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C1 Metabolism and Photorespiration of *Ficus deltoidea* based on Peptide Mass Fingerprinting Approach

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ABSTRACT

Ficus deltoidea is a popular herbal plant as ethnomedicine, especially from its leaves. The decoction of leaves is used as tonic to regain energy, strengthen uterus, improve blood circulation, treat diabetes, gout, hypertension and also to reduce water in lung disease. Therefore, the plant physiology including its photorespiration mechanism is of great importance to understand its biological properties. Plant proteins are building blocks of many bioactive secondary metabolites. The present study extracted the plant proteins using Tris-buffered phenol technique, and then crude proteins were separated by gel electrophoresis prior to peptide identification using LC-QTOF MS. The identified proteins were used to explain the C1-metabolism and photorespiration in *F. deltoidea*. Mass spectra of peptides were found to match 229 proteins, and 9 of them were strongly related to C1-metabolism. The proteins such as pentatricopeptide repeat protein, tetratricopeptide repeat protein, 5,10-methylenetetrahydrofolate dehydrogenase:5,10-methenyltetrahydrofolate cyclohydrolase and folylpolyglutamate synthase are essential in photorespiratory cycle. The detection of the proteins suggests that *F. deltoidea* perform photorespiration via C1-THF synthase/SHMT pathway which is the alternative photorespiratory pathway. The findings of this study could be used to explain the production of bioactive metabolites in *F. deltoidea*. This is also the first report to reveal the C1-metabolism and photorespiration in *F. deltoidea*.

Keywords: photorespiration, C1-metabolism, *Ficus deltoidea*, LC-MS/MS, phosphoglycolate

INTRODUCTION

Ficus deltoidea in the Moraceae family has traditionally been used as ethnomedicine. Several varieties such as var. *angustifolia*, var. *bilobata*, var. *intermedia*, var. *kunstleri*, var. *motleyana* and var. *trengganuensis* are widely used in the Malay traditional medicines. Malay women or ladies are used to consume the plant decoction for energy restoration and uterus strengthening, especially after childbirth [1,2]. Furthermore, it is believed to be effective in improving blood circulation, diabetes, gout and hypertension, as well as reducing water in lung disease [3]. The herbal drinks of *F. deltoidea* are also used for health and beauty purposes to detoxify, reduce cholesterol, improve menstrual cycle, and to assist low libido energy among men and women [4]. The combination of powdered roots and leaves of *F. deltoidea* is used to treat many ailments, particularly wound, rheumatism and sore for centuries among indigenous people in Peninsular Malaysia.

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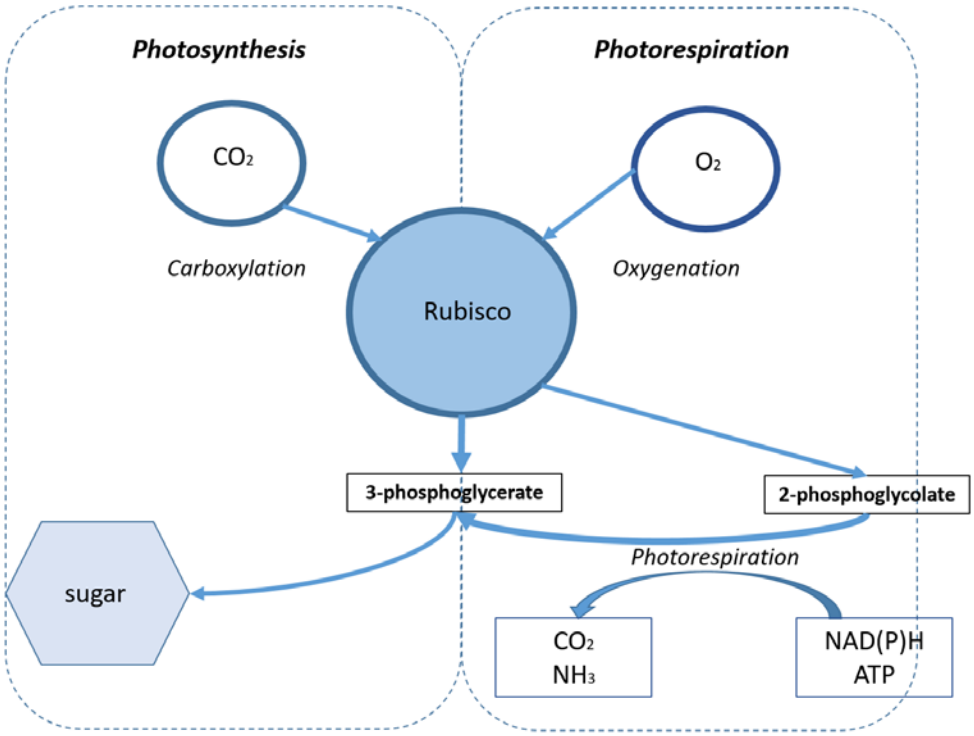

Figure 1. Schematic diagram of photosynthesis and photorespiration. CO_2, carbon dioxide; O_2, oxygen; NH_3: ammonia and Rubisco: ribulose-1,5-bisphosphate-carboxylase/oxygenase

One-carbon (C1) metabolism is very crucial to provide one carbon for methylation and other types of modifications, as well as for biomolecules such as nucleic acids and amino acids [5]. However, the regulatory mechanism of plant C1 metabolism involving enzymes is not fully understood till to date. This could be due to low abundance of enzymes, which may exist as several isoforms, mutants studies on enzyme are insufficient and C1 substituted folates are sensitive and difficult to quantify [6,7]. Nowadays, integration of molecular and genetic approaches is rapidly driving biochemical study to advance the knowledge of plant C1 enzymes and genes [7].

C1 metabolism also involves in photorespiration which is a reversed biochemical process of photosynthesis as illustrated in **Figure 1**. Photorespiration is also known as oxidative photosynthetic carbon cycle where oxygen (O_2) is fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) rather than carbon dioxide (CO_2). Photorespiration usually occurs during hot and dry conditions which are unfavourable for plant growth, and causing plants to experience drought stress [8]. Plants undergo the abiotic stress by photorespiratory process [9]. The product of photorespiration is returned to the Calvin cycle for regeneration of ribulose-1,5-bisphosphate (Rubisco), a five-carbon sugar. Thus, photorespiration is an unavoidable oxygenase reaction which covered for approximately 25% of Rubisco reaction.

Photorespiration produces 3-phosphoglycerate and 2-phosphoglycolate. The latter is converted back to 3-phosphoglycerate in the photorespiratory cycle. The photorespiratory pathway requires energy (ATP) to reduce (NAD(P)H) equivalents, release CO_2 and ammonia (NH_3) (**Figure 1**). This explains why photorespiration is considered as a wasteful pathway. Phosphoglycerate recovery is accompanied by carbon and energy losses, and therefore, photorespiration can reduce productivity of crop plants. Regulation of photorespiration is a prime subject of study for crop improvement to facilitate natural supply for world growing population and challenge from climate changes [10].

It is important to investigate the photorespiratory cycle of *F. deltoidea*, particularly dealing with stress responses to prevent reactive oxygen species accumulation under stress conditions such as drought, salinity, low CO_2 and chilling [11]. The present study was aimed to use spectrometric proteome to predict the C1 metabolism in relation to photorespiration in *F. deltoidea*. Phenol based extraction was used to extract plant proteins, separated by gel electrophoresis and trypsin digested for peptide mass fingerprinting. This is also the first report to use *F. deltoidea* as the subject of the study, since this herb has been shown to have many bioactive phytochemicals as potential medicinal leads. The plant proteome would provide insight of the underlying cellular C1 mechanism which lead to the production of those phytochemicals.

MATERIALS AND METHODS

Plant Samples

F. deltoidea was purchased from an herbal nursery located at Jalan Pontian Lama, Johor Bahru. The plant was authenticated by Universiti Kebangsaan Malaysia, Campus Bangi, Selangor. The species is *F. deltoidea* Jack var. *trengganuensis* Corner which is kept in the herbarium of Universiti Kebangsaan Malaysia with the specimen number (40213).

Protein Extraction

The frozen young leaves (1 g) of *F. deltoidea* were ground homogenously in liquid nitrogen. The ground leaves were extracted by cold extraction buffer (10 mL) for plant proteins [12]. The cold extraction buffer was consisted of 0.5 M Tris-HCl (pH 7.5), 0.1 M KCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2% β-mercaptoethanol and 0.7 M sucrose. An equal volume of Tris buffered phenol was added to the mixture, and incubated for 30 min on a shaker at 4 °C. The mixture was centrifuged for 30 min at 5000 xg after incubation. The phenol phase (top layer) was carefully harvested and put aide. The remaining mixture was extracted again using similar buffer. The collected phenol solution was topped up with 5 volumes of 0.1 M cold ammonium acetate in methanol and kept at -20 °C overnight. Precipitate could be collected by centrifugation for 30 min. The protein pellet was thricely rinsed with ice-cold methanol, and then centrifuged at 4°C for 10 min. Subsequently, the pellet was rinsed with acetone, and then air-dried *in vacuo*. The dried protein pellet was re-constituted in sample buffer consisted of 8 M urea, 20 mM dithiothreitol (DTT), 4 % 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) and 5mM Tris base for protein estimation. Protein content was estimated based on the calibration curve constructed using bovine serum albumin in the concentration range of 0 to 20 µg/mL.

One-dimensional Gel Electrophoresis

Protein separation was conducted on a 12% polyacrylamide gel with a 5% stacking gel using a Mini-Protean R Tetra Cell from Bio-Rad Laboratories GmbH (Munchen, Germany). Bio-Rad power pac was regulated at 100 V for 10 min, and followed by 150 V for 45 min. The resulting protein bands were in-gel digested by trypsin, purified and concentrated using ZipTip C18 (Merck Milipore, United States) before LC-MS/MS analysis.

Liquid Chromatography-QTOF Mass Spectrometer

A hyphenated system consisted of liquid chromatography (UltiMate 3000; Sunnyvale, CA) and QTOF mass analyzer (AB SCIEX QSTAR Elite; Foster City, California) was used to perform peptides analysis. A C18 reversed phase column (Zorbax 300SB, 150 x 0.3 mm, 5 µm) was used for separation. The mobile phase was 0.1% formic acid (Solvent A) and acetonitrile (Solvent B). The mobile phase of LC gradient was: 0-5 min, 98% A; 5-15 min, 98-55% A; 15-16 min, 55-20% A; 20 % A maintain for 3 min; 17-18 min, 20-98% A and 98% A maintain for 10 min. The total run was 30 min at 5 µL/min and the injection volume was 5 µL.

Mass spectra of peptides were searched using MS-Fit, ProteinProspector (v 5.20.0), University of California, San Francisco. The set parameters as below: maximum missed cleavage, 2; modification, carbamidomethyl; minimum match, 2; minimum peptide ion match, 1; MOWSE On, 1; and digester, trypsin. Since enzymatic digestion was sometimes incomplete with partial fragments, the number of missed cleavage was usually set at 1 or 2 in the case of trypsin digestion. Carbamidomethylation is one of the fixed post-translational modifications which considered cysteine modification and did not alter the number of potential peptides. The database used for the search is NCBInr.2013.6.17 under *Arabidopsis thaliana* with 64727 entries.

Protein Identification and Classification

The identified proteins were classified according to their biological function reported in the GoMapMan database (http://www.gomapman.org/ontology).

RESULTS AND DISCUSSION

Extraction of Plant Protein

The nature of plant tissue shall be taken into consideration during method selection for plant protein extraction. This is because plant protein presents in low concentration, heat and chemical labile. Plant protein always co-exists

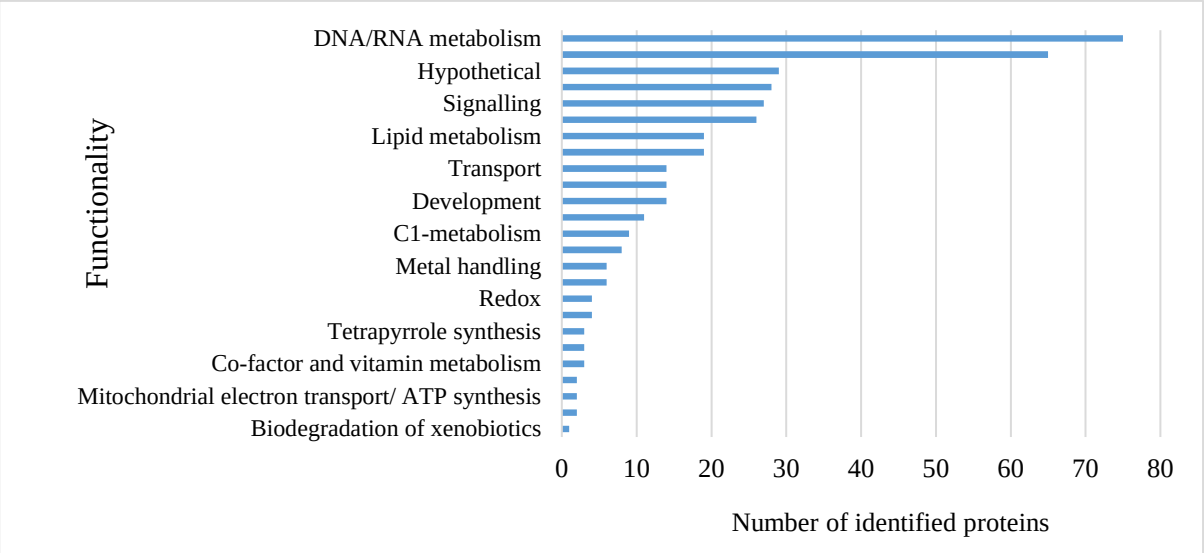

Figure 2. Functional classification of *Ficus deltoidea* leaf proteome identified by LC-MS/MS

with other components such as polysaccharides, lipids, phenolics and a vast array of secondary metabolites which cause matrix interference during protein separation and downstream analysis.

The effectiveness of plant protein extraction and contaminant removal are highly depended on the fineness of tissue powder. It was found that thick, fibrous, and lignified nature of *F. deltoidea* leaves are relatively difficult to disrupt until fine powder. Therefore, young leaves were selected for protein extraction because mature leaves always have high concentration of interference compounds, especially polyphenols. The co-existence of polyphenols in protein extracts might involve tedious rinsing step for their removal.

The ideal protein extraction method is able to capture and solubilize maximum proteome with the minimum content of contaminants. Phenol based extraction has been proven to be suitable for recalcitrant plants [13]. Recalcitrant plants were reported to have large amount of secondary metabolites such as phenolics, lipids, organic acids, carbohydrate, terpenes and pigments which could precipitate with protein and interfere proteomics analysis [14,15]. Phenol based extraction combined with ice cold methanol and acetone wash could help reduce molecular interaction between proteins and interfering compounds. Therefore, good quality and high yield (459.24 µg/mL) of protein could be extracted from *F. deltoidea* leaves. The protein pellet was white, and this explains less plant pigments and chlorophylls co-extracted by the method.

Classification of Protein

Protein extract of *F. deltoidea* was subjected to LC-MS/MS analysis. Mass spectra was matched to the protein database and found to identify 229 proteins. **Figure 2** shows the functional categories of the identified proteins. The most abundant category is belonging to DNA/RNA metabolism (75), followed by plant growth related proteins (65), hypothetical (29), miscellaneous (28), signalling (27), stress protein (26), lipid metabolism (19), cell protein (19), hormone metabolism (14), development (14), transport (14), secondary metabolism (11), amino acid metabolism (8), C-1 metabolism (9), cell wall proteins (6), metal handling (6), photosynthesis (4), redox (4), major CHO metabolism (3), co-factor and vitamin metabolism (3), tetrapyrrole synthesis (3), minor CHO metabolism (2), mitochondrial electron transport (2), nucleotide metabolism (2) and finally protein related to biodegradation of xenobiotics (1). Only proteins related to C1-metabolsim are selected and their matched peptide sequences are listed in **Table 1**.

Table 1. Identified protein related to C1-metabolism

Size	Protein Name	Location	Accession number	Mass (Da)	MOWSE Score	Sequence
20 kDA	Tetratricopeptide repeat-containing protein	cytosol, nucleus, mitochondria, and peroxisomes	NP_178157.2	102148	193526	MLQADQVPLAEK; MTLRSYVDMLK; KFPDHGETLSMK; LIQVEEPMAEASK; SEESTASGASKSGKR; HSIGTTGSFPGSDVK; RMLQADQVPLAEK; KMTLRSYVDMLK; LGAENPDSHRSLVK;
						HAGDLTAAAALADEAR; RNVKPVDPDPHGQK; AAYAEMLYILDPSK; RIPLDFLQDENFK; SHVCWHVLGLLYR; HCSLMPDSVYNSSR; MTLRSYVDMLKFQDR
	Pentatricopeptide repeat (PPR)-like superfamily protein	Mitochondria Chloroplast	NP_177755.3	87760	150087	QSIAESSAPSITNK; ANAEKVLADLENR; NSPEAILEKTSMK; LSMALEKKANAEK; AGITEGIVNGIHER; DQKDHIQTHQMK; LPGSSTGSGSQISALR; KANAEKVLADLENR; IFCEDISRMNMK; MNMKRTHDVLETK;
						EGITNDEKYMLRK; AVKEDIELEAKDQK; MNMKRTHDVLETK; MHFQKNPLAIVNVK; LTENEEEIKPRAVK; DFLPSAVSSAIEERR; VILYRGWGAEEEMK; TTEVVKIFCEDISR; RNSPEAILEKTSMK; QRNSPEAILEKTSMK;
						SLGFVEEKHDSPTRR; DQKDHIQTHQMKSR; MLLPLFHQQPLILAK; ERKSENDGLVTDLSNR; KSENDGLVTDLSNRER; DFLPSAVSSAIEERRR; MVKPMLTQGVGSPDKVR
	Tetratricopeptide repeat-containing protein	cytosol, nucleus, mitochondria, and peroxisomes	NP_182266.1	58652	88090	LCVLTTKTQKLK; YKPDRASSFIER; SAGRIAEAIADCNK; GYTAVTAIIAEEQR; CFPDSLHDLEHLK; DVADMLQDYIPSLK; HWWFTHRKLVDK; AHLLLCLRYKPDR;
						AGFNHPIPVKYEALTC; RPAPQGFLAECYMHR
	Folylpolyglutamate synthase	Mitochondria	NP_187627.3	68921	204789	ALFVPSMSTYNK; QILLFNCMEVR; FRIDGLDISEEK; MLVCGKGFLKCR; TGMFTSPHLIDVR; HGEYGTDLNRVSK; CWLQRTGNWKK;
						GDKSLSTAVNGYMR; LVTTCASSGTHFSR
50 kDA	Ribulose 1,5 biphosphate	Chloroplast	ANH54243.1	85387	760	DLAIEGNEIIR, TQFGPPHGIQVER,DSDILAAFR
	Pentatricopeptide repeat-containing protein	Chloroplast	NP_195454.1	70097	104	AYASHGK; VCSDCHTVTK; VCTGLIDMYS
	Pentatricopeptide repeat-containing protein	Mitochondria	NP_190904.1	85675	116	FNQMSDASR; ACASSSDVGLGK; TYISLICACSSSR
	Pentatricopeptide repeat-containing protein	Mitochondria Chloroplast	NP_188091.1	72712	72.1	MVFDSMR; FMSVEDAQK; GSDAMELSDVK
250 kDA	5,10-methylenetetrahydrofolate dehydrogenase: 5,10-methenyltetrahydrofolate cyclohydrolase, putative (*DHY-CYC*)	Chloroplast Cytosol Cytoplasm	AAM62762.1	31567	149	SNIVGLPVSLLLLK; HGKVPGLAVVIVGSRK; MASSSDHTAKIIDGK; EADIVIAACGQAHMIK

Proteins for C1 Metabolism and Photorespiration

This study, 3.9 % of 229 proteins identified were related to one-carbon (C1) metabolism in *F. deltoidea*. The proteins were tetratricopeptide repeat-containing protein, pentatricopeptide repeat (PPR)-like superfamily protein, folylpolyglutamate synthase and putative 5,10-methylenetetrahydrofolate dehydrogenase: 5,10-methenyltetrahydrofolate cyclohydrolase (DHY-CYC), and ribulose 1,5 biphosphate. The C1 proteins found in this study were related to photorespiration. C1 metabolism was found to be active in plant tissues producing methylated compounds such as lignin, alkaloids, and betaines [7].

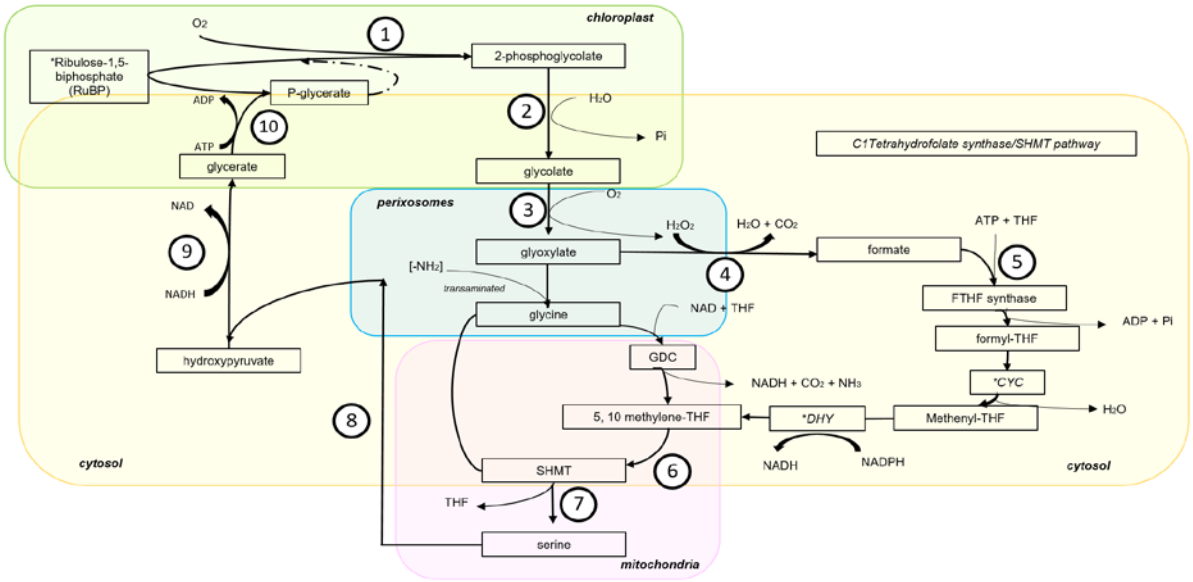

Figure 3. A suggested photorespiratory pathway via C1-THF synthase/SHMT pathway as an alternative pathway for *F. deltoidea* leaves. GDC: glycine decarboxylase; SHMT: serine hydoxymethytransferase; THF: tetrahydrofolate; FTHF synthase: 10-formyl-THF synthase; DHY-CYC: bifunctional 5,10-methylenetetrahydrofolate dehydrogenase: 5,10-methenyltetrahydrofolate cyclohydrolase. Regeneration of ribulose-1,5-biphosphate (RuPB) in Calvin cycle. **1**-Fixation of oxygen by RUBISCO resulted in 2-phosphoglycolate; **2**-Conversion of 2-phosphoglycolate to glycolate by 2-Phosphoglycolate phosphatase; **3**-Oxidation of glycolate to glyoxylate catalysed by glycolate oxidase; **4**-Nonenzymatic decarboxylation of glyoxylate to formate; **5**-Conversion of formate into 5,10 methylene-THF via C1-THF synthase pathway by 10-formyl-THF synthetase; **6 & 7**- 5,10 methylene-THF used by SHMT as a substrate for the transfer of an activated C1 moiety to another molecule of glycine resulting in the formation of serine; **8**-Serine deaminated to hydroxypyruvate by serine-glyoxylate aminotransferase (SGAT) occur in perixosomes; **9**-Hydroxypyruvate resulting from the SGAT reaction is reduced to glycerate by hydroxypyruvate reductase; **10**-chloroplastic D-glycerate 3-kinase catalyses the phosphorylation of glycerate to 3-phosphoglycerate with ATP as a co-substrate. *proteins identified from the present study.

Folylpolyglutamate synthetase (FPGS) was identified, and this enzyme can be found at the matrix and inner membrane of mitochondria involving in tetrahydrofolate (THF) synthesis. THF or collectively termed as folate, serves as donor and acceptor in one-carbon transfer reactions. THF carries one-carbon unit at various oxidation states such as methyl, methylene, methenyl, or 10-formyl THF attached to the N5 (pterin ring) and/or N10 (p-aminobenzoic acid) positions. The metabolic function is dependent upon the type of one-carbon unit loaded onto THF [16]. THF coenzymes involved in C1 metabolism are also polyglutamylated. Therefore, FPGS and dihydrofolate synthetase (DHFS) are responsible to catalyse the attachment of glutamate residues to folate during THF synthesis [17]. Folate is synthesized in dividing tissues and leaves to enable high fluxes of one-carbon unit for the accomplishment of photosynthesis and photorespiration [18]. The identification of folylpolyglutamate synthetase explains the folate metabolism in *F. deltoidea* for plant stress response [16,19]. Indeed, folate plays an important role in photorespiratory cycle at high rate in green leaves of C3 plants. Two main reactions involving serine hydoxymethytransferase (SHMT) and glycine decarboxylase complex (GDC) will result in the formation of methylenetetrahydrofolate (carbon-one unit) in the photorespiratory cycle.

Photorespiration can also occur via an alternative pathway named as C1-tetrahydrofolate synthase/SHMT pathway as presented in **Figure 3**. This pathway involves oxidation of glycolate to glyoxylate catalysed by glycolate oxidase (GO, EC 1.1.3.15) with the formation of H_2O_2. Glyoxylate which is the precursor of glycine is decarboxylated to formate, and then further converted to 5,10 methylene-THF through series of reactions. The reactions involve two enzymes which are related to one carbon-metabolism; 10-formyl-THF synthase (FTHF synthase) and 5,10-methylenetetrahydrofolate dehydrogenase: 5,10-methenyltetrahydrofolate cyclohydrolase (DHY-CYC), followed by formation of serine by serine hydoxymethytransferase (SHMT) [20]. Some organisms have 10-formyl-THF synthase (FTHF synthase), 5,10-methylenetetrahydrofolate dehydrogenase (DHY) and 5,10-methenyltetrahydrofolate cyclohydrolase (CYC) forming a single dimeric trifunctional protein [21]. However, studies on pea and spinach leaves reported that FTHF synthase activity is associated with a protein lacking of DHY and CYC activities [21,22]. FTHF synthase could be monofunction [22]. Nevertheless, FTHF synthase, CYC, and DHY were predominantly cytosolic as their activities mainly occur in cytosol [23]. Since DHY and CYC were identified in this study, it can be postulated that *F. deltoidea* is a C3 plant which does not have special feature to combat oxygenation by Rubisco. Therefore, photorespiration might occur via the alternative pathway of C1-THF synthase/SHMT.

Regulatory Proteins of Photorespiration

Another two proteins related to C1-metabolism were also identified; pentatricopeptide repeat (PPR) and tetratricopeptide repeat (TPR) proteins. Pentatricopeptide repeat (PPR) proteins which consist of modular RNA-binding proteins regulate gene expression in organelles and nucleus. PPR proteins also facilitate splicing, editing, stability and RNAs translation. PPR motif is believed to arose through divergence of a tetratricopeptide repeat (TPR) motif [24].

PPR is a canonical motif composed of 35 amino acids and repeated in tandem up to 30 times, constituting one of the largest protein families in land plants. The genome of *Arabidopsis thaliana* encodes 450 PPR proteins, and other terrestrial plant genomes sequenced encode even more PPR proteins [25]. Most PPR proteins are localised in mitochondria or chloroplasts. Their actions have significant effects on organelle biogenesis, photosynthesis, photorespiration, plant growth and other environmental responses. Thus, PPR proteins may modulate gene expression at the RNA level in relation to photosynthesis in *F. deltoidea* leaves.

The transcriptional regulators may not be adequate because promoters are limited in organelle genomes, and having extended half-lives. Therefore, organelle transcriptomes require RNA-binding proteins for functioning at the post-transcriptional level. Pentatricopeptide repeat (PPR) protein family is one of the major plant RNA-binding proteins for post-transcriptional control. The post-transcriptional processing steps involve RNA processing, editing, and splicing before translation [26]. Proteins containing PPR motifs will participate in translation initiation by binding to specific sequence elements in the 50UTR of mRNAs. PPR proteins are responsible for RNA cleavage and stability. Indeed, PPR protein binding could recruit endonucleases, and thus leading to RNA cleavage, and preventing the endonucleolytic cleavage by blocking RNases, thus stabilizing the RNA [27].

The tetratricopeptide repeat (TPR) motif involves in protein interaction. TPR can be found at various subcellular locations such as cytosols, nucleus, mitochondria, and peroxisomes [28]. The number of TPR motifs varies among proteins. TPR motif may contain 34 amino acids forming two helices; α and β connected by a short intra-repeat loop with three tandemly arranged TPR motifs. The motifs form a right-handed superhelix with an amphipathic channel that may accommodate a target protein [28,29]. TPR-containing proteins involve in many cellular functions including cell cycle control, protein transport, protein folding, regulatory phosphate turnover, transcription and splicing events.

Polypeptide transport across membrane is a cellular process. Protein complex which involves in this cellular translocation was found to have domain consisted of tetratricopeptide repeat motifs. For example, receptors containing 3-TPR domain could be Tom20 [30], Tom70 [30,31] and Tom34 [32], peroxisomal receptor Pex5 [32], and the endoplasmic reticulum translocon component Sec72 [33]. Protein transport and translocation in mitochondria, chroloplast and perixosomes systems ensure efficient exchange of proteins between cell compartments. This exchange is important for effective photosynthesis for plant survival.

CONCLUSION

The identified proteins categorized to C1 metabolism could be found in mitochondria, cytosol and chloroplast. The proteins such as Rubisco, FPGS, DHY and CYC are important in plant photorespiration to ensure continual supply of sugar for plant survival, besides providing additional benefits to support plant defence reaction during biotic and abiotic stress. Based on the detected proteins and previous findings from other researchers in literature review, the leaves of *F. deltoidea* is likely to follow the C1-THF synthase/SHMT pathway during photorespiration. The pathway is helpful for further study to investigate the secondary metabolism of the plant, especially for phytochemicals synthesis.

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