgNAR2* in different oil palm tissues**

A) *EgNRT2.3* and B) *EgNAR2* are shown as means ± SE with three independent biological replicates and three technical replicates. A significant difference between the means of each sample was analyzed by Duncan's test at $P < 0.05$. *EgeIF1* was used as an internal control. R1 + R2 = the pooled primary root and secondary oil palm root, R3 + R4 = the pooled tertiary and quaternary oil palm root, YL = oil palm young leaves and ML = oil palm mature leaves.




776 **Fig. 3 Juvenile oil palm growth responded to N fertilizer**

777 The juvenile oil palms were applied with or without N fertilizer every 2 weeks for
778 3 times within a period of 42 days. A = The physiological appearance of juvenile oil palm
779 treated with or without N fertilizer. B, C, D, and E = The bar graph showed the comparison
780 between leaf fresh weight, total chlorophyll content, total N content, and C/N ratio when
781 treated without N fertilizer (-N) or with N fertilizer (+N). Each bar graph was calculated from
782 the average of independent triplicate samples ± SE. The statistically significant values were
783 analyzed by Duncan's method at $P < 0.05$.

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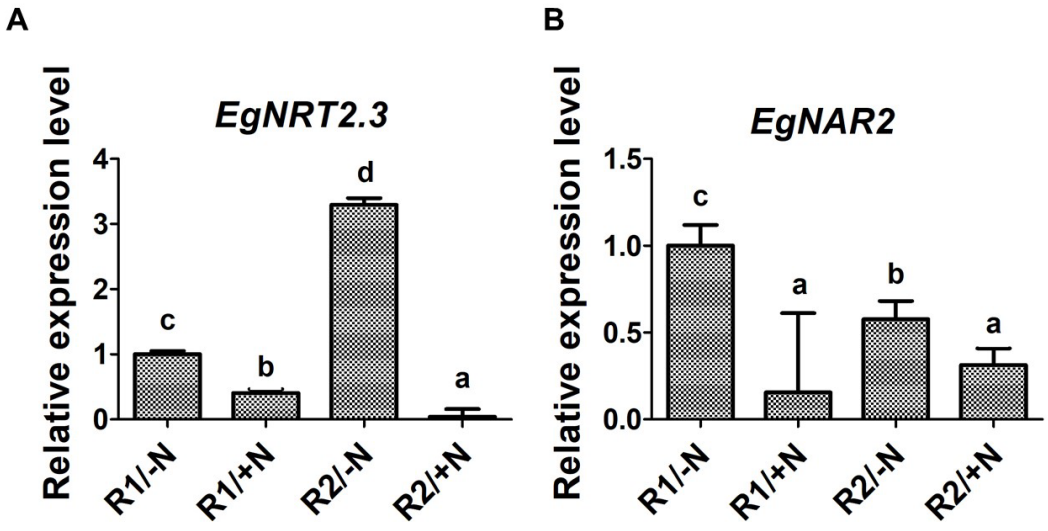


**Fig. 4 The relative expression levels of *EgNRT2.3* and *EgNAR2* in different root types responded to N deprivation**

Oil palms were applied with or without Total-N fertilizer. The different root types were observed in the expression of *EgNRT2.3* and *EgNAR2*. A) *EgNRT2.3* and B) *EgNAR2* is shown as average ± SE. Each average value was calculated with three biological replicates and three technical replicates. A significant difference between the means of each sample was analyzed by Duncan's test at $P < 0.05$. *EgeIF1* was used as an internal control. R1/-N = the primary root with unfertilized treatment, R1/+N = the primary root with fertilized treatment, R2/-N = the secondary root with unfertilized treatment, and R2/+N = the secondary root with fertilized treatment.

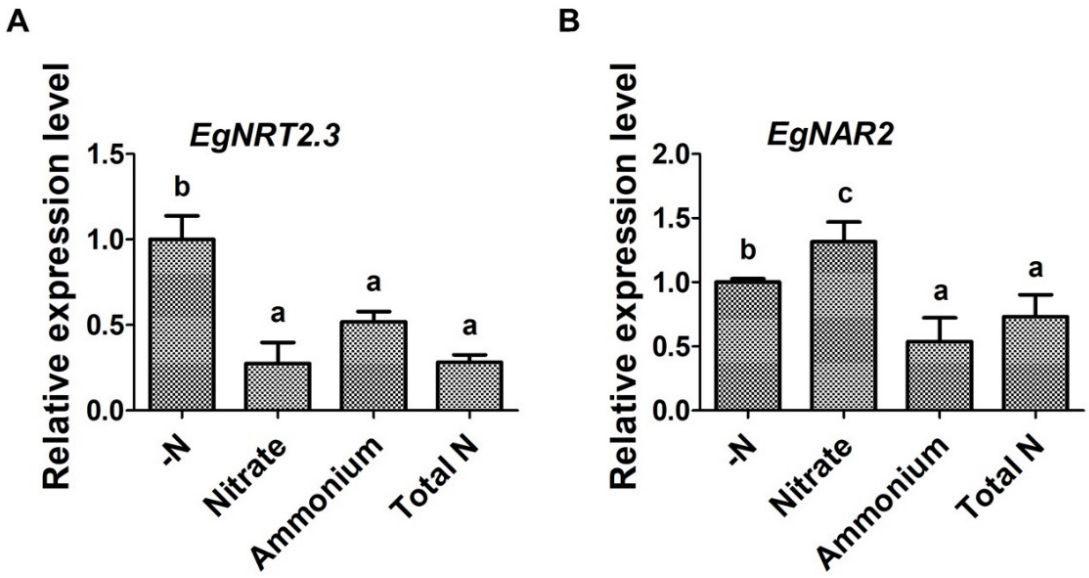


**Fig. 5 The relative expression levels of *EgNRT2.3* and *EgNAR2* in oil palm roots treated with no-N and different types of N-sources**.

Oil palms were treated with unfertilized condition (-N) and different types of fertilizer (nitrate, ammonium, and total N). A) *EgNRT2.3* and B) *EgNAR2* are shown as means ± SE with biological triplicates and technical triplicates (n=3). A significant difference between the means of each treatment $P < 0.05$; (Duncan's test). *EgeIF1* was used as an internal control.

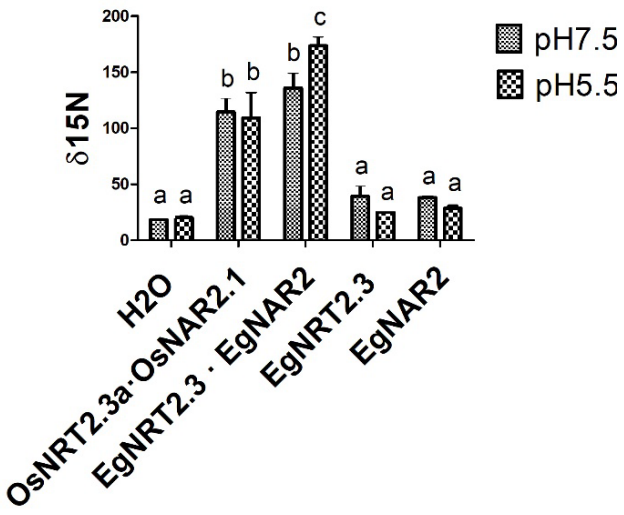


**Fig. 6 $^{15}$N-nitrate uptake study in *Xenopus* oocytes system**

The cRNA-injected oocytes were incubated with 10 mM $^{15}$N $NaNO_3$ in pH5.5 and pH 7.5. The data was the mean value of four samples with two oocytes in each sample. Significant differences were tested between the averages of each treatment $P < 0.05$; Duncan's test and the error bars are SE of means.