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Identification of gene transcripts contributing to trunking and non-trunking sago palm (*Metroxylon sagu* Rottb.)

H. Hussain[1]*, S.I.L. Kamarol[1], N. Julaihi[2] and R. Tommy[2]

*[1]Centre for Sago Research (CoSAR), Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia. [2]PELITA Mukah Sebakong Sago Plantation Sdn Bhd 1st Floor, Sublot 103, Lot 424, Mukah New Township, Jalan Orang Kaya Setia Raja, PO Box 79, 96400 Mukah, Sarawak. *E-mail: hhasnain@unimas.my*

Abstract

Sago palm (*Metroxylon sagu* Rottb.) is an economically valuable crop and cultivated widely in Sarawak, Malaysia, for its storage starch. Under certain conditions, such as deep peat areas, some sago palms do not develop well and become stunted, described as non-trunking sago palms. Morphologically, this type of sago palm is abnormal, leading to wastage of various resources and affecting starch production. The research objective was to understand the molecular process that affects non-trunking and starch synthesis in sago palm. Representational Difference Analysis was performed to identify the differences between trunking and non-trunking sago palm transcriptome. This subtractive hybridisation technique detects the differences in gene expression. After three rounds of hybridisation, the differentiated RDA products were cloned and 24 DNA sequences obtained were compared with the sequences in the NCBI database. Results showed that the differentiated genes in response to the non-trunking sago palm have similarities to genes implicated in plant growth, cell metabolism, and salt or osmotic stress. Some of these genes are related to growth, development and signaling involving neutralising the imbalance in plant stress. The information obtained through this work could be used to develop a database on related genes and enzymes contributing to trunking and non-trunking sago palm.

Key words: Representational Difference Analysis, *Metroxylon sagu*, sago palm, non-trunking

Introduction

Sago palm belongs to the palm family *Palmae* Jussieu or known as *Arecaceae*, subfamily *Calamoideae* Griffith and is classified under genus *Metroxylon* Rottboell. The common names of *Metroxylon sagu* are *sagu* or *rumbia* (Malaysia & Indonesia), *balau* (Melanau Sarawak), *saksak* (Papua New Guinea) and *ambasao* (Solomon Islands). The natural habitat of *Metroxylon* is a swampy area, and it thrives in flood, drought, acidic and wet soil. In Sarawak, the sago palm is widely cultivated since it thrives in swampy soil found in many areas there. The total area of sago palm in Sarawak by 2017 was 40,641 hectares, with Dalat, Mukah and Matu are the main areas for sago planting covering 85 % of the area (Sarawak Department of Agriculture, 2018). Being the 4th highest agricultural revenue earner for the state with a value of more than RM89 million after oil palm, pepper and rubber, sago is an important commodity. However, some difficulties were faced by the sago plantation owners. In Mukah plantation, there are sago palms with the stunted growth. This problem leads to the formation of non-trunking sago palm and this palm is unable to produce starch.

Trunk formation of sago palm takes place at 3-4 years after sucker planting and reaches the flowering stage 6-10 years after trunk formation (Yamamoto *et al.*, 2010). The accumulation of starch begin during the third year of trunk formation and is produced at a constant rate. Sago palm is considered as non-trunking when there is no trunk formation and still in a rosette stage long after it was planted as in some instances, more than ten years. The abnormal condition of the non-trunking can be seen as looking at the stunted growth of the plant. Research on sago palm is still considered in its infancy, and much research needs to be done to improve the yield of starch, to investigate the factors that are affecting the accumulation of starch. Besides looking into the morphological and molecular aspect, environmental factors are also considered affecting sago palm. The groundwater table was considered a factor influencing the starch content of sago palm where the sago palm planted at lower groundwater table gave a large amount of starch (Jong, 1995; Yamaguchi *et al.,* 1994). Type of soil did affect the trunk formation of a sago palm. Less than 10 % of sago palm produced trunk after eight years of planting at deep peat soil and more than 80 % of sago palm developed a trunk after five to six years of planting at shallow peat (Shoon *et al.*, 2006).

Morphologically, the non-trunking sago palm does not grow normally and leads to wastage of various resources and affects starch production. Fewer studies have been done regarding sago palm development, especially through the molecular biology aspects (Lim *et al.*, 2020). The most recent report related to the study in this area was the identification of proteins for the stunted growth sago (Hussain *et al.*, 2020). The importance of this research was to detect the differences in gene expression between trunking and non-trunking sago palm through cDNA profiling. Representational Difference Analysis (RDA) was chosen for this study. The RDA method is a sensitive subtractive method that can detect as low as 1 % differentially expressed gene in a test sample. The method is also less time consuming,

inexpensive and produces a low number of false positives results (Hubank & Schatz, 1994; Pastorian *et al.*, 2000). The cDNA of trunking sago palm and non-trunking sago palm were compared to identify the difference in gene expression between the two distinct phenotypes. This study describes the analysis and characterisation of the trunking and non-trunking sago palm transcriptome, by comparison through the RDA analysis as well as developing a database of potential genes that are contributing to the non-trunking phenomenon.

Materials and methods

Sampling of plant materials: The leaves of plant samples were collected from Dalat Sago Plantation in Sarawak, Malaysia (GPS: 2.852150, 111.826367). Leaves from trunking and non-trunking sago palm were used for this study. Leaves were chosen for this study due to its role in the formation of transitory starch in plants during photosynthesis. The samples were selected from the third frond of the sago palm, with three replicates for each trunking and non-trunking palms with the approximate age of 12 and 17 years respectively. The leaves were wiped with 70 % ethanol and packed in a zip lock bag covered with ice. The samples were stored in a -80 °C freezer until used.

mRNA purification and conversion to cDNA: The total RNA from the leaves samples were extracted and purified using the protocol described by Kiefer *et al.* (2000) and Gasic *et al.* (2004). Purified RNA pellets were air-dried for 10 mins and resuspended in 100 µL water. The RNA was stored at -80 °C until further use. The RNA purity was checked based on the A260/A280 ratio. Poly-(A) mRNA was prepared using MicroPoly(A) Purist mRNA Purification Kit (Ambion, USA). First strand cDNA synthesis was generated by using Revert Aid Reverse Transcriptase (Fermentas). The reaction was performed in a volume of 20 µL using 1 µg poly-(A) with the addition of 1 µL oligo (dT)18 primer and nuclease-free water. Second strand cDNA synthesis was performed in a 100 µL reaction by adding 8 µL 10X reaction buffer for DNA polymerase I, 1 U RNase H, 30 U DNA polymerase I (Fermentas) and nuclease-free water. The mixture was mixed and incubated at 15 °C for 2 h. 12.5 U T4 DNA polymerase (Fermentas) was added to the reaction mixture and incubated at 15 °C for 5 mins. The reaction was terminated with the addition of 5 µL 0.5 M EDTA followed by phenol/chloroform/isoamyl alcohol (PCI) purification.

cDNA synthesis and amplicons generation: Following the poly-(A) mRNA purification, reverse transcription of mRNA into cDNA was performed using Oligo$(dT)_{18}$ as a primer. To verify the successful synthesis of the first-strand cDNA, PCR was performed using *ELF-1* reverse and forward primer for 40 cycles at 58 °C. *ELF-1* (elongation factor 1) is a housekeeping gene found in plants. The samples were then run on 1.2 % agarose gel and viewed under UV transilluminator to confirm successful amplification and selected for amplicon preparation. The selected cDNA samples were ligated with the RBgl 12 and RBgl 24 adaptors and amplified by PCR generating amplicons for the subsequent RDA analysis.

Amplicon preparation and RDA analysis: Driver and tester preparation for the RDA started with the digestion of cDNA with restriction enzyme *Dpn*II (NEB). Five micrograms of each cDNA were mixed with 10 µL of 10 X *Dpn*II buffer and 20 units D*pn*II (10 U/µL). Sterile distilled water was added to make the final volume of 100 µL. The reaction was incubated at 37 °C for 2 h. The cDNA was then extracted with PCI for purification and resuspended in 37 µL dH_2O. Following the digestion step, cDNA was ligated with RBgl adaptors, and the RDA was performed as described in Lisitsyn *et al.* (1993) and Grohme *et al.* (2009). For identification of the overexpression of genes in non-trunking sago palm, its cDNA was designated as tester while the cDNA from trunking sago palm was designated as a driver. The common sequences would be eliminated, and the sequence of interest would be amplified exponentially. Following amplification, cloning of the differential cDNA fragments was performed, and transformed plasmids containing the differential fragments were sequenced and analysed using the BLAST program.

Results and Discussion

cDNA synthesis and amplicons generation: The total RNA extracted using both protocols yielded similar results. Following the poly-(A) mRNA purification and subsequent first strand cDNA synthesis, the samples run on 1.2 % agarose gel and viewed under UV transilluminator confirmed successful amplification. The samples that showed successful PCR amplification represented by clear bands were selected for amplicon preparation (Fig. 1).

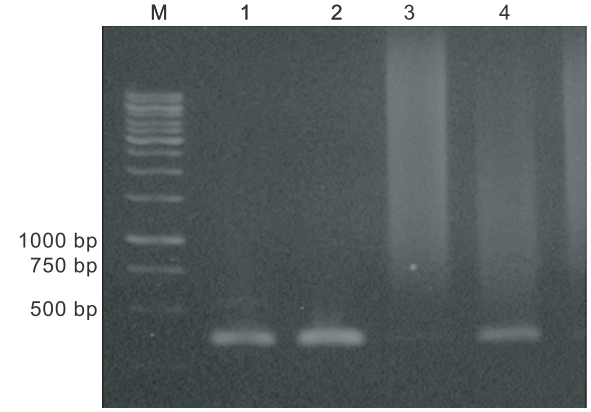
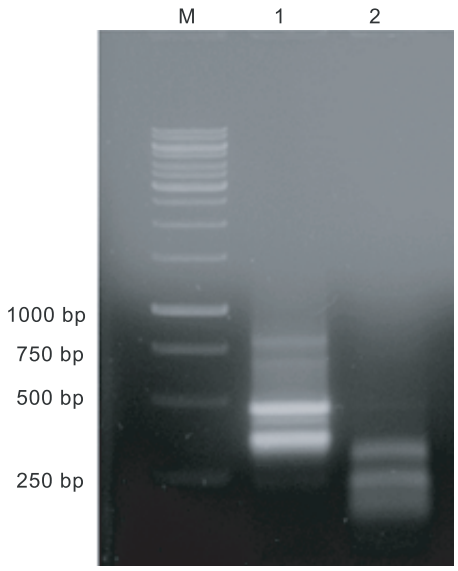


Fig. 1. Examples of PCR product from *ELF-1* amplification. The PCR products for lanes 1, 2 and 4 were selected for amplicon preparation. Lane 3 shows smearing and was not selected for amplicon preparation. M: 1 kb DNA ladder. Lane 1 and 3: trunking samples; lane 2: non-trunking sample.

Subtractive hybridisation and RDA: DNA from non-trunking and trunking samples were hybridised, and unique fragments were amplified. There were three possible combinations of hybridised fragments that were formed: driver-driver hybrids, driver-tester hybrids and tester-tester hybrids. The tester-tester hybrids were amplified exponentially due to the ligation of adaptor at both ends of fragments. Three rounds of RDA were carried out to enrich the difference fragments where the adaptor was changed for every round of RDA. The third round of RDA was conducted in a 1:40000 ratio; 0.001 µg tester : 40 µg driver. Following three rounds of RDA, the differential products were amplified exponentially, and distinct bands can be seen on a gel (Fig. 2). The size of the differential product 3 (DP3) for trunking as driver is in the range of 250 bp-760bp and non-trunking as driver is 250 bp-350 bp. The bands were excised and purified for cloning. Blue-white screening was done where the white colonies



were selected randomly and cultured. The plasmids from cultured colonies were extracted and sequenced for analysis.

Fig. 2. Agarose gel electrophoresis of the third round RDA differential product (1:40 000). Differential product 3 (DP3) was run on 1 % agarose gel. The driver for lane 1 and 2 is trunking and non-trunking, respectively. The DP3 depicted by the arrows were excised, cloned and sequenced for analysis.

Differentially expressed transcripts identified by RDA: From the final amplified differential product 3 (DP3) that were excised and cloned, 24 clones were sequenced and searched for gene similarity in the NCBI database. Table 1 shows the BLAST results for the differentially expressed transcript (DET) in non-trunking. Sixteen clones of the differentially expressed transcripts in non-trunking were analysed for the similarity matches of the gene. The lowest E-value shows the sequence has high sequence similarity match. Eight clones from the differential product in trunking have been identified, and the sequence alignment result for DET in trunking is shown in Table 2.

From the data of DET in non-trunking, there are five transcripts coding that has no functions predicted yet (unannotated proteins). The 11 DET in the non-trunking samples encoded for cell wall hydrolase (sequence similarity is 100 %), retrotransposon protein (73 %), DNA J (71 %), nucleic acid binding protein (30 %), beta-glucosidase (53 %), ATP synthase subunit beta (58 %), cytochrome P450 (93 %), glycerol kinase (81 %), PsbA (100 %) and D1 protein (100 %). The eight DET in trunking sago palms have the identity matches with S-adenosylmethionine synthase with sequence similarity 93 %, cyclophilin (84 %) and annexin (74 %). The differentially expressed transcripts were identified and classified for their functions. Table 3 shows the functional differential expressed genes in non-trunking samples. The DETs were classified for salinity stress, chemical defence, plant cell metabolism and signalling

Differential expressed genes from trunking samples (Table 4) were mostly involved in the growth development of plant and stress tolerance. There are genes coding for transcription-related processes and signalling in neutralising the imbalanced plant stress.

Differentially expressed genes in non-trunking sago palm: Results from analysis of genes in non-trunking sago palm showed that they matched with the genes for beta-glucosidase, glycerol kinase, retrotransposon protein, cytochrome P450, DNA J protein and cell wall hydrolase. The differentially expressed genes have been categorised into their functional category, stress response, plant cell metabolism, signalling and plant development and chemical defence.

Beta-glucosidase, categorised under glycosyl hydrolase, are a biologically important enzymes that are responsible for catalysing the transfer of the glycosyl group between oxygen nucleophiles (Bhatia *et al.*, 2002). These ubiquitous enzymes play a role in the hydrolysis of non-reducing beta-D-glucose residues resulting in the release of beta-D-glucose. Wong *et al.* (2006) reported the overexpression of beta-glucosidase responded to salinity stress. This protein also detected in water deficit, osmotic stress plants and activated the lignification process (Riccardi *et al.*, 1998). Cell wall lignification is initiated to overcome the water loss from the plant tissues and also affect plant growth. The function of this enzyme shows non-trunking sago palm might suffer from salinity or osmotic stress and cause a deficiency in the intake of nutrients, contributing to the non-trunking sago palm having problem in its development and growth.

Beta-glucosidase enzyme responds as a plant defence from pathogen attack where it triggers the activation of chemical defence compounds in higher plants. Nejat *et al.* (2012) showed the plant that was infected by pathogen *Spiroplasma citri* had increased expression levels of beta-glucosidase. The infected plant experienced the chlorosis of leaves, a decrease in size and numbers of flowers and stunted growth. Moreover, it plays a role in the hydrolysis of phytohormone and seed development. The data suggest the enzyme responsible for the defence against pathogen attack were affected in the non-trunking sago palm. This is further confirmed by observation of the affected plant where the leaves samples of non-trunking sago palm collected showed the chlorosis of leaves, and this might affect the growth of the plant.

Glycerol kinase is the enzyme that catalyses the reaction of glycerol and the transfer of phosphate from ATP to form glycerol-3-phosphate. A study by Aubert *et al.* (1994) showed that a high concentration of glycerol-3-phosphate does affect the activity of the glycolytic enzyme. Glucose-6-phosphate isomerases activity is inhibited by the accumulation of glycerol-3-phosphate in the cytoplasmic compartment. This enzyme catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate and vice versa. Fructose-6-phosphate is a precursor for starch synthesis in plants. Leegood *et al.* (1988) stated the increase of glycerol-3-phosphate also prevents the flowing back of carbon from triose phosphate to glucose-6- phosphate and deterioration in photosynthesis CO_2 assimilation. Glycerol fed to barley leaves showed a decrease rate of carbon dioxide intake and glucose-6-phosphate formation. Glucose-6-phosphate can further be converted to starch, which might contribute to lack of storage starch in the non-trunking sago palm.

Salt stress is believed to influence non-trunking sago palm where the BLAST result shows the sequence corresponds to retrotransposon protein in rice (*Oryza sativa*). This protein was categorised as salt stress associated gene in response to salinity stress. Salt stress has been reported to give an effect in the inhibition of photosynthesis rate with the increase of salinity in

Table 1. BLAST result for DET in non-trunking sago palm

Clone	Sequence result	Identity matches	Sequence similarity	E-value
NT01	KIITDINITVQSGEFDTEGLLAKAKFHVNYRGKPIIKPL QQVLANSLRYFLRRPTTEEGSY	Unannotated protein (*Oryza sativa Japonica*) Accession no: NP_001175463.1	90 %	2e-31
	KIITDINITVQSGEFDTEGLLAKAKFHVNYRGKPIIKPL QQVLANSLRYFLRRPTTEEAS	Amino acid binding protein (*Ricinus communis*) Accession no: XP_002523024.1	89 %	1e-29
NT02	LAAVSSCCSPPKGRFLRVTHPSATGNTTSRSTCMCKACR QRSS	Unannotated protein (*Medicago truncatula*) Accession no: XP_003599577.1	91 %	8e-26
NT03	TEAPPWADSSFRSSAYGVLAAVSSCCSPPKD--IK RTSP EGNFNVADF PS	Ribulose bisphosphate carboxylase large chain (*Medicago truncatula*) Accession no: XP_003607358.1	60 %	2e-07
NT04	APKQCFTPRCPVNCCASTHFGENQLALGSSGISPLTTTH PL	Cell wall-associated hydrolase, partial (*Medicago truncatula*) Accession no: XP_003637074.1	100 %	3e-29
	MPLEPRASWFSPKCVEAQQLTGHLGVKHCFGAGCASGTK SRQTLNTRYDPKITGVRSASETMGDKLHRREGNSPD	Retrotransposon protein (*Oryza sativa Indica*) Accession no: ABR26094.1	73 %	1e-28
NT05	KSGASGTCHGCRGAGMRTITRQIGLGMIQQMNTVCPECK GSGEIISDKDKCPSCKGNKVVQEKKVLEVHVEKGMQHNQ KIVFQG	DNA J (*Zea mays*) Accession no: NP_001136581.1	71 %	7e-32
NT06	CLHFQHQSFAEILLAEYYLKVFIKYALDKTP	Uannotated protein (*Sulfurospirillum multivorans*) Accession no: WP_025344453.1	52 %	1.7
NT07	NVVRQFGSYLPLVLKGELRGANPSTRGLGWANLWSTGCY ANSSAGLLSWYGRTAAQREILLYTSSRTRFLNRT	Unannotated protein (*Genlisea aurea*) Accession no: EPS74345.1	92 %	7e-37
NT08	LSMLSILVIGAIKICCIKLNYKDTCIGICSIFLVLNWFY LYYLILKDEHCQD	Unannotated protein (*Oxytricha trifallax*) Accession no: EJY68519.1	31 %	9.6
NT09	PASTTDEQIVPKELHAC-----MGLNACKGHDRFATNDC -----AGMGQCATQSHVCHTL	Unannotated protein (*Marinomonas mediterranea*) Accession no: YP_004313181.1	38 %	4.0
	GHVVYVSGLSCKITKENCIEMLWHEKDEKATFVNLSCLP AFLTSSCIHLISTL	Nucleic acid-binding protein (*Arabidopsis thaliana*) Accession no: NP_188328.5	30 %	4.5
NT10	MKKKAGSRIPSFTKEQSELIRGAIDFVGINHYTSVYVSD GKSGADASLRDYNADMSATFRMSRNDSGTGQFIPINMPND	Beta-glucosidase (*Triticum urartu*) Accession no: EMS64066.1	53 %	2e-21
NT11	RYLANRKPTTSLISHVVAIGSPLKGASIVGRIQDLGLGA ILGN	Alpha/beta hydrolase family protein (*Vibrio parahaemolyticus*) Accession no: EVU12253.1	40 %	0.73
NT12	WILDPLVYGDYPEIMKKKAGSRIPAFTEEQSE LIRGSIDFVGINHYTSVYVSDGKSSADAGLRDYNADLSA TFRLSKNDSGTGQFIPINMPDDPQGLQCMLQYLTDKYQN IPIYVQEN	Beta-glucosidase (*Brachypodium distachyon*) Accession no: XP_003566327.1	48 %	2e-28
	ILDPLVKGDYPEIMTKKAGSRIPSFTKEQSELIR GAIDF VGINHYTSVYVGGGVRGGGGGGGADASLRDYNADMSATFR MSRNDSGTGQFIPINMPNDPQGLQCMLQYLTDTYQNIPI YVQEN	Hydroxyisourate hydrolase (*Aegilops tauschii*) Accession no: EMT07547.1	43 %	6e-20
NT13	IFCEGFELEHACRDPKDGELCLSGAKPEETLVEARSDTD V QIVRLTWV---------- ----------GRH GCSGEPCLGIGSSKWAIFGKQNWRCGMN RKPGYG AELRAN	ATP synthase subunit beta (*Medicago truncatula*) Accession no: XP_003627732.1	58 %	7e-42
	MVRLVFRPYTQVRRTICTSVSLRASTRVSSGFAPLRHSS PSFGSRQACSNSNPSQKI--RVGQ	Unannotated protein (*Brachypodium distachyon*) Accession no: XP_003580983.1	90 %	3e-29
NT14	ILIKGFRLYSFQLPDSMSPVLLFIVTTSPCYDWVICAPA AFL	Cytochrome P450 (*Medicago truncatula*) Accession no: XP_003614380.1	93 %	2e-17
NT15	VDRNRTVSRRSKPNSRTTFIGEQPNPWDLLQPQDVMSRH RGAKRLRR*ELLGVI	Cell wall-associated hydrolase (*Candidatus* Pelagibacter sp) Accession no: ZP_05069441.1	83 %	7e-23
	TPTVDRNRTVSRRSKPNSRTTFIGEQPNPWDLLQPQDVM SRHRGAKRLRR*ELLGVI	Glycerol kinase (*Candidatus* Liberibacter americanus) Accession no: ACD87749.1	81 %	4e-22
NT16	IYPIGQGSFSDGMPLGISGTFNFMIVFQAEHNILMHPFH MLGVAGVFGGSLFSAMHGSLVTSSLI	PsbA (*Oryza sativa*) Accession no: ACS13013.1	100 %	6e-37
	IYPIGQGSFSDGMPLGISGTFNFMIVFQAEHNILMHPFH MLGVAGVFGGSLFSAMHGSLVTSSLI	D1 protein (*Coccobotrys verrucariae*) Accession no: ADQ48029.1	100 %	8e-37

Table 2. BLAST results for DET from the trunking sago palm

Clone	Sequence result	Identity matches	Sequence similarity	E-value
T01	NDPDRPIATLLFCGPTGVGKTELTKALAASYFGSE SAMLRLDMSEYMERHTVSKLIG	Unannotated protein (*Zea mays*) Accession no: NP_001169769.1	89 %	5e-28
	NPDRPIAAMLFCGPTGVGKTELTKALAASYFGEET AMLRLDMSEYMERHTVSKLIG	ATPase (*Synechococcus elongatus* PCC 7942) Accession no: YP_399279.1	82 %	2e-23
T02	DPESKVACETCTKTNMVMVLGEITTKTNVDYEKVV RDTCRSIGFTSDDVGLDADSCKVLVNIEQQSPDIA QGVHG	S-adenosylmethionine synthase (*Nicotiana tabacum*) Accession no: BAA21726.1	91 %	1e-41
T03	QTLSQYAELLWEQHHDEERASSYFEQAVQATPQDS HVLAAYAGFLWEAEDNEVESG	Unannotated protein (*Jatropha curcas*) Accession no: ADJ67173.1	63 %	2e-15
	EVLSQYGKLVWELHHDEERASSYFERAVQASPEDS HVQAAYASFLW– DTEEENDAGYNDSQCLPQHFHLGAMA	TPR domain protein (*Medicago truncatula*) Accession no: XP_003625448.1	57 %	1e-17
T04	SIYGRTFKDENFKLSHVGPGVVSMANAGPNTNGSL FM	Cyclophilin (*Triticum aestivum*) Accession no: AAP44537.1	84 %	8e-13
	SIYGRTFKDENFKLSHVGPGVVSMANAGPNTNGSLF	Peptidyl-prolyl cis-trans isomerase CYP20-2, (*Vitis vinifera*) Accession no: XP_002265030.2	94 %	5e-15
T05	DQALVRRVVMIHWDKQRLHQCKAAYKHFYKKDLAA RIASETSGDYKK	Annexin (*Sphaerulina musiva* SO2202) Accession no: EMF13133.1	74 %	2e-16
T06	DTALVRRVVMIHWDKQRLQQAKAAYKHFYKRDLAE RIKSETSGDYKK	Unannotated protein (*Pseudocercospora fijiensis* CIRAD86) Accession no: XP_007925121.1	77 %	5e-16
T07	MSLERSEVVRAVIVRTCKELKRDNGMIIRYDDNAA VVIDQEGNPKGTRVFGAI	Ribosomal protein (*Acorus calamus*) Accession no: YP_319800.1	85 %	8e-24
T08	DPDSKVACETCTKTNMVMVFGEITTKANVDYEKIV RDTCRSIGFV	S-adenosylmethionine synthase-like protein (*Eperua falcate*) Accession no: ADC80655.1	93 %	3e-21

Table 3. Functional annotation and classification of the differentially expressed genes in non-trunking sago palm

Gene product	Functional category
Cytochrome P450 (*Medicago truncatula*)	Plant signalling and development
Beta-glucosidase (*Triticum urartu*)	Stress response Chemical defence
Retrotransposon protein (*Oryza sativa Indica* Group)	Stress response Salinity
DNA J (*Zea mays*)	Plant cell metabolism
Nucleic acid-binding protein (*Arabidopsis thaliana*)	Protein binding function
Glycerol kinase (*Candidatus* Liberibacter americanus str. Sao Paulo)	Plant cell metabolism-inhibit glucose 6 phosphate activity
Hydroxyisourate hydrolase (*Aegilops tauschii*)	Plant signalling
ATP synthase subunit beta (*Medicago truncatula*)	Protein folding, unfolding and assembly process
Ribulose bisphosphate carboxylase large chain (*Medicago truncatula*)	Carbohydrate metabolism
Cell wall-associated hydrolase (*Candidatus* Pelagibacter sp)	Plant cell metabolism-plant defence

Table 4. Functional annotation and classification of the differentially expressed genes in trunking sago palm

Gene product	Functional category
Annexin (*Sphaerulina musiva* SO2202)	Signalling protein
S-adenosylmethionine synthase-like protein (*Eperua falcate*)	Growth development
Cyclophilin (*Triticum aestivum*)	Stress tolerance
Peptidyl-prolyl cis-trans isomerase (*Vitis vinifera*)	Protein modification
ATPase (*Synechococcus elongatus* PCC 7942)	Signal transduction

cotton varieties (Desingh & Kanagaraj, 2007). Salt stress can be related to the accumulation of glycerol for the regulation of osmolarity and the uptake of water (Bohnert *et al.*, 1999). When the external osmolarity increase, plants accumulate suitable solutes or metabolites such as glycerol, glycine betaine, proline and ectoine. These osmolytes act as a counterbalance in response to osmotic stress to maintain the water potential in plants. The promotion of glycerol synthesis also proved to be influenced by glucose which yeast *Zygosaccharomyces rouxii* exposed to NaCl (Ohshiro & Yagi, 1996). The synthesis of glycerol decreases when the glucose is removed from the growth medium. The amount of glycerol synthesis also increases over incubation time in the salt medium. The accumulation of glycerol might also trigger the glycerol kinase as discussed previously where glycerol kinase inhibits the formation of glucose-6-phosphate. Salt stress is thus believed to affect the non-trunking sago palm development.

Another factor affecting the growth of non-trunking sago palm is the overexpression of cytochrome P450. Cytochrome P450 forms a large superfamily of enzymes that catalyses the oxidation reaction of organic compounds. This enzyme plays a role in the synthesis of signalling compounds, lignin, defence compounds, and also control the development and growth of plants which function in synthesis and deactivation of hormone such as gibberellin, brassinosteroids and auxin (Yamaguchi, 2008). The enzyme is also involved in detoxification of herbicides such as prosulfuron and diclofop. Dasgupta *et al.* (2011) conducted a study on the overexpression of cytochrome on *Arabidopsis* resulted in abnormal growth, stunted rosettes, delayed flowering and reduction in seed yield. The growth aberrations also include thicker and darker leaves. The overexpression of cytochrome causes dwarfism and deactivation of gibberellin, acting as a plant growth regulator (Zhang *et al.*, 2011). This result shows,

the overexpression of cytochrome P450 affects the growth of non-trunking sago palm and causes stunted growth or dwarfism and delay in flowering. Sago palm ready to be harvested before flowering and at this stage, the starch production is at the maximum level. This might also affect starch production and plant maturity.

The sequence for differential expressed gene in non-trunking also shows similarity with cell wall hydrolase enzyme. Sugar starvation has been reported to influence the expression of cell wall hydrolase in *Arabidopsis* (Lee *et al.*, 2007). Three groups of enzymes categorised under cell wall hydrolase enzymes; beta-galactosidase, beta-glucosidase and beta-xylosidase of *Arabidopsis* have a role in the degradation of the cell wall. These hydrolase genes are involved in the breakdown of cell wall polysaccharides and release sugars. This suggests that non-trunking might experience sugar starvation due to the limitation in starch reserve.

Genes responsible for protein folding and unfolding, DNA J protein and ATP synthase beta-subunit have been identified in the expression of the non-trunking sago palm. DNA J protein or J-domain protein is a molecular co-chaperone and functions in protein folding, unfolding and assembly processes. One of the ATP synthase beta-subunit function is responsible for the transportation of protein through membrane fusion and protein degradation. ATP synthase beta-subunit might also have a role in activating cell death genes (Chivasa & Slabas, 2012). The study shows the enzyme regulates the expression of the gene and promote cell death. These proteins do affect the non-trunking sago palm to perform the biological process and may disrupt the functional protein in growth.

Differentially expressed genes in trunking sago palm: The expression of genes in trunking sago palm was shown to be upregulated due to various stress tolerance, salt or drought tolerance, pathogen tolerance and assist in the plant growth and promoting flowering. Annexins showed high expression in trunking sago palms. Annexins is a multifunctional family of Ca^{2+} and phospholipid-binding proteins that can be found in plants and animals (Mortimer *et al.*, 2008). These hydrophilic proteins are expressed during the lifetime of the plant. The increase of Ca^{2+} concentration has a role in activating the Ca^{2+} binding proteins which gives an effect on cellular metabolism (Clark & Roux, 1995). Plants can adapt to stresses by activating the signal pathway in the cell. Annexins gene has been reported to play a role in the signalling process due to osmotic stress or salt stress (Lee *et al.*, 2004). The study indicates the level of annexin in *Arabidopsis* increased after salt treatment. The overexpression of annexin also showed the plants were more drought tolerance and stress resistance (Konopka-Postupolska *et al.*, 2009). The transcript level of annexin mRNA was increased when the leaves were treated with NaCl and declined thereafter. This gene has an influence in regulating stress and the trunking sago palm able to adapt and respond to stress.

The other gene found responded to salt tolerance is cyclophilin. Protein cyclophilin was upregulated when rice seedlings were induced with salt stress. Ruan *et al.* (2011) compared the stress tolerance between the cyclophilin transgenic rice seedlings and wild type rice seedlings. The transgenic seedlings displayed a higher salt tolerance compared to wild type seedlings. This data was supported by Zhu *et al.* (2011), where the overexpression of cyclophilin in tobacco plants increased its tolerance to salt stress. The study also showed cyclophilin has a role in tolerance towards pathogen attack. The leaves of transgenic tobacco introduced to pathogen showed slower bacterial growth and lessened necrotic lesion symptoms. Besides, cyclophilin act as a key regulator in the signalling pathway to other plant stress such as cold, heat and ABA. It is also involved in the transition of juvenile to mature stage of plant development and influenced inflourescence in *Arabidopsis* (Berardini *et al.*, 2001). The knockout test for cyclophilin gene resulted in delayed flowering (Zhang *et al.*, 2013). Thus, this gene is responsible for the promotion of flowering in trunking sago palm, which showed plant maturity. This result shows cyclophilin influencing the tolerance of trunking sago palm to overcome stresses, salt, heat and also plant growth.

Another gene identified in the analysis is S-adenosylmethioinine synthase (AdoMetS), an enzyme that catalyses the formation of S-adenosylmethioinine (AdoMet/SAM). SAM functions as a methyl donor in numerous biochemical reactions in plant. Li *et al.* (2011) reported the knockdown of AdoMetS in rice resulted in late flowering. The result also showed rice experiencing dwarfism and low fertility. This suggests SAM can affect plant development and morphology, which suppressed growth processes. In addition, the suppression of growth due to SAM less expression can be supported by Bourjan *et al.* (1994). The silencing of SAM gene in tobacco resulted in stunted growth and causing a necrotic lesion. By comparing the gene expression in trunking sago palm, SAM regulates the normal growth of the plant and reduced expression of SAM in non-trunking sago palm can be seen where the development is abnormal.

The finding of this study showed differential activated genes in response to the non-trunking sago palm. Sequence analysis revealed that genes upregulated in non-trunking sago palm coding for proteins related to plant growth, cell metabolism genes and salt and related osmotic stress response. There was also the presence of upregulated genes related to the accumulation of sugar before transforming it into starch. Analysis using RDA technique showed that the differentiated upregulated genes in trunking sago palm code for growth development and signalling in neutralising the imbalance plant stress. Trunking sago palm has a high tolerance in stress compared to the non-trunking, where the overexpression of cyclophilin resulted in a higher tolerance to salt stress. Cyclophilin also has a role in flowering promotion. This shows the potential candidates for genes responsible for the plant maturity in trunking sago palms. The BLAST analysis also showed similarity with genes that code for hypothetical proteins with no function predicted yet. The study described here using RDA showed different expression levels of several genes in the non-trunking and trunking sago palm. Identification of these differential genes is important for the determination of contributory factor for the sago palm trunking process. There is also a potential to further study possibly with another approach for further development of a database on genes and enzymes contributing to trunking and non-trunking sago palm.

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