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Expression analysis of potential transcript and protein markers that are related to agar yield and gel strength in *Gracilaria changii* (Rhodophyta)

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ABSTRACT

The supply of agar as an important gelling and thickening agent in various industrial applications depends heavily on harvesting of natural seaweed resources and seaweed farming. To facilitate the selection of good seaweed source with higher agar yield and stronger gel strength, accurate and rapid screening method using molecular markers is necessary to replace the tedious, laborious and time-consuming conventional method which involves agar extraction and gel analysis. In this study, we characterized the expression of a number of algal transcripts and proteins from an agar producing seaweed, *Gracilaria changii* with the aim to identify potential markers for agar yield and gel strength. In total, 15 candidate transcripts that are directly or indirectly related to putative agar biosynthetic pathway were identified based on literature search. The transcript abundance of 4 and 11 of these candidates were found to be significantly ($P < 0.05$) correlated to the agar yield and gel strength of six *G. changii* samples, respectively. Among these marker genes, the transcript levels of GcFBPA and GcGALE have the highest linear correlation to both agar yield and gel strength. The protein abundance of GcFBPA and GcGALE was further examined on 13 *G. changii* samples and was found to have highly significant ($P < 0.01$) correlation to agar gel strength and agar yield, respectively. GcFBPA and GcGALE may have good potential to be used for molecular screening of yield traits and gel quality of *G. changii* at both RNA and protein levels.

1. Introduction

Agar is an important gelling and thickening agent used in processed foods, cosmetics, pharmaceutical, medical and biotechnological products [1]. The global demand for agar has increased between 2009 and 2015, at an annual average growth rate of 7% for its sales volume, outperforming other commercial hydrocolloids such as carrageenans and alginates [2]. In order to meet the high demand for agar and to overcome shortage of raw materials due to uncontrolled harvesting at natural beds [3], seaweed domestication and farming, and resource management are gaining attention from both academia and industry [4–6].

Seaweeds from the genus *Gracilaria* account for 91% of the total commercial agar production in 2015 [2]. The agar yield is an economically important factor for the selection of the right agarophyte population for agar production while the gel strength serves as the main indicator for agar quality [7]. Although seaweed species with better agar yield and gel quality have been identified for agar production previously [8,9], agar properties are also highly variable depending on environmental and physiological factors [7]. Thus, mass harvesting and processing of agarophytes without prior knowledge of their agar properties have caused poor agar yield and gel quality [5]. Failure to maximize the agar yield and gel quality from limited natural seaweed resources is not only an economic loss but also a threat to sustainable supply of agar.

In the past decades, seaweed farmers and traders evaluated the quality of their raw materials based on the following procedures: seaweed harvesting and drying, agar extraction and processing, and multiple tests to determine the agar properties [10]. However, this traditional procedure is time consuming, expensive, laborious, and requires a huge amount of sample for accurate evaluation of agar yield and gel quality. In recent years, the relationship between the expression of putative agar biosynthesis genes and agar yield in *Gracilaria* and *Gracilariopsis* species have been analyzed. Li et al. (2010) found that the

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majority of the *Gracilariopsis* (*Gs.*) *lemaneiformis* strains with a higher agar content had a higher expression for a gene encoding galactose-1-phosphate uridylyltransferase (GALT) [11], while Siow et al. [12,13] and Lee et al. [14] revealed that the expression levels of two genes encoding GDP-mannose-3,5-epimerase (GME) and GALT, were higher in *Gracilaria* samples with a higher agar yield compared to that with a lower agar yield [12–14]. In addition, Chang [15] and Hu et al. [16] reported that the gene expression of GME, UDP-glucose pyrophosphorylase and GDP-mannose pyrophosphorylase (GMP) was positively correlated with the agar content of *Gs. lemaneiformis* treated under specific conditions [15,16]. So far, gene expression that correlates to agar gel strength in agarophytes has not been reported. The transcript abundance of these genes and other related genes in the agar biosynthetic pathway can potentially be used as a fast, accurate and cost-effective way for screening and selection of the raw materials for agar production, preferably before mass harvesting of seaweeds. This will allow the harvesting of seaweeds that produce good agar yield and high gel quality, hence a better resource management for sustainable agar production.

Gracilaria changii Xia and Abbott is an agarophyte with good agar yield and gel strength in Southeast Asia [17,18] which fulfills the industrial requirements for the first-grade food agar set by Japanese Specifications for Processed Agar (JPSA). The availability of genomics and transcriptomics information of this species [14,19–21] facilitates expression studies of candidate genes related to agar biosynthesis. In this study, we characterized the expression of a number of algal transcripts and two proteins from *G. changii* for the identification of genes with transcript and protein abundance that correlated to agar yield and gel quality. These candidate markers will facilitate future screening and selection of *G. changii* samples with good agar yields and high gel strengths for agar production.

2. Materials and methods

2.1. Sample collection and treatments

Fresh and intact bunches of *Gracilaria changii* (Xia and Abbott) Abbott, Zhang and Xia were collected during low tides from a tropical mangrove estuary at Morib, Selangor, Malaysia (02°45.878′ N; 101°25.976′ E) periodically from October 2012 to May 2016 (see Table 1). Seaweeds without cystocarpic and adelphoparasitic structure were carefully cleaned from mud, epibionts and epiphytes. The cleaned seaweeds were acclimatized overnight in synthetic seawater (ASW: Instant Ocean Synthetic Sea Salt, Aquarium Systems Inc., Mentor, OH, USA) at 32 parts per thousands (ppt).

To obtain seaweeds with different agar yields and gel strengths under salinity stresses, approximately 300–350 g of seaweeds were treated in 30 L ASW at 10 ppt, 30 ppt and 50 ppt for 5 days as described in [22]. Sulfate deprivation treatment was performed on *G. changii* for 5 days as described in [23]; while pH treatment was performed on *G. changii* at three different pHs (i.e. 6.61, 8.04 and 9.30) for 3 and 6 days, as described in Lee et al. (unpublished data). These treatments were conducted under natural photoperiod (12:12 light/dark cycle with approximately 12.5–15 μmol photons $m^{-2} s^{-1}$) and continuous aeration in the laboratory while other environmental parameters such as water temperature (25 °C) were kept constant throughout the experiment. Approximately 40 g of each harvested sample was stored at −80 °C for RNA and protein extractions while the remaining samples were dried at 65 °C for agar extraction.

2.2. Agar extraction and determination of agar properties

The extraction of agar was performed as described in [23]. Agar yield was calculated in triplicates as the average percentage of agar dry weight in relation to dry weight of seaweed. Measurement of gel strength was conducted based on [17]. Briefly, 1.5% (w/v) agar gel was prepared in a 100 mL beaker (with a depth of 15–20 mm) using a texture analyser (TA-XT2, Stable Micro Systems Ltd., Survey, UK) with a 10 mm diameter cylindrical plunger (P/10 CYL Delrin probe), a 5 kg load cell and operating speed of 1 mm s^{-1}. Gel strength (expressed in g cm^{-2}) was calculated based on the maximum force applied to break the gel at a distance of 5 mm.

2.3. RNA extraction and first strand cDNA synthesis

RNA was isolated from 10 g of each thalli sample using a conventional extraction method described by [24]. Genomic DNA was removed from the extracted RNA samples using RNase-free DNase I (New England Biolabs, Beverly, MA) following the manufacturer's instructions. The purity and quality of the RNA were evaluated using Nanodrop spectrophotometer (Implen UK Ltd., Essex, UK). The samples that met the following criteria were processed for qRT-PCR analyses: absorbance ratios $A_{260/280}$ and $A_{260/230}$ that were between 1.9 and 2.1 and > 2, respectively, and 25S rRNA:18S rRNA ratio near to 2:1. Approximately 2.5 μg of DNA-free total RNA was reverse transcribed into cDNA with Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies, USA) by following the manufacturer's instructions. The synthesized cDNA was stored at −20 °C until use.

2.4. Protein extraction and quantification

Approximately 0.1 g seaweed was ground into fine powder and resuspended in 1 mL prechilled Extraction Buffer I consisting of 10% (w/v) trichloroacetic acid (TCA), 1% (w/v) polyvinylpyrrolidone (PVP), and 2% (v/v) 2-methanol (2-ME) in acetone. The tube was incubated on ice and sonicated at 60% ultrasound amplitude for 30 s, before cooling

Table 1
Agar yield and gel strength of *Gracilaria changii* samples. Data are shown as mean ± SD, $n = 3$. Different letters indicate significant differences ($P < 0.05$) among samples analyzed by Duncan's Multiple Range Test.

No.	Samples	Abbreviation	Date of collection	Agar yield, % dw	Gel strength, g/cm^2	References
1	GC-10PPT	GC 10 ppt	July 2013	23.5 ± 0.9^{ab}	392.6 ± 19.6^{ab}	This study
2	GC-30PPT	GC 30 ppt		22.6 ± 0.6^{ac}	360.3 ± 22.5^{ac}	
3	GC-50PPT	GC 50 ppt		24.3 ± 0.4^{b}	351.1 ± 18.3^{ac}	
4	GC-Sulfate control 1	GC SC1	October 2012	18.7 ± 0.3^{d}	251.3 ± 51.5^{d}	[23]
5	GC-Sulfate treatment 1	GC ST1		19.9 ± 0.2^{e}	267.6 ± 10.0^{d}	
6	GC-Sulfate control 2	GC SC2	March 2013	21.3 ± 1.2^{f}	261.0 ± 11.8^{d}	
7	GC-Sulfate treatment 2	GC ST2		21.7 ± 1.4^{af}	274.8 ± 37.5^{d}	
8	GC-Day 3 pH 6.5	GC_D3_6.5	May 2016	19.2 ± 0.4^{de}	381.4 ± 32.0^{bc}	Lee et al. (unpublished data)
9	GC-Day 3 pH 8.0	GC_D3_8.0		18.1 ± 0.2^{d}	420.4 ± 8.2^{be}	
10	GC-Day 3 pH 9.5	GC_D3_9.5		19.1 ± 0.2^{de}	331.1 ± 26.8^{c}	
11	GC-Day 6 pH 6.5	GC_D6_6.5		24.2 ± 0.2^{b}	452.9 ± 42.9^{e}	
12	GC-Day 6 pH 8.0	GC_D6_8.0		23.0 ± 0.4^{c}	426.3 ± 18.6^{be}	
13	GC-Day 6 pH 9.5	GC_D6_9.5		23.5 ± 0.3^{bc}	359.0 ± 19.6^{ac}	

for 30 s. The sonicating and cooling process was repeated for 30 min. After centrifugation at 10,000 ×*g* for 15 min at 4 °C, the supernatant was discarded and the pellet was rinsed three times with acetone supplemented with 0.07% (v/v) 2-ME. Five hundred microliter of Tris-buffered phenol solution and 500 µL of Extraction Buffer II [30% (w/v) sucrose, 2% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-ME, 0.1 M Tris, pH 8, 0.02% (v/v) sodium azide, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF)] was added. The mixture was mixed and incubated on ice for 5 min. The sample was centrifuged at 10000 ×*g* for 15 min, 4 °C and the upper phenolic phase was collected. The residual pellet was extracted twice, and the phenolic phase collected was combined. Then, the solution was precipitated with five volumes of 80% (v/v) methanol supplemented with 0.1 M ammonium acetate at −20 °C for overnight. The mixture was centrifuged and the pellet was rinsed with 3 mL acetone supplemented with 0.07% (v/v) 2-ME. After washing, the pellet was resuspended in 150–250 µL solubilizing buffer consisting of 2% (w/v) SDS. 40 mM Tris-HCl buffer pH 8, 0.02% (v/v) sodium azide, 1 mM EDTA and 1 mM PMSF and then incubated at room temperature for 60 min. The proteins were quantified using Bradford method. The absorbance was measured at OD_{595} using spectrophotometer (Eppendorf, Germany) and the concentration of each sample was estimated by comparing the absorbance to a standard curve generated using Bovine Albumin Serum (BSA). The protein samples were stored at −80 °C prior to further use.

2.5. qRT-PCR analysis

The sequences of housekeeping genes and candidate markers (Fig. 1) were retrieved from the genome and transcriptome of *G. changii* [14,19] to identify the open reading frame (ORF), 3′ untranslated regions (UTRs) and 5′UTRs. The PCR primers were designed using online tool Primer3 software ver. 0.4.0 (http://frodo.wi.mit.edu) [25]. The reverse primer was designed in such a way that it annealed to the 3′UTR which was specific to each cDNA sequence, to avoid amplification of gene family members (Supplementary Table 1). The following criteria were applied for primer design: a product size of 100–350 bp, a primer length of 16–24 bp, GC content between a 40–60% and a primer melting temperature which was close to 60 °C. The PCR primers which showed a single melt peak, PCR efficiency between 90 and 105% and a coefficient of linear correlation (R^2) larger than 0.98 were used for subsequent gene expression analysis.

Two housekeeping genes for qRT-PCR normalization in *G. changii* (encoding histone and uncharacterized transporter YJL193W) were selected among the three candidate housekeeping genes that showed stable gene expression in previous studies [14,20], based on their stability ranking and pairwise variation as analyzed by geNorm version 3.5 [26].

All qRT-PCR analyses were performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, USA) and iQ™5 real time detection system (Bio-Rad, USA). The reaction mixture (20 µL) consisted of 100 ng of first-strand cDNA template, 1× Brilliant III Ultra-Fast SYBR Green QPCR master mix, 200–400 nM of each respective primers and molecular grade H_2O (bioWORLD, USA). The PCR amplification was performed with the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s; and a melt curve analysis with temperature increasing from 55 °C to 95 °C with an increment of 0.5 °C/10 s. A control sample without template was included in all experiments to rule out the presence of contaminants and primer-dimer. The primer concentrations were between 100 and 400 nM for respective primer (Supplementary Table 1). All the PCRs were repeated in triplicates.

The results obtained were analyzed and normalized to the geometric mean of the housekeeping genes using $2^{-\Delta\Delta Ct}$ method as described by [27,28]. All normalized gene expression values were rescaled to the expression value of the genes on GC-Sulfate Control 1 (SC1), to ease the comparison of gene expression levels across different *Gracilaria* samples.

2.6. Generation of polyclonal antibodies

Polyclonal antibodies for GcFBPA and GcGALE were ordered from GenScript (USA, New York). The GcFBPA and GcGALE peptides for the production of polyclonal antibodies were designed using GenScript custom peptide synthesis service (GenScript, USA, New York) by

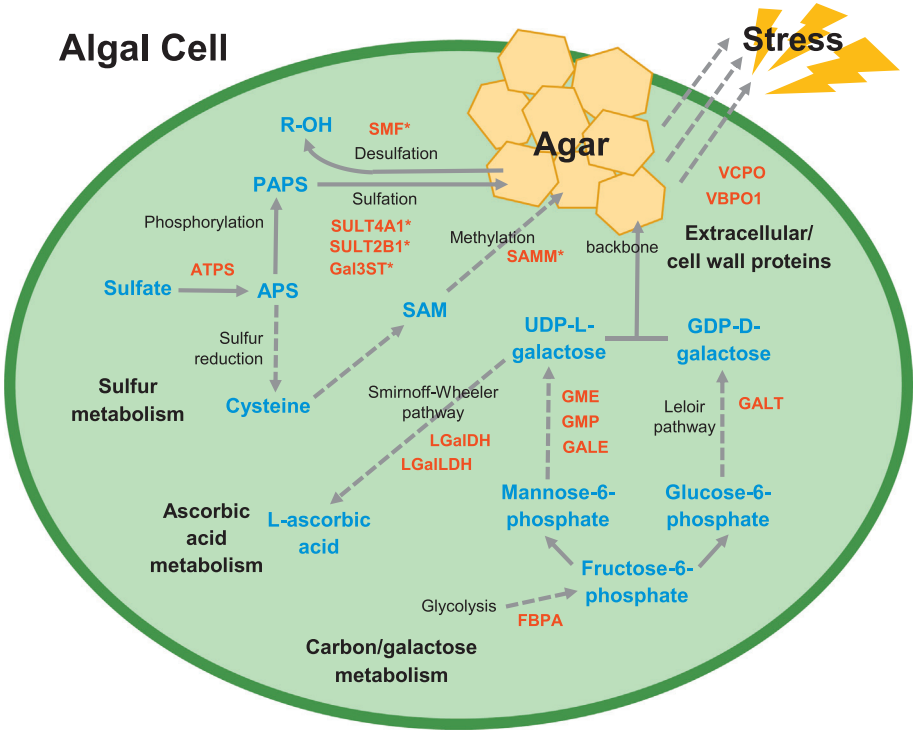

Fig. 1. Proposed agar biosynthesis in red seaweeds. It is divided into (1) biosynthesis of agar precursors, UDP-L-galactose and GDP-D-galactose derived from the carbon metabolism or galactose metabolism (which is closely linked to ascorbic acid metabolism), (2) elongation of agar monomers to polysaccharide chain via galactosyltransferases, and (3) modification of agar polysaccharide by major side chain substituents, sulfate and methoxyl groups derived from sulfur metabolism [34]. The agar as well as other extracellular/cell wall proteins are putative components related to cellular stress response. Asterisk (*) indicates putative enzymes for the indicated pathway or metabolic process. Solid line and dotted line arrows indicate single and multiple enzymatic steps, respectively. Please see Table 2 for complete abbreviation of candidate markers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary of candidate genes, putative functions and mRNA expression.

Candidate markers	Source of selection	Abbreviation	Putative function	mRNA expression in red algae
Fructose 1,6 bisphosphate aldolase	1, 2	GcFBPA	Catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceroldehyde-3-phosphate and dihydroxytacetone phosphate in glycolysis; and condensation of erythrose-4-phosphate and dihydroxytacetone phosphate to form sedoheptulose-1,7-bisphosphate in Calvin cycle.	• In *Pyropia* (*Py.*) *tenera*, the transcript abundance of FBPA increased 2–3 folds following treatment under desiccation stress for 0.5–8 h [51]. • In *Py. haitanensis*, the transcript abundance of two FBPAs increased 6.2 and 117.8 folds, respectively, while the transcript abundance of another FBPA decreased by 3.1 folds, when the seaweeds were exposed to severe dehydration stress [52]. • In *Gracilaria* (*G.*) *lemaneiformis*, the transcript abundance of FBPA decreased by 3.5–7.9 folds within 2–4 days exposure to nitrogen deprivation [53]. • In *G. changii* and *G. salicornia*, the transcript abundance of several FBPAs decreased 1.7–5.4 folds upon sulfate deprivation [14]. • In *Sheathia* (*Sh.*) *arcuata*, the transcript abundance of two FBPAs were increased 2.6 and 5.3 folds, respectively, under low irradiance condition [54].
UDP-galactose-4-epimerase	1, 2	GcGALE	Catalyzes the reversible conversion of UDP-D-glucose to UDP-D-galactose in final step of the Leloir pathway.	• In *Gelidium pristoides*, the transcript abundance of GALE increased during night time, coincident with the breakdown of floridean starch [43]. • In *Py. tenera*, the transcript abundance of GALE increased 4–6.5 folds following treatment under desiccation stress for 0.5–8 h [51]. • In *Py. haitanensis*, the transcript abundance of FBPA increased 4.4 folds when the seaweeds were exposed to severe dehydration stress [2].
Galactose-1-phosphate uridylyltransferase	1, 2, 5	GcGALT	Catalyzes the reversible conversion of UDP-glucose and galactose-1-phosphate to glucose-1-phosphate and UDP-galactose as the second step in the Leloir pathway.	• In *G. changii*, *G. salicornia* and *G. edulis*, the transcript abundance of GALT was affected by salinity stresses and species. The differences of GALT expression in the three species were correlated to the differences in their agar yields [12]. • In *G. salicornia* and *Gracilariopsis* (*Gs.*) *lemaneiformis*, the transcript abundance of GALT was higher in samples/strains with higher agar yield compared to those with lower agar yield [11,14].
GDP-mannose 3,5-epimerase	1, 2, 5	GcGME	Involves in L-galactose biosynthesis and ascorbic acid biosynthesis in Smirnoff-Wheeler pathway by converting GDP-D-mannose to GDP-L-galactose	• In *G. changii*, *G. salicornia* and *G. edulis*, transcript abundance of GME varied among species and correlated to the differences in their agar yields [13]. • In *G. salicornia*, the transcript abundance of GME was higher in samples with higher agar yield compared to those with lower agar yield [14]. • In *Gs. lemaneiformis*, the transcript abundance of GME was higher in selected samples with high agar yield treated under abiotic stresses for 3 weeks, but has no correlation to the agar yield of the same samples treated for 2 and 4 weeks [16].
GDP-mannose pyrophosphorylase	2	GcGMP	Catalyzes conversion of GTP and alpha-D-mannose-1-phosphate to diphosphate and GDP-mannose.	In *Gs. lemaneiformis*, the transcript abundance of GMP was higher in selected samples with high agar yield treated under abiotic stresses for 3 weeks, but has no correlation to the agar yield of the same samples treated for 2 and 4 weeks [16].
Sulfatase modifying factor	2	GcSMF	A member of the sulfatase-modifying factor family responsible for post-translational modification of sulfatase into functional protein.	Not reported.
L-Galactose dehydrogenase	1, 2	GcLGalDH	Catalyzes the conversion of L-galactose and NAD^+ to L-galactono-1,4-lactone, H^+ and NADH for ascorbate biosynthesis.	In *Py. tenera*, the transcript abundance of LGalDH increased 2–4.9 folds following treatment under desiccation stress for 0.5–8 h [51].
L-Galactono-1,4-lactone dehydrogenase	2	GcLGalLDH	Catalyzes the last step in ascorbate biosynthesis by converting of L-galactono-1,4-lactone to L-ascorbate.	Not reported.
Vanadium chloroperoxidase	1, 3, 4, 5	GcVCPO	Catalyzes the vanadium-dependent halogenation of organic compounds in the presence of inorganic chloride and hydrogen peroxide.	In *G. changii*, the transcript abundance of VCPO increased 4 folds when the seaweeds were exposed to light deprivation for 7 days [33].

(*continued on next page*)

Table 2 (*continued*)

Candidate markers	Source of selection	Abbreviation	Putative function	mRNA expression in red algae
Vanadium bromoperoxidase	1, 3, 4, 5	GcVBPO1	Catalyzes the oxidation of bromide (0.0067% of sea water) by hydrogen peroxide in the presence of vanadium.	• In *Py. yezoensis*, the transcript abundance of VBPO increased 5.5 folds when the seaweeds were exposed to high temperature (24 °C) compared to those maintained at normal temperature (8 °C) [55]. • In *G. changii*, the transcript abundance of VBPO decreased 3.4 folds and 2.7 folds when the seaweeds were exposed to light deprivation for 7 days [33]. It also increased when the seaweeds were exposed to high salinity stress (50 parts per thousands; ppt) for 7 days compared to the control maintained at 30 ppt [56]. • In *G. changii* and *G. salicornia*, the transcript abundance of VBPO decreased 2.7 folds and 2.4 folds, respectively, when the seaweeds were exposed to sulfate deprivation for 5 days [14].
ATP sulfurylase	1, 2	GcATPS	Catalyzes the activation of sulfate into adenylyl sulfate with the presence of ATP.	• In *Py. tenera*, the transcript abundance of two ATPSs increased and decreased 2–2.8 and 1.4–2.1 folds, respectively, following treatment under desiccation stress for 1–8 h [51]. • In *Sh. arcuata*, the transcript abundance of ATP was increased 5.7 folds under low irradiance condition [54].
SAM-dependent methyltransferase	2	GcSAMM	Transfers the methyl group from the universal biological cofactor SAM to proteins, small molecules, lipids, and nucleic acids.	In *Py. tenera*, several SAMMs were either up- or down-regulated when the seaweeds were treated under desiccation stress for 0.5–8 h [51].
Galactose-3-O-sulfotransferase	2	GcGal3ST	Involves in 3′-sulfation of *N*-acetyllactosamine in both O– and N-glycans.	Not reported.
Sulfotransferase 2B1-like	2	GcSULT2B1	A member of the sulfotransferase family that transfers sulfate group from a donor molecule to hydroxyl group of an acceptor.	Not reported.
Sulfotransferase 4A1	2	GcSULT4A1	A member of the sulfotransferase family that transfers sulfate group from a donor molecule to hydroxyl group of an acceptor.	Not reported.

[1]Literature study.
[2]Mining of genome data from [19].
[3]DEGs from [22].
[4]DEGs from [33].
[5]DEGs from [14].

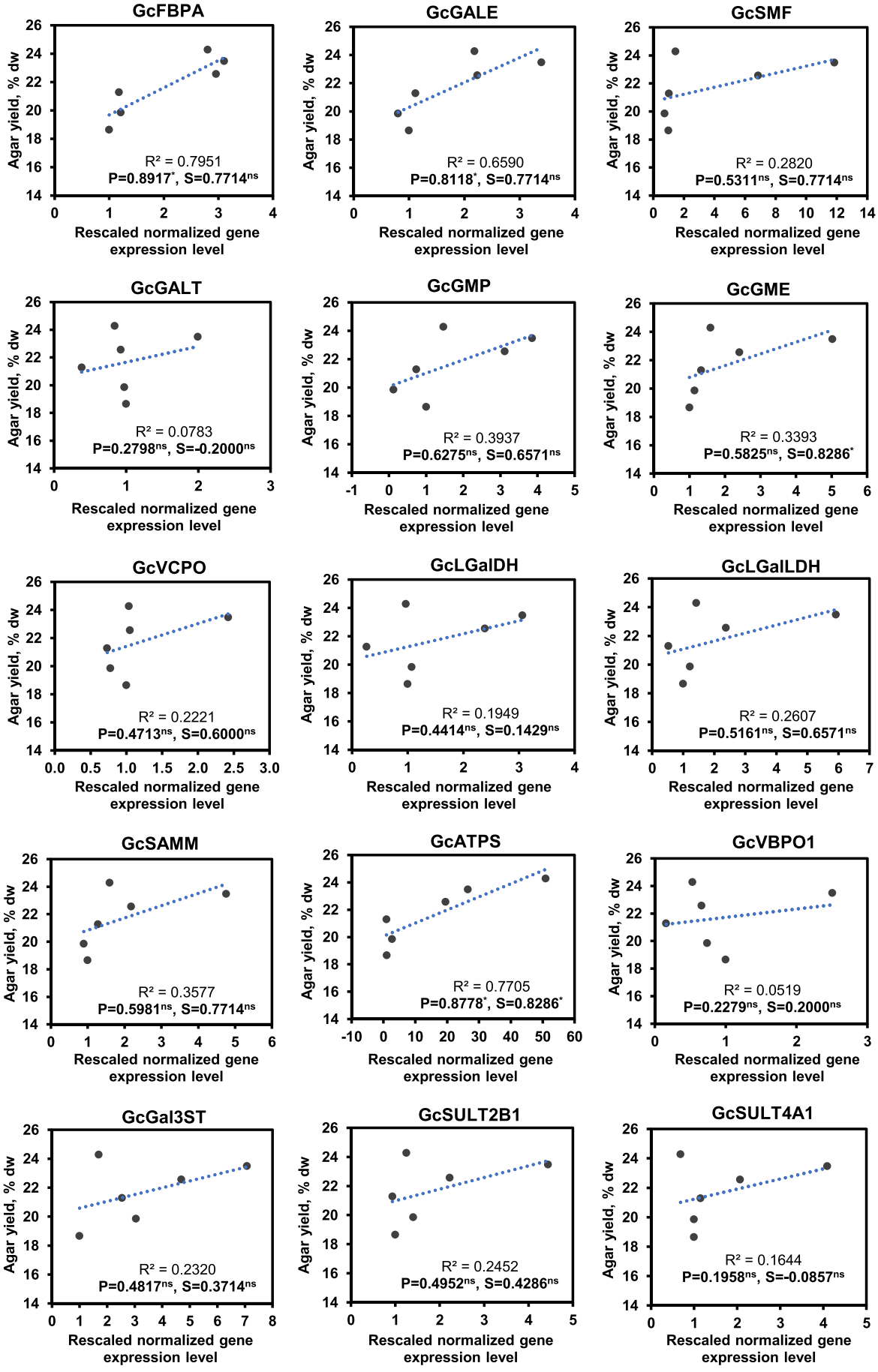

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Fig. 2. Correlation between mRNA expression levels of candidate genes from *G. changii* and agar yield. dw, dry weight; R^2, coefficient of determination (which is the square of coefficient of correlation). P, Pearson correlation; S, Spearman correlation. Asterisk * and ** indicate statistically significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively. Each dot represents the transcript abundance of a *Gracilaria* sample (data are shown as mean, $n = 3$; please see Table 1 and Supplementary Table 3 for mean ± SD) while the blue dotted line is the best-fit trendline for all data points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

OptimumAntigen™ Design Tool (GenScript, USA, New York) (Supplementary Table 2). The selection of peptides were conducted based on the following criteria: 1) The peptide size is > 4 kDa with 10–15 amino acid residues; 2) The peptide location is near to C or N terminus; 3) The peptide has high score in hydrophilicity (> 0), antigenicity (> 1.0), surface probability (> 0); and disordered score; and 4) The peptide has the lowest similarity (< 90%) to the host which is *Oryctolagus cuniculus* (rabbit).

2.7. SDS-PAGE and western blot

Total protein sample (15 µg) was separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride (PVDF) Transfer membrane 0.2 µM (Millipore Corporation, Massachusetts) in TBST buffer with 5% (w/v) BSA. The TBST buffer consisted of 1× Tris Buffered Saline (0.14 M NaCl, 0.003 M KCl, and 0.025 M Tris base) supplemented with 0.1% (v/v) Tween 20. The protein blot was incubated with 0.5 µg/mL of either anti-GcFBPA or anti-GcGALE for 2 h at room temperature. The blot was washed in TBST buffer for 10 min before it was incubated with anti-rabbit horseradish peroxidase (HRP) at 1:10000 dilution for 1 h at room temperature. The washing step was repeated twice. The blot was then incubated in 3,3′-diaminobenzidine (DAB) solution (Amresco, USA) for 2 to 5 min with shaking. The blot was air-dried and scanned using HP Deskjet Ink Advantage 2135 All-In-One Printer (HP, US). The scanned image was converted into JPEG format with 600 dots per inch (dpi) and 32-bit in grayscale. By using ImageJ 1.45 s (Wayne Rasband, National Institutes of Health, Bethesda, USA; http://imagej.nih.gov/ij), all protein bands were marked by rectangular box so that each formed a peak in the profile plot. The background signals were eliminated by setting a baseline at the base of each peak. The size of the peak of interest was calculated as a percentage of the total size of all the measured peaks in the same western blot image. The protein abundance was quantified as relative band intensity of peak of interest to the total protein using ImageJ 1.45 s. The experiment was performed in three replicates.

2.8. Statistical analyses

All statistical analyses were performed with the SAS statistical software version 9.3 (SAS Institute Inc., Cary, North Carolina, USA), with *P*-values < 0.05 and 0.01 considered as statistically significant and highly significant, respectively. Duncan Multiple Range Test (DMRT) test was used to analyze the differences between the means among agar yields and gel strengths of different *G. changii* samples at $P < 0.05$. Pearson's and Spearman's correlation coefficients were computed to determine the linear and monotonic correlations of re-scaled normalized transcript/protein abundance to the agar properties, respectively.

3. Results and discussion

3.1. Agar properties of Gracilaria samples

The agar yields and gel strengths of 13 *G. changii* samples that were collected at different years/periods and treated under different conditions in the laboratory varied significantly among samples (Table 1). For examples, the untreated samples collected in July 2013 had significantly higher agar yield compared to those collected at other times (i.e. October 2012, March 2013 and May 2016); while untreated samples from October 2012 and March 2013 had significantly lower agar gel strength compared to other samples (Table 1). The effects of dry and rainy seasons on the agar quality had been well documented [29,30], and were often correlated to the water temperature [31] and amount of rainfall [18]. The effects of treatments (i.e. salinity, sulfate deprivation and pH) induced substantial changes to the agar yield and gel strength but the variations were smaller compared to the seasonal effects, suggesting that complex and multiple environmental stresses could be the main cause for the heterogeneity in agar quality. Intraspecific variation of the agar yield and gel quality of agarophytes (including *Gracilaria* spp.) grown under different abiotic and biotic conditions had been well documented [7]. The agar yields of these samples ranged from 18.1 to 24.3% dry weight (dw) while their gel strengths were in the range of 251.3–452.9 g/cm^2 (Table 1). These values were comparable to previous findings on the same species collected from Malaysia (12.1–25.1% dw and 205.9–562.5 g/cm^2 for agar yield and gel strength, respectively) [17,18] and Philippines (12–26% dw and 360–630 g/cm^2) [32]. The *G. changii* samples with significant differences ($P < 0.05$) in agar yield and gel strength were deemed suitable as testing materials for the expression of candidate transcripts and proteins.

3.2. Selection of candidate transcripts and proteins

A total of 15 candidate markers were identified based on: literature search, mainly on differentially expressed genes (DEGs) of *G. changii* in response to abiotic stresses [14,19,22,33]. These candidates are involved in pathways such as sulfur metabolism, carbon/galactose metabolism, ascorbic acid metabolism, additional of sulfate/methoxyl/pyruvate groups and extracellular/cell wall proteins, which could be directly or indirectly involved in agar biosynthesis pathway (Fig. 1).

The agar biosynthesis pathway remains poorly studied with fragmented information on the steps and enzymes involved [34]. Although the transcript abundance of candidate genes associated to agar biosynthesis i.e. fructose 1,6-bisphosphate aldolase, UDP-galactose-4-epimerase, galactose-1-phosphate uridylyltransferase, GDP-mannose-3,5-epimerase and UDP-glucose pyrophosphorylase, and a few other candidate markers listed in this study, had been analyzed by real-time PCR and RNA-sequencing approach in different red algal species under different conditions (Table 2), only the expression of a few genes had been related to agar yield and gel quality [11–16]. Despite several genes for enzymes involved in step (1) of agar biosynthesis had been identified and characterized, the genes for enzymes that are responsible for steps (2) and (3) such as galactosyltransferases, sulfotransferases, sulfatases, pyruvyltransferases and methyltransferases have not been investigated in agarophytes. A comprehensive analysis of the expression of the proposed agar biosynthesis genes in relation to agar yield and gel strength could help to identify the key genes that determine these agar properties.

3.3. Gene expression profiles of candidates in relation to agar yield and/or gel strength

The relative gene expression levels of the 15 candidate markers were analyzed in six different *G. changii* samples which have the most significant differences in their agar yields and/or gel strengths (i.e. SC1, ST1, SC2, 10PPT, 30PPT and 50PPT; Supplementary Table 3). When the transcript abundance of sample SC1 was set as 1.00 (to ease the comparison as SC1 which has the lowest expression level for majority of the

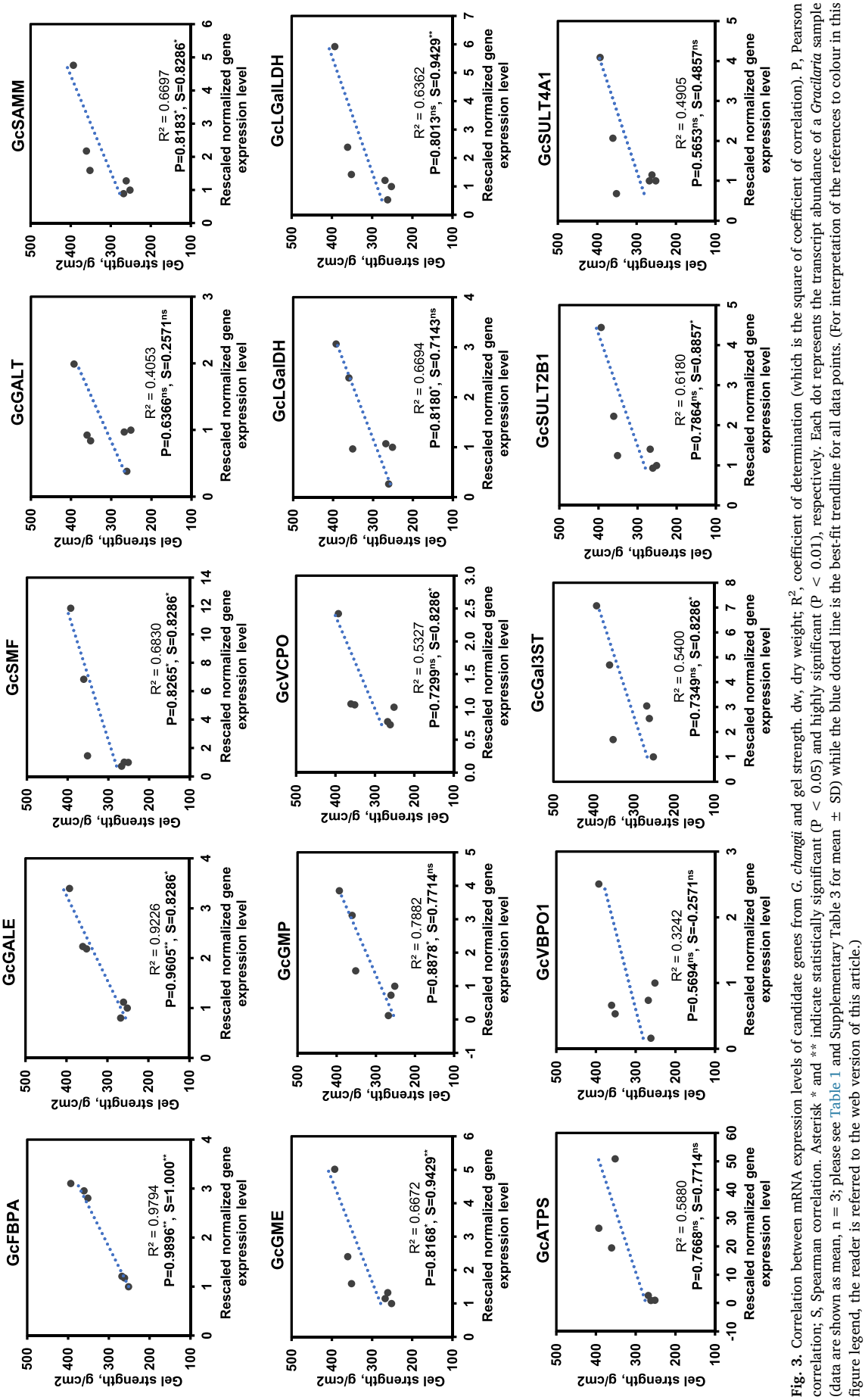

Fig. 3. Correlation between mRNA expression levels of candidate genes from *G. changii* and gel strength. dw, dry weight; R^2, coefficient of determination (which is the square of coefficient of correlation). P, Pearson correlation; S, Spearman correlation. Asterisk * and ** indicate statistically significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively. Each dot represents the transcript abundance of a *Gracilaria* sample (data are shown as mean, n = 3; please see Table 1 and Supplementary Table 3 for mean ± SD) while the blue dotted line is the best-fit trendline for all data points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

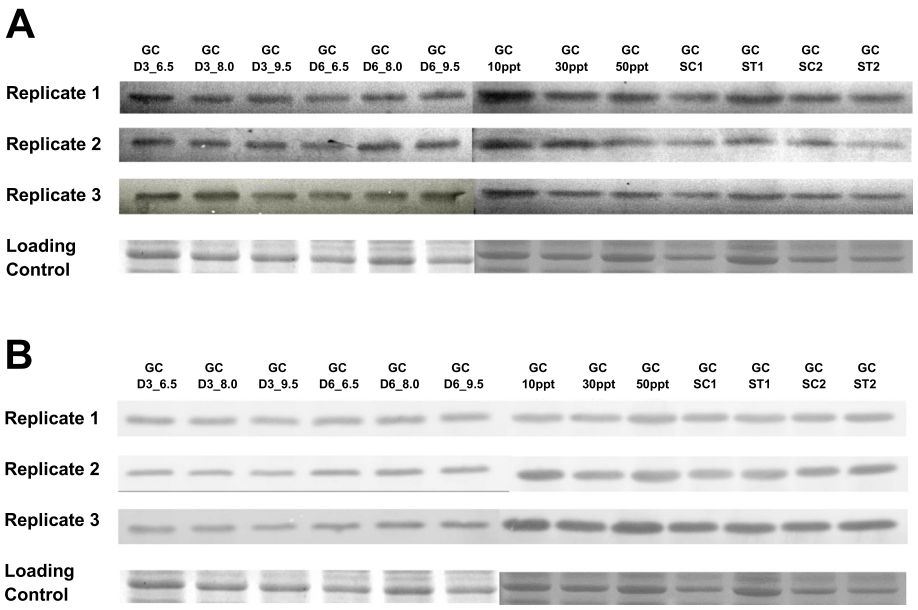

Fig. 4. Hybridization signals of total proteins of different *G. changii* samples to anti-GcFBPA (A) or anti-GcGALE (B) polyclonal antibodies. Total protein extracted from *G. changii* (~15 μg) was used for western blot with three technical replicates. Please refer to Table 1 for the abbreviation of samples.

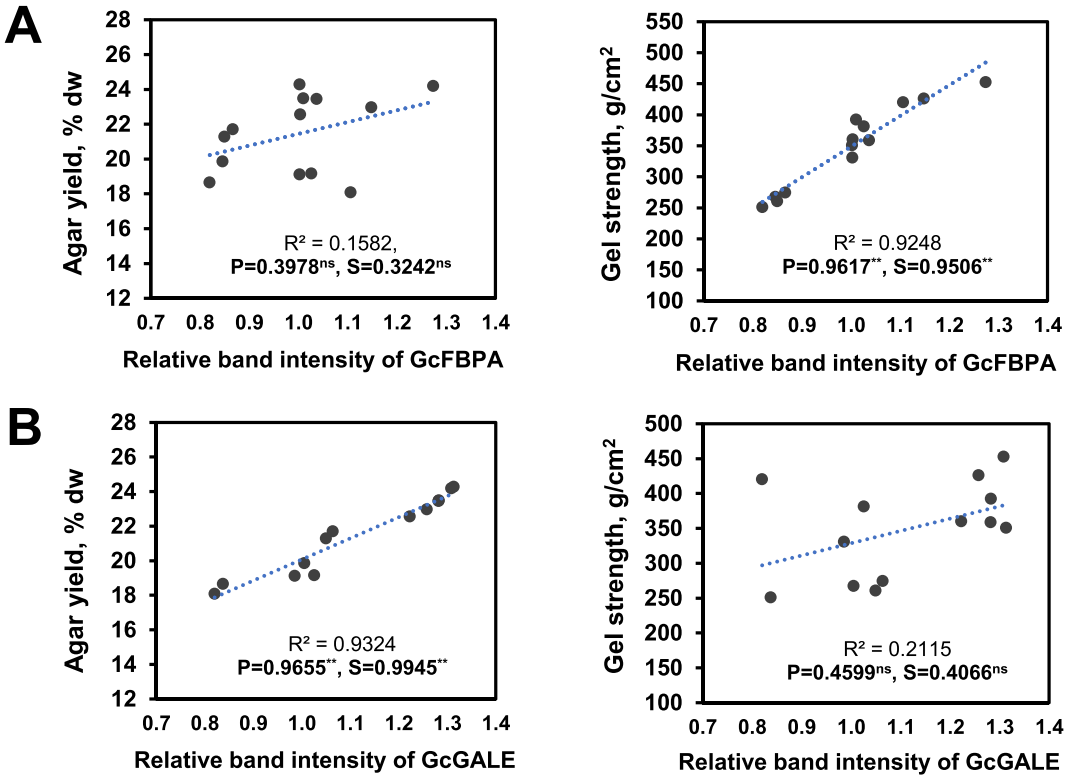

Fig. 5. Correlation between protein abundance of (A) GcFBPA and (B) GcGALE from *G. changii* and agar yield or gel strength. dw, dry weight; R^2, coefficient of determination (which is the square of coefficient of correlation). P, Pearson correlation; S, Spearman correlation. Asterisk * and ** indicate statistically significant ($P < 0.05$) and highly significant ($P < 0.01$), respectively. Each dot represents the transcript abundance of a *Gracilaria* sample (data are shown as mean, n = 3; please see Table 1 and Supplementary Table 4 for mean ± SD) while the blue dotted line is the best-fit trendline for all data points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genes tested), the transcript abundance of other samples ranged from 0.1 to 50.9-fold of that in the SC1 sample, with a majority of the tested genes having a 50% increase or decrease in transcript abundance (i.e. 0.5- and 1.5-fold changes) in at least one treated sample. The findings suggested that the transcript abundance was affected by treatments or collection time. Most of the candidates tested (except for GcATPS and GcPPT1) showed the highest transcript abundance in samples treated at 10 ppt salinity, while the lowest transcript abundance was detected in either ST1 or SC2 for most genes except for GcGal3ST and GcPPT1.

The correlation between the transcript abundance and agar yield (Fig. 2) or gel strength (Fig. 3) was determined using both Pearson and Spearman correlation tests, which investigate the linear and monotonic

relationships between two variables. Among the 15 candidates tested, the transcript abundance of 4 (i.e. GcFBPA, GcGALE, GcGME and GcATPS) and 11 (i.e. GcFBPA, GcGALE, GcSMF, GcGME, GcGMP, GcVCPO, GcLGalDH, GcLGalLDH, GcSAMM, GcGal3ST and GcSULT2B1) genes were significantly correlated ($P < 0.05$; either Pearson or Spearman or both) with agar yield and gel strength, respectively (Figs. 2 and 3). Among these genes, two candidate markers (i.e. GcFBPA and GcGALE) that had the most significant linear correlation ($P < 0.05$) with both agar yield and gel strength could be the most promising transcript markers to be used for the screening of seaweed raw materials.

GcFBPA encodes fructose-bisphosphate aldolase (FBPA), which is important for glycolysis and Calvin cycle [35]. GcFBPA might play a role in controlling the photosynthetic carbon flux and amount of fructose 1,6-bisphosphate to be channeled into the biosynthesis of agar precursors. The transcription of FBPA was affected by abiotic stresses such as temperature, drought and salinity stress in *Dunaliella salina* [36], *Arabidopsis thaliana* [37], wheat *Triticum aestivum* [38], mangrove plant *Sesuvium portulacastrum* [39] and algae (Table 2), suggesting its crucial role in environmental stress response. Meanwhile, UDP-galactose-4-epimerase (GALE) catalyzes the reversible conversion of UDP-D-glucose to UDP-D-galactose. The increase of agar yield during dark condition was reported previously [40–42]. The breakdown of floridean starch during night time was reported to be associated with increased expression of the gene encoding GALE In *Gelidium pristoides* [43], suggesting its key regulatory role in providing UDP-D-galactose for agar production. However, it is not clear how GcFBPA and GcGALE that are involved in fructose and galactose metabolisms could directly affect the agar gel strength. The agar yield may increase the molecular weight of gel which then affects the agar gel strength [44].

3.4. Protein abundance of GcFBPA and GcGALE in relation to agar yield/gel strength

Since the mRNA expression level of a gene does not always correlate with its protein abundance, we also analyzed the protein abundance of GcFBPA and GcGALE in 13 *G. changii* samples (Supplementary Table 4) i.e. with additional samples resulting from pH stress treatments performed at a later stage. The collection time and treatments were found to have remarkable effects on the protein abundance of GcFBPA and/or GcGALE (Fig. 4), except for salinity and sulfate deprivation which had less effect on the protein abundance of GcFBPA. The highest protein abundance for both GcFBPA and/or GcGALE was shown by GC_D6 samples (i.e. GC_D6_6.5, GC_D6_8.0 and GC_D6_9.5) whereas the lowest protein abundance was found in sulfate-deprivated samples or GC_D3 samples (i.e. GC_D3_6.5, GC_D3_8.0 and GC_D3_9.5).

The protein abundance of GcFBPA was found to be not significantly correlated with the agar yield (in contrast to its RNA expression levels), but it had a highly significant correlation (Pearson and Spearman; $P < 0.01$) with agar gel strength (Fig. 5). On the other hand, the protein abundance of GcGALE was highly significant (Pearson and Spearman; $P < 0.01$) to agar yield but had no correlation to gel strength despite its transcript abundance was significantly correlated to gel strength (Fig. 5). Lack of correlation between transcript abundance and protein abundance could be partly due to the limitation of detection methods (e.g. RNA-based quantitative PCR versus western blotting), bias of quantification methods, background noise and errors [45]. Besides, the differences between transcript and protein abundance of GcFBPA could also be due to post-transcriptional modification, mRNA stability and protein translational efficiency [45–48].

4. Conclusions

In conclusion, we have analyzed the transcript abundance of 15 genes related to agar biosynthesis, and identified four and eleven candidates that have significant correlation to agar yield and gel strength of six *G. changii* samples. These candidates have the potential to be further developed as transcript markers for the selection of seaweed materials. The protein abundance of two candidates i.e. GcGALE and GcFBPA with their mRNA expression levels tightly correlated to both agar yield and gel strength, were also found to be significantly correlated to gel strength and agar yield, respectively.

In future, these candidates could be developed into user-friendly markers for the screening of *G. changii*. The ability to identify wild or cultivated seaweeds with high agar yield and quality will help to ensure sustainable supply of commercial grade starting materials and maximum economic returns from the seaweed resources. With the development of genetic transformation methods in algae [49,50], the future research could include knockout or overexpression of the candidate genes in *G. changii* to shed lights on the functional roles of GcGALE and GcFBPA, as an important step in deciphering the agar biosynthesis mechanism.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Author contributions

CLH conceived the research and designed the experiments. PEL and SMP provided technical and logistic support in obtaining biological samples. YYL and WKL performed the experiments. CLH, YYL and WKL analyzed and interpreted the data. CLH, ATCL, PM and JOA supervised the experiment. CLH, YYL and WKL wrote the original draft. All authors edited and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101532.

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