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Proteomics of Sago Palm Towards Identifying Contributory Proteins in Stress-Tolerant Cultivar

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Abstract

Metroxylon sagu Rottb. or locally known as sago palm is a tropical starch crop grown for starch production in commercial plantations in Malaysia, especially in Sarawak, East Malaysia. This plant species accumulate the highest amount of edible starch compared to other starch-producing crops. However, the non-trunking phenomenon has been observed to be one of the major issues restricting the yield of sago palm starch. In this study, proteomics approach was utilised to discover differences between trunking and non-trunking proteomes in sago palm leaf tissues. Total protein from 16 years old trunking and non-trunking sago palm leaves from deep peat area were extracted with PEG fractionation extraction method and subjected to two-dimensional gel electrophoresis (2D PAGE). Differential protein spots were subjected to MALDI-ToF/ToF MS/MS. Proteomic analysis has identified 34 differentially expressed proteins between trunking and non-trunking sago samples. From these protein spots, all 19 proteins representing different enzymes and proteins have significantly increased in abundance in non-trunking sago plant when subjected to mass spectrometry. The identified proteins mostly function in metabolic pathways including photosynthesis, tricarboxylic acid cycle, glycolysis, carbon utilization and oxidative stress. The current study indicated that the several proteins identified through differentially expressed proteome contributed to physical differences in trunking and non-trunking sago palm.

Keywords *Metroxylon sagu* · Trunking · Sago palm · Differential expression

1 Introduction

Metroxylan sagu Rottboell, known as the true sago palm, has the highest starch yields among sago species [19]. During the flowering stage, the trunks of the sago plant accumulate the highest amount of edible starch at about 150–300 kg of dry starch per tree [35]. The productivity of sago plant was calculated to be approximately three to four times of rice, corn or wheat. Compared to cassava, the productivity of sago is about 17 times higher [33]. Therefore, sago remains among the highest yielding starchy crops in the world [24, 55].

This species can be found across Southeast Asia (Thailand, Malaysia, Brunei, Indonesia, Philippines) as well as major regions in the Solomon Islands and Papua New Guinea [17, 20]. The tree's trunk or bole contains a large quantity of starch which is used to produce food-based products such as bread, cakes, vermicelli and noodles [32, 33, 43, 47]. The different parts of the plant can also be used as walling materials and hut building, animal feeds, production

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of edible sago worm, basketry [35]. Moreover, the sago pith waste can be utilized as a substrate for production of bioplastic [35, 55], bio-ethanol [10, 15, 35, 55], medicine, pesticide, poison for fish [9] and sugar [15, 55].

Sago palm can grow well in underutilized wetlands where almost no other food crops can grow. The plant can undergo salt-stress tolerance due to its good distribution and growth in brackish water areas [21]. However, the occurrence of the non-trunking sago palm in deep peat area is a challenging problem in large-scale sago palm plantations in Mukah, Sarawak. Sago palm growing in shallow peat or mineral soil starts trunk formation around the fourth-year of growth. However, the sago palm in deep peat area will remain at the rosette stage, even after 10 years. This non-trunking sago palm phenomenon occurs in 80% of the total plantation area [56] and has been highlighted as one of the major production constraints. This problem directly affects starch productivity, the plantation time and wastes land area. Research has reported that the abnormality of the plant growth may be caused by environmental factors and lack of space or nutrients [31]. Meanwhile, the study from Edward and Hussain [18] found that the failure of trunk formation and stunted in height is caused by stress-related factors. In addition, another study found that insufficient nutrients; primarily nitrogen, phosphorus and potassium in deep peat soil contributes to the lack of trunk formation [25].

This study was conducted to investigate the changes of protein expression, which may directly or indirectly related to the non-trunking sago palm occurring in the plantation area in Sarawak, Malaysia. Major emphasis is on comparative proteomics of different samples under similar conditions of plant growth stage, leaf coordination and soil. Thus, the objective of this research was to identify proteins that may provide critical information for understanding the sago palm tolerance mechanism leading to the non-trunking phenomenon. The knowledge may be utilised in the development of molecular markers for selection of stress-tolerant cultivars and creating tolerant crops through genetic manipulation. The development process will also take into account the clearly consistent genes revealed by other relevant approaches.

2 Materials and Methods

2.1 Plant Materials

The third frond of trunking and non-trunking sago plant leaves at age of 16-years-old, grown in deep peat area, were collected from Pelita Dalat Sago Palm Plantation in Mukah, Sarawak, Malaysia. Plant leaves were cleaned manually with distilled water and surface sterilized with 70% ethanol. They were immediately frozen in liquid nitrogen and stored at − 80 °C until further analysis.

2.2 Protein Extraction and Quantification

The extraction protocol used was based on Alam et al. [6], with some modifications (Triton X-100 was used instead of NP-40). Frozen sago leaves were ground using chilled mortar and pestle, in the presence of liquid nitrogen. About 2 g of powdered sample were incubated in 20 ml of extraction buffer [0.5 M Tris–HCl (pH 8.3), 2% (v/v) Triton X-100, 20 mM $MgCl_2$, 2% (v/v) β-ME and 1 mM PMSF]. The mixture was vortexed for 1 min, then centrifuged at 5000*g* for 15 min at 4 °C. The supernatant was collected and subjected to PEG fractionation, added with 50% (w/v) stock solution of PEG 4000 to a final concentration of 15% (w/v) solution. The mixture was let incubated on ice for 30 min and pelleted by centrifugation at 5000*g* for 15 min at 4 °C. The supernatant was collected again and directly precipitated with three volumes of 100% acetone at − 20 °C for overnight. The pellet obtained by centrifugation at 5000*g* for 15 min at 4 °C was further extracted by well-dissolved it in 20 ml extraction buffer. An equal volume of Tris-buffered phenol (pH 8.0) was added and mixed. After that, the mixtures were added with 0.7 M sucrose and mixed thoroughly. The sample was centrifuged at 5000*g* for 15 min at 4 °C to obtain the phenolic phase. After separation, the phenolic phase was precipitated by four volumes of 0.1 M ammonium acetate in methanol and kept at − 20 °C for overnight. The new pellet was obtained through centrifugation at 5000*g* for 15 min at 4 °C. Then the pellet was washed three times with 0.1 M ammonium acetate in methanol with centrifugation in the interval. The protein pellet was air-dried for several minutes and suspended in solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 0.002% bromophenol blue). The concentration of total protein was determined using a standard Bradford protein assay [14, 37]. Bovine serum albumin (BSA) was prepared in several dilutions in the range between 0 and 100 µg/ml. Meanwhile, the protein samples were assayed in triplicate. Then, 1 ml of Bradford reagent was added into all samples and the mixture was incubated at room temperature for at least 5 min. The absorbance value was measured at 595 nm using a spectrophotometer within 1 h. The protein concentration was calculated by comparison with the BSA standard curve.

2.3 Two-Dimensional SDS-PAGE and Image Analysis

Two-dimensional electrophoresis (2D-PAGE) of extracted protein was performed according to the method of Al-Obaidi et al. [3]. Protein sample was liquified in isoelectric focusing buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% (immobilized pH gradient, pH 4–7), and 0.002%

bromophenol blue. A total volume of 250 µl diluted sample containing 300 µg proteins was let rehydrated for 24 h in IPG strip (13 cm, pH 4–7). Isoelectric focusing (IEF) was performed and focused on Ettan IPGphor platform (GE Healthcare) by applying following parameters: 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 9 h, and 50 V for 6 h [28]. The strip then was equilibrated with reducing agent buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) for 15 min. Followed by alkylation with buffer containing 6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue, and 2.5% iodoacetamide for another 15 min as described by Al-Obaidi et al. [5]. The electrophoresis was performed at 12% polyacrylamide gel using SE600 Ruby vertical electrophoresis system (GE Healthcare) and followed the parameters; 15 min at 10 mA/gel for the first step and 3 h 30 min at 20 mA/gel for the second step. Full range rainbow molecular weight marker was used as a protein standard. After that, the gel was stained using Coomassie staining method for overnight. The gel was destained using destaining solution (10% acetic acid) until spots were clearly visible. The gel was scanned using the Densitometry system (Bio-Rad Laboratories) and analyzed using Progenesis Same Spots software (Nonlinear Dynamics, Durham, NC, USA). Four biological replicates were used for each sample comprising of trunking and non-trunking sago (Supplementary material 1).

2.4 In-Gel Digestion, Protein Identification and Database Search

Selected protein spots with fold changes of more than 1.5 times, for at least two timeframes, were excised from the 2-DE gels. The proteins were subjected to in-gel digestion with trypsin as described by Al-Obaidi et al. [2]. Excised gels were washes for 30 min with 100 mM ammonium bicarbonate, then destained with a solution containing 15 mM $K_2Fe(CN)_6$ and $Na_2S_2O_3$ in water. Next, the gel plugs were incubated in 10 mM DTT dissolved in 100 mM NH_4HCO_3 for 30 min at 60 °C for protein reduction. The gel plugs then undergo incubation in 55 mM iodoacetamide with 100 mM NH_4HCO_3 at room temperature (26 °C) in the dark for 20 min, dehydrated with 100% (v/v) acetonitrile (ACN) for 5 min and dried. Trypsin solution (12.5 ng/ml trypsin in 25 mM ammonium bicarbonate) was added to the dried gel plugs for hydration for 10 min at room temperature (26 °C), and follow by overnight incubation for digestion at 30 °C. After that, the digested proteins were further desalted with Zip-Tip Cµ18 (Millipore, Bedford, MA, USA) to obtain the peptides. Trypsin-digested peptides were analyzed and identified using an Ultraflextreme MALDI-ToF/ToF mass spectrometer (Bruker, Bremen, Germany). Peptide calibration standard was used to calibrate the spectra. Data analysis was performed using MASCOT peptide sequence matching software (Matrix Science Ltd., London, UK) against taxonomy *viridiplantae*—green plants entries in the SwissProt database using MASCOT search engine (Last update: April 2015, containing 21, 306, 855 sequences). The search was performed using the following parameters: Fixed modification included was carbamidomethylation of cysteine whilst variable modification included was oxidation of methionine; peptide tolerance was ± 0.2 Da, MS/MS tolerance was 0.5 Da, peptide charge $+1$, $+2$, $+3$ and only monoisotopic mass were included in the search. Trypsin was used as a proteolytic enzyme with 1 allowed missed cleavage per peptide. For a confident ID, only proteins with a minimum of 2-matched peptides and MASCOT scores greater than 55 or extensive homology ($p < 0.05$) were considered.

3 Results

3.1 Image Analysis of Sago Protein Profiles by 2-DE

Different level of protein expressions in 16 years old trunking and non-trunking sago palm were analysed using 2-DE gels. Four gels were run from each trunking and non-trunking leaf samples to obtain four independent experiments (picture of gels provided as Supplementary Material). The 2-DE analysis of both sago leaf samples revealed a total of 865 protein spots. Out of the total number of protein spots, 34 spots were detected as significantly altered in abundance between trunking and non-trunking samples with $p < 0.05$ and fold change > 1.5. Fig 1 shows the representative image analysis of each sample and labelled with their protein ID.

3.2 Proteins Analysis by Mass Spectrometry

Based on the MALDI-ToF/ToF MS/MS analysis, 19 proteins were successfully identified out of 34 selected proteins (Table 1). In addition, there are 15 additional proteins that were identified but fall within the non-significant groups (Table 2). All the 19 identified proteins were upregulated in non-trunking sago compared to the trunking sago leaves. Among the proteins are enolase (spot 428), phosphoglycerate kinase (spot 516), ribulose bisphosphate carboxylase/oxygenase activase (spot 553 and 590), glutamine synthetase (spot 558), phosphoribulokinase (spot 580), malate dehydrogenase 1 (spot 582), malate dehydrogenase (spot 592), oxygen-evolving enhancer protein (spot 649, 652 and 664), ascorbate peroxidase (spot 702), putative cytosolic ascorbate peroxidase protein (spot 717), 23 kDa water-oxidizing complex of photosystem II protein (spot 746), carbonic anhydrase (spot 753), cytochrome b6-f complex

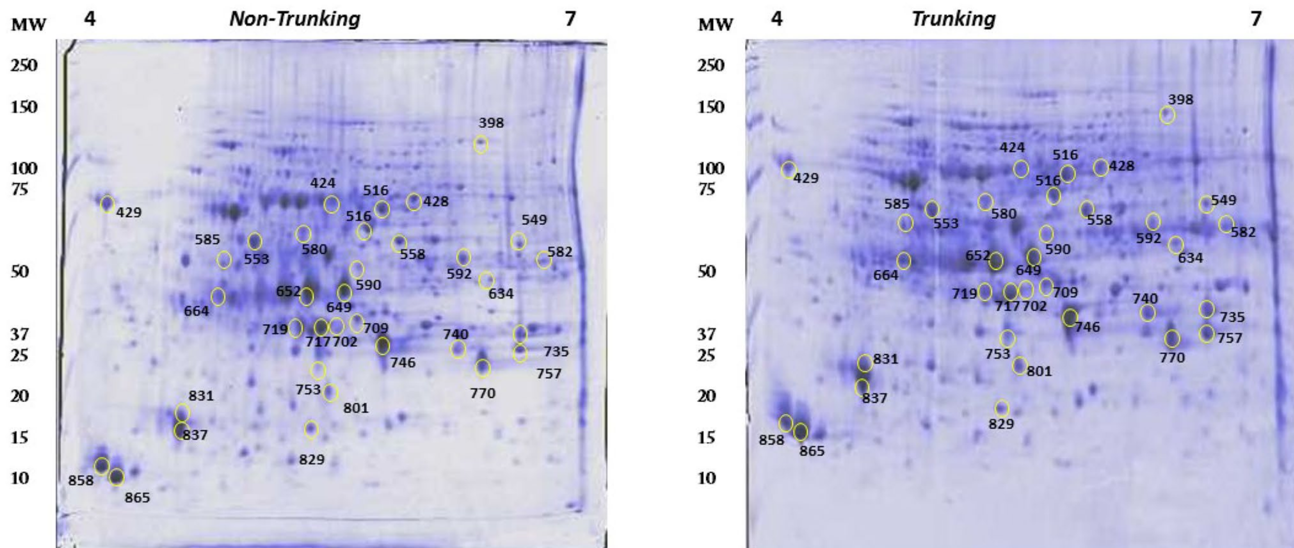

Fig. 1 Representative 2-DE profiles of trunking and non-trunking sago leaves. Thirty-four proteins were significantly different in abundance are labelled with their protein ID in the gel

iron-sulfur subunit (spot 829) and hypothetical proteins (spot 549, 709 and 770).

4 Discussion

4.1 Proteomics Analysis

The study of proteome has widely been used in order to understand plant response/adaptation through changes in the environment for plant growth and development [36]. This study was intended to identify differentially expressed proteins that can be potential candidates towards the non-trunking phenomenon of the sago palm. Several databases such as UniProt and NCBI were used to identify the functional and cellular process of the identified proteins. At the same time, to understand the role of the proteins towards the occurrence of stunted growth and physical changes of the sago plant. This is due to the fact that proteome in the plant is altered ahead before changes in characteristics are shown on plant tissue [7]. The identified proteins can be categorized into several categories such as photosynthesis, energy carbohydrate, defence and stress response, and unknown protein.

The identified proteins were mainly involved in energy metabolism and carbohydrate processes such as enolase (spot 428), phosphoglycerate kinase (spot 516), glutamine synthetase (spot 558), phosphoribulokinase (spot 580), malate dehydrogenase 1 (spot 582) and malate dehydrogenase (spot 592). Meanwhile, some of the proteins contributed to the photosynthetic activity such as ribulose bisphosphate carboxylase/oxygenase (RuBisCO) activase (spot 553 and 590), oxygen-evolving enhancer protein (spot 649, 652 and 664), carbonic anhydrase (spot 753), 23 kDa polypeptide of water-oxidizing complex of photosystem II (spot 746), cytochrome b6-f complex iron-sulfur subunit (spot 829) and unknown proteins (spot 549 and 770). The other proteins response to oxidative stress like ascorbate peroxidase (spot 702) and putative cytosolic ascorbate peroxidase protein (spot 717), while one more protein (spot 709) with unknown function.

Both enolase and phosphoglycerate kinase (PGK) is involved in the glycolytic process of the plant. Enolase is an abundant enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway [41, 59]. It is generally expressed in several plant species in response to a number of abiotic stresses such as salt, drought and cold [40, 49, 62]. It is also reported that enolase was expressed during fruit ripening in tomato [59]. Meanwhile, phosphoglycerate kinase (PGK) is a monomeric enzyme that catalyses the formation of ATP and 3-phosphoglycerate via the transfer of phosphate group of 1,3-bisphosphoglycerate to ADP [41]. This is the first reaction in the reduction step of Calvin cycle which is related to glycolysis, one of the respiration pathways. The enzyme found was related to biological stress in the plant [26]. A recent study showed the upregulation level of PGK enzyme when exposed to phosphorus deficiency using maize leaves [63]. High level of enolase and PGK enzymes in non-trunking sago may reflect a rapid stimulation of glycolysis and rate of metabolism. It also indicates an increased in photosynthetic carbon assimilation following exposure to a long-term abiotic stress which happened to non-trunking sago palm.

Table 1 List of identified proteins that showed higher expression level in non-trunking sago in comparison to trunking sago palm

Spot ID	Accession	Protein name	FC	*p* value	MW	pI	Score	MP	Cov (%)	Reference organism	Biological process	Molecular function
428	KVH99923.1	Enolase	2.6	0.001	42,441	7.63	67	33	8	*Cynara cardunculus var. scolymus*	Glycolytic process	Magnesium ion binding, phosphopyruvate hydratase activity
516	XP_014501683.1	Phosphoglycerate kinase, cytosolic	2.0	0.01	42,442	5.89	162	34	8	*Vigna radiata var. radiata*	Glycolytic process	ATP binding, phosphoglycerate kinase activity
549	XP_017178386.1	Uncharacterized protein LOC108169472	4.0	6.23E−04	36,787	5.95	73	22	6	*Malus domestica*		RNA–DNA hybrid ribonuclease activity, nucleic acid binding
553	XP_021650402.1	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic-like	2.4	9.87E−04	52,089	6.06	187	36	7	*Hevea brasiliensis*		ATP binding
558	AJD14835.1	Glutamine synthetase	3.3	0.018	39,247	5.64	123	31	8	*Boehmeria nivea*	Glutamine biosynthetic process	ATP binding, glutamate-ammonia ligase activity
580	XP_010933400.1	Phosphoribulokinase, chloroplastic-like	2.7	0.002	46,062	5.91	95	53	12	*Elaeis guineensis*	Carbohydrate metabolic process	ATP binding, phosphoribulokinase activity
582	KHN00213.1	Malate dehydrogenase 1, mitochondrial, partial	1.9	0.005	27,461	6.22	104	23	8	*Glycine soja*	Carbohydrate metabolic process, malate metabolic process, tricarboxylic acid cycle	L-malate dehydrogenase activity
590	XP_021650402.1	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic-like	2.8	0.025	52,089	6.06	103	36	7	*Hevea brasiliensis*		ATP binding
592	XP_010920478.1	Malate dehydrogenase	3.0	4.89E−06	35,909	6.01	150	57	17	*Elaeis guineensis*	Malate metabolic process, oxidation–reduction process	Malate dehydrogenase activity
649	AEN02470.1	Oxygen-evolving enhancer protein	7.0	0.00001078	35,542	8.13	140	38	11	*Camellia sinensis*	Photosystem II assembly, photosystem II stabilization	Oxygen evolving activity
652	AEN02470.1	Oxygen-evolving enhancer protein	3.5	2.53E−05	35,542	8.13	126	38	11	*Camellia sinensis*	Photosystem II assembly, photosystem II stabilization	Oxygen evolving activity
664	AEN02470.1	Oxygen-evolving enhancer protein	3.7	0.00006106	35,542	8.13	58	21	6	*Camellia sinensis*	Photosystem II assembly, photosystem II stabilization	Oxygen evolving activity
702	ACJ38537.1	Ascorbate peroxidase	2.5	0.04	27,595		62			*Oncidium hybrid cultivar*	Response to oxidative stress	Heme binding, peroxidase activity

Table 1 (continued)

Spot ID	Accession	Protein name	FC	*p* value	MW	pI	Score	MP	Cov (%)	Reference organism	Biological process	Molecular function
709	XP_008784609.1	Uncharacterized protein At5g02240-like isoform X1	3.8	0.006	33,185		103	17		*Phoenix dactylifera*		
717	AEZ00894.1	Putative cytosolic ascorbate peroxidase protein	6.0	2.07E−04	27,564	5.51	80	38	15	*Elaeis guineensis*	Response to oxidative stress	Heme binding, peroxidase activity
746	CAA45700.1	23 kDa polypeptide of water-oxidizing complex of photosystem II, partial	14.5	1.07E−05	22,015	5.28	58	21	10	*Nicotiana tabacum*	Photosynthesis	Calcium ion binding
753	XP_008801824.1	Carbonic anhydrase, chloroplastic isoform X2	5.6	4.16E−04	22,731	6.12	87	27	13	*Phoenix dactylifera*	Carbon utilization	Carbonate dehydratase activity, zinc ion binding
770	KFK31259.1	Hypothetical protein AALP_AA6G089000	10.0	1.58E−05	28,679	8.60	76	25	9	*Arabis alpina*	Photosynthesis	Calcium ion binding
829	OAY82086.1	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	23.0	7.53E−07	25,452	9.25	132	30	12	*Ananas comosus*	Photosynthesis	2 iron, 2 sulfur cluster binding; electron transporter, transferring electrons within cytochrome b6/f complex of photosystem II activity; plastoquinol-plastocyanin reductase activity

Table 2 List of the non-significant proteins

Spot ID	Accession	Protein name	FC	*p* value	MW	pI	Score	MP	Cov (%)	Reference organism	Biological process	Molecular function
398	XP_001696129.1	Predicted protein	1.5	5.04E−04	272,063	8.84	23	22	0	*Chlamydomonas reinhardtii*		
424	AHI88624.1	3-hydroxy-3-methylglutary CoA reductase	1.8	0.041	60,684	8.09	29	19	3	*Dendrobium chrysotoxum*	Coenzyme A metabolic process, isoprenoid biosynthetic process	Hydroxymethylglutaryl-CoA reductase (NADPH) activity, NADP binding
429	XP_006419784.1	Hypothetical protein CICLE_v10005759mg	6.1	2.91E−04	25,686	5.81	31	18	7	*Citrus clementina*		Hydrolase activity
459	XP_010696390.1	Uncharacterized protein LOC104908918	2.9	8.39E−04	63,524	7.65	23	25	4	*Beta vulgaris*		Microtubule binding
585	KZV34967.1	Sedoheptulose-1,7-bisphosphatase, chloroplastic-like	3.2	3.79E−04	128,958	7.27	34	13	1	*Dorcoceras hygrometricum*	Carbohydrate metabolic process	Phosphatase activity
634	XP_002273627.2	Diphthamide biosynthesis protein 2 isoform X1	1.7	0.037	56,040	5.15	22	21	4	*Vitis vinifera*	Peptidyl-diphthamide biosynthetic process from peptidyl-histidine	Transferase activity
719	At1g35710	Leucine-rich repeat receptor-like protein kinase	6.0	0.003	64,326	9.05	31	23	3	*Ipomoea nil*		Protein binding
735	XP_008380167.1	Oxygen-evolving enhancer protein, chloroplastic-like	9.4	1.12E−04	28,842	8.67	39	10	3	*Malus domestica*	Photosynthesis	Calcium ion binding
740	EOY00479.1	Carbonic anhydrase 2, CA2 isoform 1	12.0	2.01E−05	31,111	5.48	48	16	5	*Theobroma cacao*	Carbon utilization	Carbonate dehydratase activity, zinc ion binding
757	EMT19451.1	Carbonic anhydrase, chloroplastic	7.7	0.002	22,653	5.97	37	17	8	*Aegilops tauschii*	Carbon utilization	Carbonate dehydratase activity, zinc ion binding
801	XP_011072147.1	Putative late blight resistance protein homolog	12.0	2.58E−05	101,371	5.77	31	23	2	*Sesamum indicum*		ADP binding
831	OIW14372.1	Hypothetical protein TanjilG_15726	30.0	1.04E−06	45,854	9.05	20	25	6	*Lupinus angustifolius*		Methyltransferase activity
837	XP_010941216.1	Glycine cleavage system H protein 3, mitochondrial-like	34.0	5.00E−03	23,306	5.41	38	16	7	*Elaeis guineensis*	Glycine decarboxylation via cleavage system	
858	XP_019238609.1	Uncharacterized protein LOC109218693	22.0	0.002	32,242	5.69	31	23	8	*Nicotiana attenuata*		
865		3-isopropylmalate dehydratase large subunit					35			*Malus domestica*		

A higher level of protein expression related to photosynthetic activity has been identified in non-trunking sago proteome. They are ribulose bisphosphate carboxylase/oxygenase activase, also known as RuBisCO activase (2 spots), oxygen-evolving enhancer proteins (3 spots), carbonic anhydrase, 23 kDa water-oxidizing photosystem II protein complex, cytochrome b6-f complex iron-sulfur subunit and hypothetical protein AALP_AA6G089000. RuBisCO is considered the most abundant protein in plant leaves and is important for ATP binding during carbon fixation [8, 23]. Activation of RuBisCO is mediated by a specific protein known as RuBisCO activase, and dependent on the concentrations of both ATP and ADP [50] in the response of photosynthesis to temperature [46]. RuBisCO activase is one of a new type of chaperone, functions to promote and maintain the catalytic activity of RuBisCO [46]. Due to differential expression of this protein in sago palm, it is suggested that RuBisCO isoforms contributing to the trunking and non-trunking in sago palm. The presence of protein isoforms with different expression patterns has also been reported in date palm and other species such as *Arabidopsis* [54]. Interestingly, these isoforms are observed in both trunking and non-trunking sago palm with an upregulated level in the non-trunking palm. RuBisCO activase consistently increased under both high temperature and drought stress which concludes its crucial role to continue CO_2 fixation under stress, as well as protecting the plant photosynthetic capacity. In addition, it is expected that a higher RuBisCO content may be related to the quick restoration of the protein function during recovery [16, 27]. The increased level of this photosynthetic protein reinforced the suggestion that non-trunking sago palm requires additional energy to activate various resistance mechanisms under abnormal and stressful conditions [41], hence requiring the higher level of this protein.

For oxygen-evolving enhancer (OEE) proteins, it is the most identified photosynthetic enzyme in non-trunking sago plant with three upregulated spots. The OEE proteins are mostly present in the plant to cope with salt stress including water deficient, ion imbalance and toxicity [57]. This protein was also observed in relation to the accumulation of salt in the leaves, which reduces photosynthetic efficiency in the plant before the leaves die [1]. Peripherally bound to photosystem II (PSII), it is essential for oxygen-evolving activity and PSII stability. Enhanced expression of OEE2 and other enzymes in rice had indicated protective responses due to low doses and short term NaCl stress. Study on mangrove *Bruguiera gymnorrhiza* was resulted to the increased on this protein level after NaCl treatment using northern analysis, and also by two-dimensional gel electrophoresis to support the finding [57]. Therefore, the presence on the numbers of OEE proteins in non-trunking leaves indicated the salt tolerance of the sago palm due to the decreased on the rates of transpiration, as the mangrove habitat is quite similar to the non-trunking sago plantation.

The highly expressed protein of carbonic anhydrase (CA) in non-trunking sago palm compared to its expression in the trunking palm, is involved in many biological processes including acidity regulation, CO_2 transfer, ion transfer, respiration and photosynthetic CO_2 fixation [58]. This protein is a highly abundant soluble protein, after RuBisCO, in the C3 plant chloroplast [44, 48]. Functionally, it can rapidly interconvert the major forms of C1. With this function, the plant is able to maintain the supply of CO_2 for RuBisCO by speeding the dehydration of $HCO\text{-}_3$ in the stroma [42]. In other words, CA acts as a primary enzyme in the CO_2 fixing pathway and without it, CO_2 can still diffuse at a reasonable rate and only marginally affected photosynthesis [11]. Similar results were obtained from studies of *Halogeton glomeratus*, where increasing level of photosynthetic enzymes was related to CO_2 assimilation and light reactions under salt stress, showing growth inhibition under salt stress condition [60]. This is similar to the condition where the non-trunking of sago palm is located.

Glutamine synthetase (GS) was also observed to have differential expression in trunking and non-trunking sago. This enzyme catalyses the construction of glutamine from glutamic acid, NH_3 and ATP during glutamine biosynthetic process [41]. There are two isoforms of GS which are cytosolic GS (GS_1) occurring in the cytosol and chloroplastic GS (GS_2) located in chloroplasts/plastids [34]. GS_1 is the predominant isoform found in non-photosynthetic tissues and has complex function due to its numerous isoforms [13, 39] while GS_2 assimilates ammonia from nitrate reduction and reassimilates ammonia during photorespiration in leaves [45]. A study on tea plant has ascertained that GS is used for removal of toxic caused by excess ammonium via glutamine synthesis reaction. When the excess concentration of ammonium accumulates under cold stress condition, the theanine synthesis pathway of the tea plant is activated to remove ammonium stored in theanine [41]. During the growth of transgenic tobacco plants stressed by low nitrogen level, increased activity of GS enhanced the activities nitrogen absorption and other enzymes involved in nitrogen and carbon metabolism [61]. In addition, a study on *Halogeton glomeratus* plant also suggested that the accumulation of GS protein and other compatible organic solutes may be associated to the salt stress tolerance [60]. Thus, the increased level of GS protein in non-trunking leaves can be linked to stress tolerance to enhance the primary metabolism of the plant.

Phosphoribulokinase, chloroplastic-like protein and malate dehydrogenase (MDH) are also upregulated in non-trunking sago in comparison to trunking sago palm. Phosphoribulokinase, chloroplastic-like is essential in carbohydrate metabolic process and plays very important roles in

photosynthesis [51]. Overexpression of this protein in non-trunking leaves is another evidence of environmental stress like previously reported on the study of proteomics of date palm during drought and salinity stress [22], and also on maritime pine during gravitropism and phototropism stimuli [29]. Malate dehydrogenase (MDH) plays an important role in cellular metabolism in non-trunking sago leaves. MDH is involved in the synthesis of malate through the reversible reduction of oxaloacetate to malate [52, 53] and has a crucial role in removing radicals that were formed during oxidative stress [38]. A study on oil palm had found an altered abundance of MDH after being infected with *Ganoderma boninense* and reported that overexpression of MDH may be an indication of metabolic disorder of the plant tissues [4]. As the non-trunking sago plant is affected by environmental stress condition in a swampy area, therefore, become an essential need in attempts to improve nutrient deficiency during plant development. Moreover, presence of MDH enables plants to adapt to various stress conditions, as well as to control growth and development [53].

Another enzyme showing differential expression level between trunking and non-trunking sago palm, ascorbate peroxidase, is usually associated to plant responses to stress. In photosynthesizing eukaryotes, the balanced state of a redox reaction is maintained by a gentle balance between energy consumption and energy production. The balanced state is affected by the need to avoid increased production of reactive oxygen species (ROS). The low-level photosynthesis rate in the chloroplast may increase the ROS production under salt stress condition [12]. High antioxidant activity is suggested to be linked to the plant's ability for stress tolerance [30]. Based on the proteomics analysis of *H. glomeratus,* the elevated expression level of antioxidative enzymes was observed under salt stress. The situation happened in order to regulate the balance of ROS formation as well as for removal and defence against cell oxidative damage [60]. The up-regulated presence of ascorbate peroxidase protein in the non-trunking sago palm indicated that the protein plays crucial roles in defending against oxidative stress.

Based on the 2D-PAGE coupled with MALDI-ToF-ToF MS analysis, sago palm developed an increase of protein expressions in leaves of non-trunking sago as a response to thermotolerance, salt tolerance and other environmental stress factors. The proteins affect the photosynthesis and metabolism process in adaptation to stay alive, however, causes a physical abnormality of the sago plant. This interesting finding might become the first trigger on sago palm research in understanding the factor that causes the non-trunking phenomena in sago plantation.

In conclusion, the proteomic study showed upregulation in many proteins and enzymes related to energy and metabolism in stressed non-trunking sago palm leaves in comparison to the trunking trees. The study showed that changes in the proteins and metabolites related to energy, metabolism and growth due to the environmental stress could lead to the non-trunking phenomena of sago palm growth. Further investigation on the changes of those enzymes and metabolites at the molecular level is required in order to fully understand the trunk formation and elongation process under abiotic stress, as well as starch biosynthesis process in the trunk of the sago palm.

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