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ABIOTIC STRESS RESPONSE-ASSOCIATED PROTEINS IN A SALT TOLERANT STRAIN OF THE CYANOBACTERIUM FREMYELLA DIPLOSIPHON: A POTENTIAL BIOFUEL AGENT

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ABSTRACT
Cyanobacteria have gained great significance as a clean green alternative to fossil fuels as they are renewable and sustainable. Fremyella diplosiphon is a potential biofuel-producing cyanobacterium that efficiently captures light energy for photosynthesis. Efforts in our laboratory have been aimed to identify salt response pathways in this organism to develop strategies to enhance halotolerance in this model organism. In this study, protein expression in wild type and halotolerant mutant F. diplosiphon strains was compared using two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time of flight mass spectrometry. Of the 16 up-regulated proteins identified in the mutant, tripartite ATP-independent periplasmic (TRAP) transporter solute receptor was found to assist in salt-stress response with a significant hit to a corresponding spot with a score of 669 and 21% sequence coverage. Another protein, elongation factor Tu, linked to enhanced abiotic stress tolerance was significantly matched to a spot with a score of 1358 and 52% sequence coverage. Results of our study indicate that TRAP transporter solute receptor likely assists in enhancing halotolerance of the mutant, while elongation factor Tu is up-regulated in response to heat shock. These findings enable better understanding of F. diplosiphon salt-stress response paving the way for novel approaches in enhancing its halotolerance, leading to viable options leading to biofuel production.

Keywords: renewable energy, heat mutagenesis, polyacrylamide gel electrophoresis, MALDI/TOF mass spectrometry

1. INTRODUCTION
The use of fuels in a productive and economic way is a major challenge that we face today. While fossil fuels such as oil, coal and natural gas provide about 85% of our energy needs, the main concern of using fossil fuels is that it is a non-renewable resource, which will be depleted in the near future. In addition, continuous use of petroleum-based fuels is unsustainable because of the contribution of these fuels to atmospheric pollution and climate changes. Biofuels, which includes fuels derived from biomass conversion, as well as those from vegetable oils or animal fats, is one of the most important alternatives to fossil fuels that has gained great importance in recent years. These biofuels can be used as a fuel for vehicles in its pure form or can also be used as an additive to reduce levels of particulates, carbon monoxide and hydrocarbons in diesel-powered vehicles.

As third generation biofuels agents, photosynthetic cyanobacteria, the oldest known group of organisms on Earth offer immense potential for 'green' energy. These organisms

are amazingly productive and produce roughly 100 times the amount of clean fuel per acre compared with other biofuel crops (Abed et al. 2009) and convert light energy into chemical energy through photosynthesis using special pigments called phycobilisomes. To inhabit a wide range of environmental conditions, these organisms have evolved different mechanisms to sustain their photosynthesis (Gutu and Kehoe 2012). Thus, cyanobacteria have been found unique environmental applications such as the production of photosynthesis-derived biofuel (Ducat et al. 2010).

Recently, the potential of Fremyella diplosiphon as a production-scale biofuel agent has been recently unearthed in our laboratory. Theoretical biodiesel properties in F. diplosiphon transesterified lipids have revealed extremely high cetane number and oxidative stability, exceeding the minimum fuel standards (Tabatabai et al. 2018). Values for density, viscosity, iodine cold filter plugging point, cloud point and pour point were also above the minimum or within the acceptable range for both American and European fuel standards, with abundant C16:1 and C18:1 fatty acids indicating high-quality biodiesel. This breakthrough along with other unique capabilities of the organism such as fast growth in 7-9 days in a wide range of light wavelengths makes biofuel derived from F. diplosiphon attractive for large-scale production.

Salinity of the hydrosphere caused by man-made and environmental factors is a significant limitation for the survival of this organism which has an immense potential as a biofuel agent (Bhadauriya et al. 2007; Srivastava et al. 2008, Tabatabai 2017a). Genetic manipulation of F. diplosiphon to increase salt tolerance through biotechnological approaches has enabled our team to uncover unique environmental applications of this species (Tabatabai 2017b). Adaptation strategies in cyanobacteria in response to high salinity have been identified as well. Prior studies in cyanobacteria have reported that a major salt stress response pathway in these organisms is divided into three phases. First, an immediate rise in cellular concentration of sodium chloride (NaCl) occurs, including an accelerated invasion of sodium and chloride into the cytoplasm after turgor collapse. This is followed by the exchange of sodium with potassium resulting in the elimination of toxic impacts of high sodium concentration on cell metabolism. In the final phase, the synthesis of compatible solutes occurs to further stabilize osmotic potential in the cytoplasm and maintain the structure of cellular proteins and membranes. In short, these microorganisms adapt to salt stress by protecting the mechanisms for repair of the respiration process in photosystem I and cytochrome oxidase activity (Allakhverdiev et al. 2000).

The impact of salt stress on photosynthesis, growth, and pigmentation in F. diplosiphon has been documented (Tabatabai et al. 2017a; Singh and Montgomery 2013a,b). Our effort to enhance salt tolerance in this organism via heat mutagenesis has resulted in a mutant that thrives in 20 g L-1 NaCl (Tabatabai et al. 2017a). A three-fold increase in tripartite ATP-independent periplasmic (TRAP) solute receptor gene was detected in this mutant suggesting that it assists in cellular salt stress response in F. diplosiphon. However, further analysis of additional proteins involved in halotolerance will provide a better understanding of the molecular basis in this species. In cyanobacteria, synthesis of proteins which induces by the salt stress is distinguished to the specific salt stress and general proteins groups Berntsson et al. 2010). The objective of this study was to identify up-regulated proteins in the halotolerant strain Fd33-M25 via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI/TOF/TOF MS) for potential use as a biofuel agent.

2. METHODS

2.1 Strain and Culture Conditions

Halotolerant F. diplosiphon strain (Fd33-M25) isolated by subjecting the short filamentous wild type strain (WT-Fd33) to heat mutagenesis (Tabatabai et al. 2017a) were used in this study. Cultures were grown in liquid BG-11 medium (Allen 1968) containing 20 mM HEPES (hereafter BG-11/HEPES) under fluorescent white light with continuous shaking at 170 rpm at 28 °C for seven days. Light fluence rate was adjusted to 30 µmol m-2 s-1 using a LI-190SA Li-Cor quantum Sensor connected to a LI-250 Li-Cor light meter (Li-Cor, USA). Wild type strain WT-Fd33 not treated with heat mutagenesis served as the control in this study (Tabatabai et al. 2017a).

2.2 Sodium Dodecyl sulfate–polyacrylamide Gel Electrophoresis (SDS-PAGE)

Total proteins in the wild-type and mutant strains were separated using SDS. Proteins were isolated using Cell Lytic B reagent (Sigma-Aldrich, USA) supplemented with lysozyme, benzonase, and protease inhibitors according to the manufacturer's protocol. SDS-PAGE was performed in a Mini-Protean Tetra gel system using a 10% precast polyacrylamide gel (Bio-Rad, USA). Samples were mixed with Laemmli sample buffer supplemented with ß-mercaptoethanol (BME), heated to 95 °C for 5 min and run on a 10% polyacrylamide gel at 150V for 60 min. The gel was washed three times for 5 min in distilled water to remove excess SDS and protein bands were visualized using Simply Blue stain (Life Technologies, USA).

2.3 Two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Further separation of proteins was carried out using 2D-PAGE according to the carrier ampholine method of isoelectric focusing (IEF) of O'Farrell (1975) by Kendrick Labs (USA). WT-Fd33 and mutant F. diplosiphon cultures (300 mL) grown at culture conditions mentioned above were centrifuged at 5,515 x g for 8 min. Pelleted cells (100 mg) were lysed in 500 µl of osmotic lysis buffer (10 mM Tris, pH 7.4, and 0.3% SDS) containing 10X nuclease (50 µg mL-1 RNase and 100 µg mL-1 DNase in 5 mM MgCl2 and 10 mM Tris-Cl, pH 7.0), phosphatase inhibitors I and II (EMD Millipore, USA) and 100X protease inhibitor (20 mM AEBSF, 1 mg mL-1 leupeptin, 0.36 mg mL-1 E-64, 500 mM EDTA, and 5.6 mg mL-1 benzamidine) stocks. SDS boiling buffer without BME (5% SDS, 10% glycerol and 60 mM Tris, pH 6.8) (500 µl) was added and samples heated in a boiling water bath for 5 min before protein concentrations were determined using the BCA Assay (Thermo Fisher Scientific, USA). Samples were lyophilized and re-dissolved at 4 mg mL-1 in 1:1 ratio of diluted SDS boiling buffer (5% SDS, 5% BME, 10% glycerol and 60 mM Tris, pH 6.8.): urea sample buffer (9.5 M urea, 2% w/v IGEPAL CA-630, 5% (BME), and 2% ampholines consisting of 1.6% pH 5-7 and 0.4% pH 3.5-10) prior to loading on a 10% acrylamide gel. Isoelectric focusing was performed in a glass tube using 2.0% Servalyt at pH 3-10 (Serva, Germany) for 9600 volt-hours. Tropomyosin (1 µg) was added to each sample as an IEF internal standard. Tropomyosin migrates as a doublet with the lower observed spot exhibiting a molecular weight (MW) of 33,000 Da and a pI of 5.2. Six proteins, myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000) were used as MW standards in IEF (Sigma, USA). These standards appear as bands at the basic edge of the acrylamide gel stained with Coomassie Brilliant Blue R-250.

2.4 Identification of Proteins Using MALDI/TOF Mass Spectrometry

Differential protein expression in the WT-Fd33 and mutant was identified using MALDI-TOF mass spectrometry (MS). Protein spots from two biologically replicated 2D-PAGE gels were manually excised, digested using modified porcine trypsin (Promega, USA) in ammonium bicarbonate, and purified as described by Natarajan et al. (2014). Samples were spotted on a MALDI plate and co-crystallized with a 5 mg mL-1 concentration of α-

cyanohydroxycinnamic acid matrix prepared in 70% acetonitrile containing 0.1% trifluoroaceticacid.

2.5 Identification of Proteins in Wild Type and Mutant F. diplosiphon

Protein identification was performed using the Mascot search engine (http://www.matrixscience.com) against the NCBI non-redundant database with the taxonomy filter "other bacteria". The parameters for database searches included: monoisotopic mass, trypsin as the digestive enzyme with allowance for 1 missed cleavage, peptide tolerance of 50 parts per million (ppm), MS/MS tolerance of 0.6 Da, allowance of 1+ peptide charge, fixed modification for carbamidomethylation of cysteine residues, and variable modifications for oxidation of methionine residues as well as N-terminal pyroglutamic acid resulting from glutamic acid or glutamine. Positive identification of proteins by MS/MS analysis required a single peptide having a significant ion score.

3. RESULTS AND DISCUSSION

3.1 SDS-PAGE of Wild Type and Mutant F. diplosiphon Total Protein Extracts

Comparison of total proteins using SDS-PAGE revealed differential expression in the wild type and mutant strains ranging from 35 to 130 kDa, indicating widespread changes in the proteome associated with salt tolerance in Fd33-M25. SDS-PAGE has been used to identify alterations in protein expression induced by heat mutagenesis and salinity stress (Sato et al. 2010).

3.2 Identification of Substrate Binding Proteins (SBPs)

Up-regulation of proteins was further confirmed by 2D-PAGE (Fig. 1), which identified proteins representing 32 spots in the wild type (Table 1) and 24 spots in the halotolerant strain (Table 2). Of these spots, 16 were found to be up-regulated in the mutant strain, indicating that heat mutagenesis significantly altered expression of these proteins. Several overexpressed proteins in the mutant were found to be substrate-binding and associated proteins (SBPs), a class of protein domains that are often associated with membrane protein complexes for transport or signal transduction (Berntsson et al. 2010). Specifically, a SBP exclusive in the mutant was identified as a TRAP type transporter solute receptor by MALDI-TOF MS (spot #1) (Fig. 1).

Differentially expressed proteins were identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Substrate-binding and associated proteins up-regulated in Fd33-M25 are marked with black arrows representing the following: spot #1: Tripartite ATP-independent periplasmic TRAP-type transporter solute receptor, spot #2: bicarbonate-binding protein, spot #3: phosphoglycerate kinase, spot #4: $Fe(3+)$ ABC transporter substrate-binding protein, spot #5: phosphogluconate dehydrogenase. A Mascot search of NCBI non-redundant databases revealed a TRAP-type protein of interest (GI: 662703514) as the most significant match with a MOWSE score of 669. Other Mascot information for this protein revealed a theoretical isoelectric point (pI) of 4.9, molecular weight (Mr) of 41206 Da, 6 peptides matches, and 21% sequence coverage. TRAP solute receptors could enhance the ability of the mutant to uptake compatible solutes when grown in 20 g L-1 NaCl. The uptake of these solutes is a preferred mechanism since it is less energy-intensive than de novo synthesis of osmoprotectants (Mulligan et al. 2011). In Synechocystis sp. strain PCC 6803, the TRAP transporter complex GtrABC is a Na^+-dependent transporter for the uptake of glutamate in response to osmotic stress.

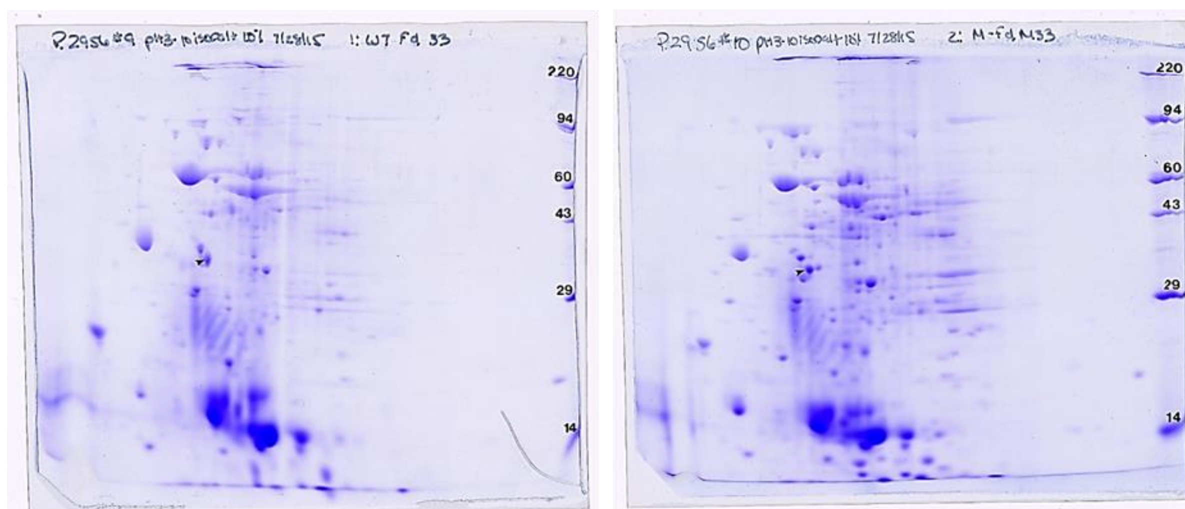

Figure 1. Two-dimensional polyacrylamide gel electrophoresis of Fremyella diplosiphon (A) wild type (WT-Fd33) and (B) halotolerant mutant (Fd33-M25) run on a 10% acrylamide gel.

Another known compatible solute is glycine betaine which has been reported in several phototrophic bacteria (Grammann et al. 2002). A glycine betaine/choline ATP-binding cassette transporter system in F. diplosiphon has been reported to assist in glycine betaine uptake (Singh and Montgomery, 2013a) suggesting that this organism can uptake compatible solutes in response to salinity stress. Accordingly, the TRAP transporter system could serve as a secondary glycine betaine transporter in concert with an ABC transporter. These results indicate that alterations in the regulation of the TRAP transporter complex could be a potential source of halotolerance in the mutant. In addition, a six-fold overexpression of a TRAP-type C4-dicarboxylate transport system resulted in the exclusion of sodium ions from the cytoplasm at high salinity in the halotolerant bacterium Tistlia consotensis (Rubiano-Labrador et al. 2015). Similar systems have also been found in the moderately halotolerant cyanobacterium Synechocystis sp. strain PCC 6803, where a Na+-dependent TRAP transporter for L-glutamate was reported (Quintero 2011).

Table 1: Proteins identified in Fremyella diplosiphon wild type strain (WT-Fd33) via 2D-PAGE and MALDI-TOF/TOF.

Identified Protein	Accession #	MW	pI	# PM	% Seq cov	MOWSE
Membrane protein	gi|655653826	67591	4.52	10	22	1148
S-layer protein	gi|655729225	65072	5.09	8	20	851
Elongation factor Tu	gi|655669450	43764	5.1	11	49	1073
Fructose-bisphosphate aldolase	gi|499175825	39119	5.46	5	30	710
Elongation factor Ts	gi|655690303	24414	5.5	7	34	573
Phosphate ABC transporter substrate-binding protein	gi|655646357	35287	9.89	7	34	785
Chain B, X-ray Crystal structure of phycocyanin	gi|459358656	18283	4.98	5	50	684
Membrane protein	gi|655653826	67591	4.52	7	19	656
ATP synthase subunit beta	gi|655670889	51841	4.95	12	32	938
Elongation factor Tu	gi|655669450	43764	5.1	9	41	740
Fe(3+) ABC transporter substrate-binding protein	gi|655641475	38156	5.82	10	47	1047
Phosphate ABC transporter substrate-binding protein	gi|655646357	35287	9.89	6	27	629

Elongation factor Ts	gi|655690303	24414	5.5	3	30	385
Molecular chaperone groel	gi|499174454	57731	5.01	9	29	874
ATP synthase subunit beta	gi|655670889	51841	4.95	12	37	1490
Elongation factor Tu	gi|662705204	43764	5.1	9	43	750
Hypothetical protein D082_28000	gi|662705353	60433	5.47	9	31	1114
ABC transporter substrate-binding protein	gi|655741293	41206	4.9	6	21	669
Elongation factor Tu	gi|655669450	43764	5.1	15	52	1358
Bicarbonate-binding protein	gi|655714980	47823	5.57	7	25	722
Elongation factor Tu, partial	gi|909637040	34530	5.06	3	23	500
Fructose-bisphosphate aldolase	gi|499175825	39119	5.46	8	33	912
Chain B, X-ray crystal structure of phycocyanin	gi|459358656	18283	4.98	3	46	456
Phosphoglycerate kinase	gi|655666522	42010	4.99	7	37	899
Phosphoribulokinase	gi|655688149	38101	5.14	9	37	791
Fe(3+) ABC transporter substrate-binding protein	gi|655641475	38156	5.82	12	47	1265
Photosystem I reaction center subunit XII	gi|655705579	32581	9.45	10	37	903
Phycocyanin alpha subunit	gi|2673718	11319	9.3	1	19	140
Photosystem I reaction center subunit XII	gi|655705417	30870	9.51	9	46	1010
Phosphate ABC transporter substrate-binding protein	gi|655646357	35287	9.89	9	34	958
Phosphate ABC transporter substrate-binding protein	gi|655646357	35287	9.89	4	39	751
Elongation factor Tu	gi|655669450	43707	5.1	7	43	893

In addition to TRAP solute receptors, other SBP spots were identified as bicarbonate-binding protein (spot #2), phosphoglycerate kinase (spot #3), Fe(3+) ABC transporter substrate-binding protein (spot #4), and phosphogluconate dehydrogenase (spot #5) (Fig. 1) suggesting that heat mutagenesis significantly affects SBP regulation. This is interesting to note since SBPs are not only involved in TRAP-type transporter systems, but several mechanisms of salt-stress response. Members of this protein class are key components of ABC transporter systems that uptake compatible solutes such as glycine betaine that can be used as osmoprotectants in bacteria such as Listeria monocytogenes and Escherichia coli (Roeßler and Müller 2001; Ko and Smith 1999). This indicates that heat mutagenesis could result in enhanced robustness of multiple salt-stress response pathways. This is supported by recent transcriptomic analysis in mutant halotolerant strains of the microalgae Chlorella sp., which revealed up-regulation of sorbitol/mannitol transport system substrate-binding proteins relative to wild type salt-sensitive strains (Li et al. 2018).

3.3 Elongation Factor Tu

In addition to SBPs, several proteins that differentially accumulated in Fd33-M25 and WT-Fd33 spanning a wide range of isoelectric points and molecular weights were identified. One of these up-regulated proteins identified was the elongation factor Tu, which is known to be highly conserved in bacteria (Jiang et al. 2016). MALDI-TOF/TOF revealed this protein of interest (GI: 655669450) as the most significant match with a MOWSE score of 1358 via Mascot search of NCBI non-redundant databases. Other Mascot information for this specific protein revealed a theoretical pI of 5.1, Mr of 43764 Da, 15 peptides matches, and 52% sequence coverage (Table 1). This membrane-bound protein is involved in translation and protein biosynthesis and plays a role in various abiotic stress response pathways (Barbier et al. 2013; Fu et al. 2013). Specifically, overexpression of this protein in E. coli has been

reported to enhance heat tolerance, enabling survival when exposed to high temperatures up to 55 °C (Bhadula et al. 2001). This suggests that up-regulation of the elongation factor Tu in F. diplosiphon is a response to heat shock treatment in the mutagenesis process. While accumulation of this protein has been linked to a more robust heat, drought, and UV light stress response (Shrivastava et al. 2015; Fu et al. 2013; Bhadula et al. 2001), to our knowledge there exist no studies to discuss its potential effect on salinity stress. Thus, additional studies to determine if elongation factor Tu up-regulation assists in salt stress response in addition to heat tolerance.

4. CONCLUSIONS

Protein expression in wild type and heat-transformed F. diplsosiphon strains using 2D-PAGE and MALDI-TOF MS was ascertained in this study. The results indicate up-regulated SBPs in theFd33-M25 strain obtained by heat mutagenesis, which are typically key components of bacterial salt-stress response mechanisms such as compatible solute uptake via TRAP-type and ABC transporters. While the elongation factor Tu was also overexpressed likely in response to heat stress, further studies are required to determine its role in halotolerance. In future studies, comprehensive characterization of proteins associated with salt-stress response pathways in F. diplosiphon will be pursued. This will enable development of efficient strategies to further enhance halotolerance of this strain as a potential biofuel agent.

5. ACKNOWLEDGEMENTS

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