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Original Article

Isolation, expression analysis and characterization of *EgNDL*, a NDR-like protein in oil palm (*Elaeis guineensis* Jacq.)

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Abstract

A novel cDNA of a SF21-like protein or NDR-like protein (*Eg*NDL) from *Elaeis guineensis* Jacq. is 1,044 bp in length and encodes a putative protein with a 347-amino-acid open reading frame. The *Eg*NDL showed 93% identity to the pollen-specific SF21-like protein of *Phoenix dactylifera* and also showed 79% identity to the NDL protein of *Theobroma cacao*. Expression analysis of the *EgNDL* gene in various tissues showed that *EgNDL* was expressed in the anthers, pistils, mesocarp and leaves. The high expression of *EgNDL* among three oil palm varieties was significantly expressed in *Pisifera* ($P<0.05$), which is commonly used as a male parent in crosses. Computational tools were used to predict the protein and concluded that *Eg*NDL is a putative membrane protein that may function in a signal transduction pathway during pollen development. In this context, knowledge regarding *EgNDL* and its potential role in plant developmental processes will benefit oil palm breeding programs.

Keywords: *Pisifera*, anther, pollen, NDR, NDL

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the highest yielding vegetable oil source, making it the most efficient oil-bearing crop in the world. There are three varieties, *Tenera*, *Dura* and *Pisifera*, which are different in their fruit characteristics. *Tenera* is a commercially preferred, thin-shelled, heterozygous (Sh^+/Sh^-) palm obtained by crossing the thick-shelled homozygous dominant (Sh^+/Sh^+) *Dura* and the shell-less homozygous recessive (Sh^-/Sh^-) *Pisifera* (Beirnaert and Venderweyen, 1941). Oil palm is a unisexual plant; thus, male and female flowers are borne on separate inflorescences in leaf axils of the same tree. The gender of inflorescences alternates in a series of varying periods, thereby reducing the possibility of self-pollination. Most breeding programs produce *Tenera* hybrid progeny by fertilizing maternal *Dura* plants with pollen from *Pisifera*. Although, *Pisifera* has the lowest demand for resources due to abortive fruit, while *Dura* and *Tenera* produce fertile flowers. As a consequence, obtaining a sufficient quantity and quality of *Pisifera* pollen is necessary for a successful cross (Durand-Gasselin *et al.*, 1999).

In this study, a putative gene from *E. guineensis* that encodes a protein similar to SF21 or NDL proteins family

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has been isolated and was designated as *EgNDL*. *SF21* is a group of genes that share sequence homology with several human genes but their precise function is not confirmed. Members of *SF21* gene family proteins have been identied in stigma, pollen, and pistil of *Arabidopsis thaliana*, *Nicotiana tabacum*, and *Oryza sativa* (Kräuter-Canham *et al.*, 1997, 2001; Nas *et al.*, 2005).

Apart from being one of the pollen-specific protein, SF21 also belongs to the N-myc downregulated (NDR)-like or NDL proteins family (Kräuter-Canham *et al.*, 1997, 2001; Lazarescu *et al.*, 2006, 2010). Animal NDR genes consist of Ndr1/RTP/Drg1, Ndr2, and Ndr3 which are involved in tumor progression and cellular differentiation (Okuda and Kondoh, 1999; Mudgil and Jones, 2010). In *Arabidopsis*, the NDL proteins family contains NDL1, NDL2 and NDL3 which play a role in signaling pathway, auxin transport and auxin gradients (Mudgil *et al.*, 2009). The expression level of NDL1 effects the proper development of vegetative and reproductive organ (Mudgil *et al.*, 2013). The finding of *E. guineensis* gene homologous to *SF21* or *NDL* family is the first step in exploring the molecular mechanisms underlying oil palm reproductive processes and could be useful in oil palm breeding programs.

2. Materials and Methods

2.1 Plant material

Elaeis guineensis Jacq. materials were grown in the field at the Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The tissues analyzed consisted of leaves, stem, root, mesocarp and male and female inflorescences that were harvested from 3-year-old *Tenera* hybrids. The leaves were collected at the 17-leaf position of the fronds. The mesocarp tissues were collected 12 weeks after anthesis. The male and female inflorescences were collected from the axils of the fronds before anthesis.

2.2 Isolation of *EgNDL* from oil palm

The cDNA of *EgNDL* gene was collected from oil palm EST library (Phongdara *et al.*, 2012). Genomic DNA was extracted from the mesocarp using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR was performed using 100 ng of DNA template, 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer pair and 0.2 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Four gene-specific primers, *EgNDL*-F1, *EgNDL*-F2, *EgNDL*-R1 and *EgNDL*-R2 (Table 1) were designed according to *EgNDL* cDNA. The PCR procedure was initiated with a 95°C pre-denaturation step for 4 min followed by 35 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 1 min. The fragment was purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. The sequences were analyzed by comparing the genomic DNA and cDNA sequences. The alternative splicing from the cDNA to genomic DNA was predicted using Splign program (Kapustin *et al.*, 2008).

2.3 Sequence analysis

The Basic Local Alignment Search Tool BLASTX and BLASTP from the National Center for Biotechnology Information were used to search for protein sequence identity (Altschul *et al.*, 1997). The amino acid sequence of *Eg*NDL was aligned with those of its homologs by ClustalW (Thompson *et al.*, 1997). The translated sequences of the *EgNDL* gene were analyzed by the Conserved Domain Database (Marchler-Bauer *et al.*, 2015). The phosphorylation sites of the *EgNDL* protein were predicted using Motif Scan to evaluate their performance (Pagni *et al.*, 2007).

2.4 Semi-quantitative RT-PCR analyses of the tissue distribution and variety-specific expression

The tissue expression of *EgNDL* was determined using total RNA from six different tissues of hybrid *Tenera* variety, leaves, stem, root, mesocarp, anthers, and pistils from unpollinated flowers. The variety-specific expression was examined using leaves material from three varieties; an F1 *Tenera* hybrid resulting from a crossed between maternal *Dura* and paternal *Pisifera* as from a parental oil palm trees. All the total RNA samples were extracted using the RNeasy Plant Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 200 ng total RNA using the SuperScript III First-Strand

Table 1. List of primers used for PCR amplification.

Primers	Nucleotide sequences (5′ - 3′)
EgNDL-F1	GAG TTA GGA GCT GCT CCA ATT TC
EgNDL-F2	CCA TAC TAT TCT GAT GCT GTC CAC ATG AC
EgNDL-R1	TGT CAT GTG GAC AGC ATC AGA AT
EgNDL-R2	GTC CAT TGA CAC CCT TGT CTT GAT AGG C
EgNDL-R3	GCC TGT AGA GCC CAT ACC CTA TGA AGA AG
Eg18S-F	CAA AGC AAG CCT ACG CTC TG
Eg18S-R	CGC TCC ACC AAC TAA GAA CG

Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The amplication reactions were performed using gene-specific primers (*EgNDL*-F2 and *EgNDL*-R2) and *18S rRNA* primers (Table 1), which served as an internal control. The PCR reaction was performed using three biological replicates including no-template controls. The expression levels were analyzed the densitometric image by Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

2.5 Quantitative real-time PCR analysis of the variety-specific expression

The gene expression of different oil palm varieties was confirmed by using quantitative real-time PCR (qRT-PCR). Oil palm leaves were used due to the results of tissue expression. The reactions were performed in a 12.5-µl volume containing 0.4 pmol of each primer (*EgNDL*-F2 and *EgNDL*-R3, Table 1), 100 ng of the cDNA template and 1x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on the MX300P Real-Time Detection System (Stratagene, Santa Clara, CA, USA). The *18S rRNA* gene was used as an internal control. The thermal profile was as follows: 95°C for 5 min, 45 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Each PCR reaction was performed in three biological replicates with no-template controls. For the relative quantification of gene expression, the threshold cycle (C_T) values were used to compare between the *EgNDL* and *18S rRNA* using the $2^{-\Delta\Delta CT}$ method.

2.6 Statistical analysis

The relevant gene expression values in the experiments were analyzed using SPSS version 17. A one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were conducted to compare the groups. Statistical significance was considered as $P<0.05$.

2.7 Protein structure and modeling

The modeling of the 3D strucutural *Eg*NDL protein was performed using the Protein Data Bank (PDB) by I-TASSER (Zhang, 2008). The generated 3D structure was visualized by PyMOL (DeLano, 2002). The quality of the structure models was assessed through Ramachandran plot calculation in PROCHECK (Laskowski *et al.*, 1993). The membrane topology was predicted using the HMMTOP (Tusnady and Simon, 2001), TMHMM server version 2.0 (Krogh *et al.*, 2001), PRED-TMR (Pasquier *et al.*, 1999) algorithms. A transmembrane topology model was visualized using the TMRPres2D program (Spyropoulos *et al.*, 2004).

3. Results

3.1 Isolation of *EgNDL* from *Elaeis guineensis*

The cDNA of *EgNDL* derived from the EST library was 1,427 nucleotides, including a 21-bp 5′ untranslated region (5′-UTR), a 1,044-bp open reading frame (ORF) encoding 347 amino acids and a 362-bp 3′-UTR with a poly-adenylation (poly A) tail. The *EgNDL* cDNA was submitted to GenBank under the accession number JQ886411. The genomic and cDNA sequence comparisons predicted 11 exons (60, 100, 98, 89, 61, 87, 78, 57, 56, 136 and 222 bp in length) and 10 introns (1,498, 270, 129, 118, 80, 83, 93, 96, 97 and 124 bp in length) (Figure 1A). The alignment of the genomic DNA and cDNA sequences revealed the intron-exon junctions of the *EgNDL* with a conserved GT-AG type at their ends (Figure 1B). The constitutive splicing of *EgNDL* was shown in Figure 1C.

3.2 Sequence analysis of *EgNDL*

Based on the BLASTP results, the amino acid sequences with the highest percent identities to *Eg*NDL

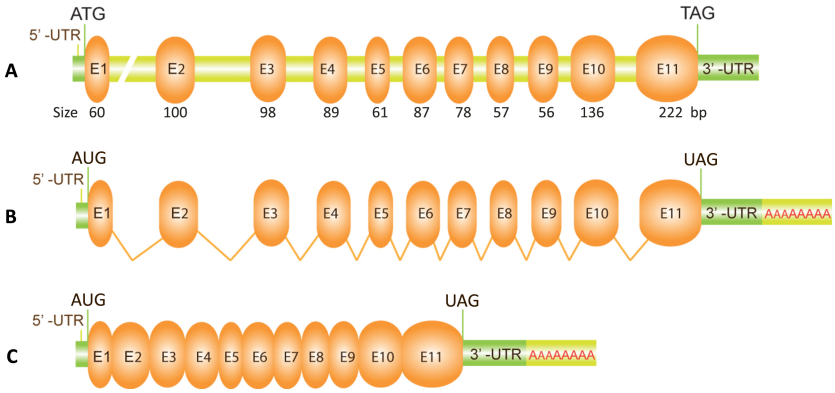

Figure 1. Diagram representing the structural *EgNDL*. (A) Genomic DNA sequence of the gene showing introns (bar in light green) and exons (ellipsein orange color). The 5′ and 3′ UTRs are shown in dark green. The exons are numbered in the ellipses and the sizes are noted below the exon. The transcription start site and stop codon are shown above the drawing. (B) The *EgNDL* mRNA structure showing the splicing sites in RNA processing. (C) Mature mRNA.

(AFH75336) were the pollen-specific proteins SF21-like of *Phoenix dactylifera* (XP_008795469.1), SF21-like of *Setaria italica* (XP_004951412.1) and SF21C1 of *Helianthus annuus* (ABB79742.1) with 93%, 82% and 74%, respectively. *Eg*NDL also had 79% homology to NDL of *Theobroma cacao* (XP_007012258.1), Figure 2. All sequences have an NDR domain (Figure 2, underlined residues), which is involved in cell differentiation. The presence of a protein kinase-specific phosphorylation site showed that the *Eg*NDL amino acid sequence conserved various protein kinases. The TYPD and SWME of the casein kinase II phosphorylation sites were found, including TSK and SPR of the protein kinase C phosphorylation sites and RRYS of the cAMP-dependent protein kinase phosphorylation site were present in *Eg*NDL (Figure 2, boxed).

3.3 Expression of *EgNDL*

The expression of the *EgNDL* was detected by semi-quantitative RT-PCR analysis in all of the tissues. An up-reglulation of the *EgNDL* was observed in both vegetative and reproductive tissues. The expression of *EgNDL* was high in the anthers, pistils and mesocarp with no statistically significant differences between the tissues (P>0.05, Figure 3). Moderate expression level was found in leaf but there was no detectable RT-PCR product in stem and root. When comparing the level of gene expression in leaves among varieties using *18S rRNA* as the internal control in RT-PCR analysis, the highest level of *EgNDL* expression was statistically significant in *Pisifera* followed by *Dura* and *Tenera* at P<0.05 (Figure 4). This observation was confirmed by qRT-PCR.

3.4 Molecular modeling and topology prediction of *Eg*NDL

Comparative modeling was used to predict the 3D structure of *Eg*NDL using I-TASSER (Zhang, 2008). The result showed 31% identity to the human N-MYC down-stream-regulated gene 2 (NDRG2) protein in *Mus musculus* (PDB ID: 2QMQ chain A) and 29% identity to the human NDRG2 protein in *Homo sapiens* (PDB ID: 2XMQ and 2XMR chain A) (Figure 5A). We also propose a computerized trans-membrane protein topology model of *Eg*NDL; one definite transmembrane helix based on the PRED-TMR result (Figure 5B). Our results suggest that *Eg*NDL may function in signal transduction.

4. Discussion and Conclusions

A sequence alignment analysis revealed that *Eg*NDL is highly similar to *SF21-like* from *P. dactylifera* (Singh *et al.*, 2013) and the *SF21C* gene from sunflower (Lazarescu *et al.*, 2006). *SF21* genes in angiosperms belong to the *NDR* gene family commonly found in animals (Kräuter-Canham *et al.*, 2001). Both sequence alignment and 3D structure modeling also demonstrated that *Eg*NDL belongs to the NDR family. Although the functions of SF21 and NDL proteins family still remain unclear, many lines of evidence have described the function of proteins in signaling pathway and associate with the development of reproductive organ. Our attempt here aims to investigate a potential role for reproductive development of *Eg*NDL by compare our results with the results of other studies. Many reports have described the molecular characterization of anther/pollen-specific genes. For example, *Zm401* from maize and *Si401* from foxtail millet were demonstrated

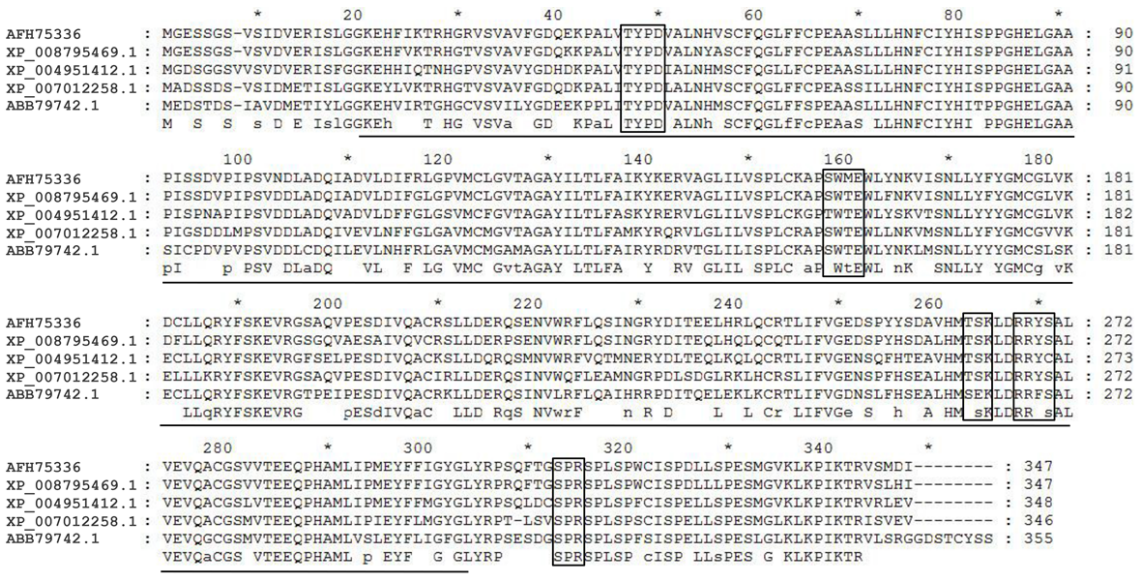

Figure 2. Deduced amino acid sequences of *Eg*NDL (AFH75336) show homology to pollen-specific protein SF21-like of *P. dactylifera* (XP_008795469.1), SF21-like of *S. italica* (XP_004951412.1), SF21C1 of *H. annuus* (ABB79742.1) and NDL of *T. cacao* (XP_007012258.1), respectively. The underlined of amino acids represents the conserved NDR domain, including the predicted protein kinases (boxed).

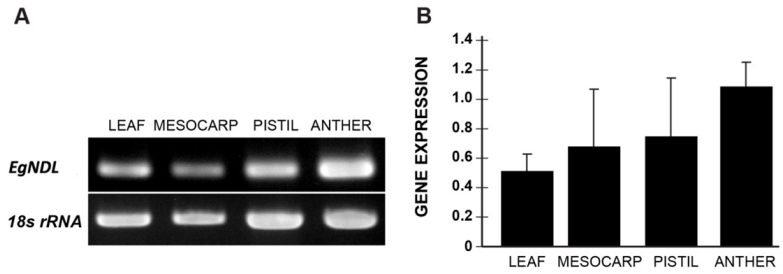

Figure 3. *EgNDL* mRNA expression determined in various tissues of hybrid *Tenera* by semi-quantitative RT-PCR. (A) Gel electrophoresis showing gene expression. (B) The graph represents the *EgNDL* expression in various tissues with no significant differences among the groups (P>0.05).

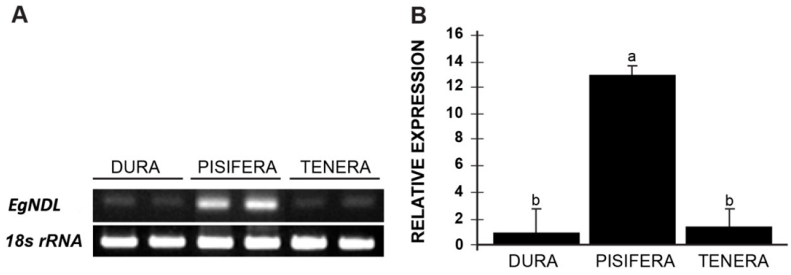

Figure 4. Variety-specific gene expression of *EgNDL*. (A) The qRT-PCR of *EgNDL* was specifically performed in *Pisifera*, followed by hybrid *Tenera* and *Dura* as shown in the gel electrophoresis and graph. (B) The graph illustrates the fold change in the relative gene expression. The data are shown as means ±SE. Different lower case letters indicate significant differences between treatment groups (P<0.05).

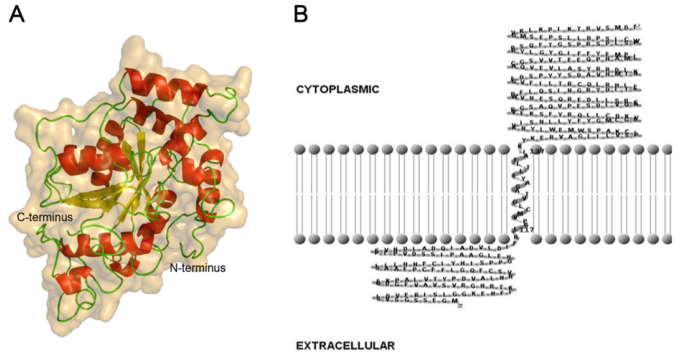

Figure 5. Protein prediction (A) *Eg*NDL 3D predicted structure; surface representation (light brown) of the atomic model of *Eg*NDL with overlaying flap. Shown are the N- and C-ternini including the 12 helices (red) and 8 beta-strands (yellow). (B) Schematic representation of the possible transmembrane topology of *Eg*NDL.

to play an important role in anther and pollen development, respectively (Ma *et al.*, 2008; Qin *et al.*, 2008). In contrast to other anther/pollen-specific genes, the original *SF21* genes of sunflower were found in various tissues, including pollen and stigmas from unpollinated florets as well as styles and ovaries from pollinated florets (Lazarescu *et al.*, 2006). *ORSF21* of *Senecio squalidus* is expressed exclusively in floral tissues, but no expression was performed in root, leaf, stem and capitulum bud. However, two distinct sequences of *ORSF21* were detected in different expression pattern. *ORSF21B* is expressed exclusively in mature pollen, while *ORSF21A* is expressed in the transmitting tissue of the pistil (Allen *et al.*, 2010). The *SF21* of tobacco is expressed in pistil and pollen tissues (Kräuter-Canham *et al.*, 1997, 2001). In *Arabidopsis*, there are three of the *SF21* orthologues, At5g56750 (NDL1) and At5g11790 (NDL2) are expressed in pollen whereas the At2g19620 (NDL3) is found in pistil (Allen *et al.*, 2010). The different expression patterns of the members of SF21 gene family indicate different roles of protein among variant tissues. In this work, the *EgNDL* gene was expressed not only in the flower but also in the leaves and mesocarp, suggesting that *EgNDL* is expressed during

vegetative and reproductive development. The highest *EgNDL* expression level appeared to occur in the anther, where a large amount of pollen is produced; however, this expression level was not significantly different compared to the expression levels in pistil and mesocarp. The existence of variant transcripts of *EgNDL* must be further investigated before the tissue-specific gene expression pattern can be conclusively determined.

In the model plant *Arabidopsis*, over expression of *NDL1* disrupts vegetative and reproductive organ development (Mudgil *et al.*, 2013). NDL1 proteins, together with AGB1 positively regulate basipedal inflorescence auxin transport; it also regulates the expression of auxin responsive genes such as PIN proteins and AUX1 proteins (Okuda and Kondoh, 1999; Mudgil *et al.*, 2009). It is known that auxin is the phytohormone highly accumulated in pollen and anthers and plays a key role in gametogenesis development. Intracellular auxin homeostasis was reported to be associated with the development of male gametophytes via a specifically expressed protein, PIN8 (Dal Bosco *et al.*, 2012).

Oil palm sex determination is a complex process. Currently available data proposed four types of factor that might participate in sex determination and differentiation: abiotic factors (e.g. water stress), metabolic factors (e.g. carbon reserves), hormone status and genetic factors. The possible involvement of hormones in oil palm sex determination was obtained from the indirect effect of auxin NAA (a-naphthyleneacetic acid) on female inorescence production (Corley, 1976; reviewed by Adam *et al.*, 2011). The commercial oil palm, *E. guineensis* (*Tenera*), derives from hybridization of variety *Dura* × *Pisifera*. A high percentage of the *Pisifera* are more or less female sterile. The high expressed level of *EgNDL* in *Pisifera* when compare to *Dura* and *Tenera* might imply the function of our *EgNDL* similar to *Arabidopsis NDL1*. A transmembrane topology of *Eg*NDL by molecular modeling also supports the idea of involvement in the G protein signaling pathway. However all of these observations require further intensive experiments.

In conclusion, this is the first report on *EgNDL* in oil palms. The knowledge gained from closely related plants, suggests that *EgNDL* may play an important role in reproductive development through its involvement in signal transduction cascades. In future studies, we aim to identify *EgNDL* variant transcripts, determine the specific organ expression pattern of each variant and study the signal transduction pathways related to the sex determination process in oil palms.

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