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Genome-wide identification of the *GT7* and *GH16* genes and their correlations with agar metabolism in the agarophyte *Gracilariopsis lemaneiformis*

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The economically important seaweed *Gracilariopsis lemaneiformis* (*Gp. lemaneiformis*) is the main resource for agar production and abalone bait. Few studies have focused on the functions of glycosyltransferases (GTs) and glycoside hydrolases (GHs) involved in saccharometabolism, including that of agar. To investigate the possible functions of glycosyltransferase family 7 (GT7) and glycoside hydrolase family 16 (GH16) members in agar metabolism, analyses of these gene families were conducted using bioinformatics-based and physiological methods. In total, five *GlGT7* genes and four *GlGH16* genes from the Gp. lemaneiformis genome were identified and analyzed. These GT7 and GH16 members are absent in higher plants, and the majority of GlGT7s and GlGH16s have no introns. Catalytic pocket residue analysis revealed that GlGT7s may function as β-1,4-galactosyltransferases and that GlGH16s play roles as agarases. Promoter prediction and qRT-PCR experiments verified that these genes can be regulated by light and phytohormones. GlGT7s were predicted to interact with carbohydrate sulfotransferases (STs), whereas yeast two-hybrid (Y2H) assays revealed no interactions between these proteins. Under heat stress, no significant difference was found in agar content; however, the expression of *GlGT7s* fluctuated and that of *GlGH16* increased. Low nitrogen stress significantly increased the agar content, and the expression of *GlGT7*s increased, whereas that of *GlGH16s* decreased. In three cultivars of *Gp. lemaneiformis*, the expression of *GlGT7* genes in cultivars 981 and Lulong No. 1 was upregulated relative to that in the wild type, whereas *GlGH16* expression levels were significantly decreased. Fluorescence microscopy further showed that β-1,4-galactose accumulation was consistent with increases in agar content and *GlGT7* expression. Pearson correlation analysis confirmed that the expression levels of *GlGT7s* and *GlGH16s* were positively and negatively correlated with agar accumulation, respectively. Taken together, these results demonstrated that GlGT7 and GlGH16 are intimately correlated with agar metabolism; in particular, *GlGT7-2*, *GlGT7-5*, and *GlGH16-4* could act as molecular markers to indicate agar yield. This study will provide a valuable basis for breeding new cultivars with high agar content.

KEYWORDS

***Gracilariopsis lemaneiformis*, agar metabolism, glycosyltransferase family 7 (GT7), glycoside hydrolase family 16 (GH16), correlation analysis**

Introduction

Agar, a sulfated polysaccharide that is abundant in the cell walls of agarophytes, is industrially produced from various kinds of red seaweeds, such as members of the families Gelidiaceae, Gracilariaceae, Gelidiellaceae, and Pterocladiaceae. Because of its excellent rheological properties, bioactive functions, and good compatibility with other bioactive compounds, agar has been widely applied in the fields of food, pharmaceutical, and chemical industries (Lee et al., 2017a; Kim et al., 2021). In addition, as the main component of cell walls in red seaweed, agar can be synthesized and remodeled to protect against extreme environments occurring in the intertidal zone, enabling seaweeds to remain flexible enough to withstand the physical challenges imposed by waves and tides. The biosynthesis of agar from red seaweeds represents an important strategy to address environmental variability (Ficko-Blean et al., 2015). Despite the great importance of agar in applications and algal physical processes, this material cannot be synthesized artificially and the complete synthesis pathways and regulatory mechanisms of agar are still unknown. The basic structure of agar, a type of conglomerate of linear glucan chains, involves the cross-linking of D-galactose and L-galactose through α-1,3- and β-1,4-glycosidic bonds, which are catalyzed by galactosyltransferases (GalTs) and hydrolyzed by glycoside hydrolases (GHs), respectively (Lee et al., 2017a; Hu et al., 2018). However, studies related to the effects of candidates of GalTs and GHs on agar metabolism are still rare.

Glycosyltransferases (GTs) constitute a large multigene family that consists of more than 110 subfamilies whose members are categorized according to the similarity of their amino acid sequences, substrate specificity, reaction mechanism, and three-dimensional structure. GTs are responsible for catalyzing the synthesis of various glycans, which play important roles in plant growth, reproduction, and stress resistance. Among GTs, members of the glycosyltransferase 7 family (GT7) are purportedly involved in the biosynthesis of sulfated polysaccharides such as chondroitin and heparin (Cantarel et al., 2008). In humans, GT7 sequences were annotated as N-acetylgalactosaminyltransferases, which are used for chondroitin synthesis (Brawley et al., 2017). In Florideophytes and Bangiophytes of red algae, N-acetylgalactosaminyltransferases are sulfate chondroitin synthases and can transfer acetyl galactosamine to core tetrasaccharides to extend chondroitin sulfate chains (Sato et al., 2011). GT7 sequences have been annotated in the genomes of *Chondrus crispus* and *Porphyra umbilicalis* (Collén et al., 2013; Ho, 2020). Although the knowledge of genomes of red macroalgae addresses the possibility of GT7 being involved in sulfated galactan synthesis, few studies have discussed the connection between agar and GT7.

GHs, which are responsible for hydrolyzing the glycosidic bond between a sugar molecule and other moieties, are likely to be involved in cell wall remodeling and recycling (Cantarel et al., 2008). In higher plants, xyloglucan endotransglucosylase/hydrolase (XTH), which belongs to the glycoside hydrolase family 16 (GH16), is responsible for cleaving cellulose and hemicellulose cross-linkages and is necessary for cell wall loosening and cell expansion (Ezquer et al., 2020). In marine environments, macroalgae, especially red and brown seaweeds, are important carbon sources for microbial hydrolysis and utilization (Rhein-Knudsen and Meyer, 2021). Agar can be cleaved into agar oligosaccharides by agarases from marine bacteria for industrial use and carbon recycling (Jiang et al., 2020). To date, large numbers of microorganisms have been identified and isolated for cleaving agar or agarose for commercial production (Kim et al., 2010; Seo et al., 2014; Gao et al., 2019). The red algae *C. crispus* and *P. umbilicalis* harbor genes that appear to encode GH16 enzymes such as carrageenase and porphyranase, and these GH16s are believed to participate in cell wall expansion and recycling (Lipinska et al., 2020). However, no studies have revealed the connection between agar and GH16 *in vivo*.

The agarophyte *Gracilariopsis lemaneiformis* (*Gp. lemaneiformis*) is mainly used for agar production and for abalone feed and food industries. Wild thalli of *Gp. lemaneiformis* have gradually become scarce because of human disturbance and difficulty surviving heat stress (Zhou et al., 2016). To meet the increasing demand of the agar industry, the cultivars *Gp. lemaneiformis* 981 and Lulong No. 1, which have excellent high temperature tolerance, are highly productive and produce high agar yields, have been bred. The cultivation of *Gp. lemaneiformis* has become the second largest algal industry in China (Wang et al., 2016). However, problems such as germplasm degradation, unstable agar production, and changing environments still hinder the development of the *Gp. lemaneiformis* industry. To identify molecular markers indicating agar content, we characterized the GT7 and GH16 members in *Gp. lemaneiformis* and analyzed their sequence features, promoter elements, phylogenetic relationships, interacting proteins, morphology, and changes in transcription profiles under heat and low nitrogen stresses. In this study, the candidates for agar polymerization and modification were identified from *Gp. lemaneiformis*, which provides clues for the clarification of the functions of agar metabolism-related enzymes and their metabolic pathways. Taken together, these findings will provide a valuable basis for breeding new cultivars with high agar content.

Materials and methods

Seaweed collection and culture

Wild-type *Gp. lemaneiformis* was collected from Zhanshan Bay in Qingdao, Shandong Province, China (36° 03′ N, 120° 22′ E), in May 2020. *Gp. lemaneiformis* cultivars 981 and Lulong No. 1 were collected from the coasts of Xiapu (26° 42′ N, 119° 59′ E) and Lianjiang (26° 39′ N, 119° 72′ E), Fujian Province, China, respectively. In the laboratory, the thalli were cultivated in transparent aquaria at an irradiance of 40 μmol photons m^{-2} s^{-1}, under a 12h: 12h light: dark photoperiod, and at 23°C for 1 week of adaptation. The tank containing 5 l of natural seawater (salinity of 25‰) was filled with filtered air, and the seawater was changed every day. The algae used for treatments were mainly those of cultivar 981.

Heat and low nitrogen treatments

The cultivar 981 was exposed to heat and low nitrogen stresses for agar and related gene correlation analysis in the laboratory.

Approximately 10 g of fresh thalli of each biological replicate was placed into 8 l of seawater, and each treatment was replicated three times. During the 11 days of heat-stress treatment, the thalli were cultured with normal Provasoli medium at 23°C (the control treatment, CK) or at 28°C (high temperature treatment, HT). During the 21 days of the nitrogen treatment, the thalli were cultured with normal Provasoli media (CK) and Provasoli media without ammonium nitrate (low nitrogen treatment, LN). After the experiments, samples were collected for agar and gene expression analyses. Apart from these factors, the other culture conditions were consistent with the adaptive culture conditions.

Light and hormone treatments

To explore the responses of different treatments according to promoter predictions, the thalli were cultivated under 40 μmol photons m^{-2} s^{-1} (CK) and 180 μmol photons m^{-2} s^{-1} light intensity (high light treatment, HL). For the hormone treatments, the thalli were cultivated in media without hormone addition (CK), with 50 μmol·l^{-1} abscisic acid treatment (ABA), and with 50 μmol·l^{-1} methyl jasmonate treatment (MeJA) (Hou et al., 2018; Sun et al., 2022). Apart from the light and hormone factors, the other culture conditions were the same as the adaptation culture conditions. After 6 h of treatment, samples were collected for relative expression analyses of *GlGT7s* and *GlGH16s*.

Agar extraction

Approximately 5 g of fresh thalli was used to assay the agar content according to the alkali treatment method (Chen Q. L. et al., 2022). The agar content was calculated using the following formula: agar content (%) = (dry weight of agar/dry weight of thalli) × 100.

Observation of thalli after treatments

The *Ricinus communis* agglutinin I (RCA_{120})-fluorescein staining method was used but was modified according to the methods of Lin et al. (2020). The thalli were cut into approximately 0.1 mm slices and then washed twice with phosphate-buffered saline (PBS) solution. The slices were resuspended in staining buffer and incubated for 1 h in the dark, with inverting occurring every 20 min. After incubation, the stained slices were collected and washed three times with PBS solution to remove the free RCA_{120}-fluorescein. The stained slices were ultimately observed under a Nikon NI-U Normal Fluorescence Microscope (Japan).

Identification of GlGT7 and GlGH16 members in *Gp. lemaneiformis*

Based on the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org/), the protein sequences of GT7 and GH16 were downloaded from UniProt (https://www.uniprot.org/) and used to generate the GT7- and GH16-BLAST databases, respectively, as referenced by Ulvskov et al. (2013). To identify the predicted GT7 and GH16 sequences in *Gp. lemaneiformis* (GlGT7s and GlGH16s), the genomic sequence of this alga reported by Sun et al. (2018) was used as the query sequence to search against the GT7- and GH16-BLAST databases using the BLASTP tool with an e-value ≤ e^{-10}. After obtaining the predicted GlGT7s and GlGH16s, the Pfam database (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) were then used to confirm the conserved domains, and those sequences lacking the conserved domains were excluded.

Sequence analysis and structural characterization

The open reading frames (ORFs) of the *GlGT7* and *GlGH16* genes were predicted using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The theoretical molecular weight (MW) and isoelectric point (pI) of GT7 and GH16 proteins were calculated by ProtParam (https://web.expasy.org/protparam/). The MEME program (http://alternate.meme-suite.org/tools/meme) was used to identify the conserved motifs, with the following parameters: any number of repetitions, maximum of 10 misfits and an optimum motif width of 6–200 amino acid residues. The exon–intron structures were determined *via* Gene Structure Display Server (GSDS, http://gsds.cbi.-pku.edu.cn/).

Promoter response elements

The template DNA from *Gp. lemaneiformis* cultivar 981 was isolated *via* a RaPure Plant DNA Mini Kit (Magen, Guangzhou, China) according to the manufacturer's protocol. The predicted promoter region of each sequence was limited to 2,000 bp upstream from the transcription start site of each sequence. The actual sequence used for validating the promoter region was 2,015–2,135 bp, including the beginning 15–135 bp of the ORFs, so that we could ensure that the promoter region of each sequence was sufficient. The primers used for each sequence are listed in Supplementary Table S1. Promoter elements were predicted using the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Phylogenetic analysis

GT7 sequences from the genomes of Ochrophyta and Rhodophyta were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/), JGI (https://genome.jgi.doe.gov/portal/), and Ensembl (https://plants.ensembl.org/index.html) databases. The other protein sequences annotated as belonging to the GT7 family were retrieved from CAZy and downloaded from UniProt. GH16 sequences, considered to encode β-agarases from marine bacteria, were retrieved from the NCBI database. The other GH16 sequences were retrieved

from the genomes of Ochrophyta, Rhodophyta, and terrestrial plants in the NCBI, JGI, and Ensembl databases. These sequences are presented in Supplementary Tables S2, S3. Multiple-sequence alignment involving different species was performed using Clustal X (Dublin, Republic of Ireland). A phylogenetic tree was subsequently constructed using the maximum likelihood method with 1,000 bootstrap replicates by MEGA 7 (Auckland, New Zealand).

Interaction network analysis and yeast two-hybrid assays

Interaction networks comprising chondroitin synthase and other proteins and comprising β-agarase and other proteins were constructed using the STRING database v11.0 (https://string-db.org/). To verify the interactions between GlGT7 proteins and carbohydrate sulfotransferase (GlST) proteins, putative GlST sequences were retrieved by searching the genome of *Gp. lemaneiformis* using the hidden Markov model (HMM) of sulfotransferase (ST) (PF03567), with an e-value ≤ e^{-10}. The full-length sequences of GlGT7s and GlSTs were obtained by PCR with gene-specific primers (Supplementary Table S4) and then cloned and ligated into pGBKT7 and pGADT7 vectors (Clontech), respectively. Autoactivation and toxicity tests were performed following the protocols of the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA, USA). Then, one-to-one verification assays were performed. The recombinant vectors pGADT7-GlST-1, pGADT7-GlST-2, and pGADT7-GlST-3 were co-transformed with pGBKT7-GlGT7-1, pGBKT7-GlGT7-2, pGBKT7-GlGT7-3, pGBKT7-GlGT7-4, and pGBKT7-GlGT7-5 into the yeast two-hybrid (Y2H) yeast. The resulting yeast cells were subsequently incubated on SD/-Trp/-Leu/-His/-Ade/x-α-gal/AbA media to validate their interactions.

Total RNA extraction and expression analysis of the *GlGT7* and *GlGH16* genes

After the different treatments of high light, hormone, heat, and low nitrogen, the total RNA was extracted using a Plant RNA kit (OMEGA, Georgia, USA). The extracted RNA was then quantified by a NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). First-strand cDNA was synthesized using HiScript II Q RT SuperMix for qPCR with a gDNA wiper (Vazyme, Nanjing, China) and stored at -20°C for subsequent analysis. All the procedures were performed following the manufacturers' instructions.

Primer Premier 5 was used to design primers specific to the full-length sequences of the GlGT7 and GlGH16 genes (Supplementary Table S5), and the specificity of the primers was determined by NCBI Primer-BLAST. qRT-PCR was carried out using PerfectStart Green qPCR SuperMix (TransGen, Beijing, China) on a Mastercycler RealPlex2 real-time PCR system (Eppendorf, Germany) with the following program: 95°C for 30 s followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 10 s. For each treatment, three biological replicates, each with two technical replicates (n = 6), were evaluated to acquire reliable results. The housekeeping gene β-actin was used as an internal reference gene (Ding et al., 2014). The relative expression levels of the GlGT7s and GlGH16s were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The correlations between the expression profiles of *GlGT7s* or *GlGH16s* and agar contents under heat and low nitrogen treatments were determined by Pearson correlation analysis (n = 6 of each treatment, SPSS 16.0, USA). The normal distribution of all the data under each treatment and the homogeneity of variance were confirmed by a Shapiro–Wilk test ($p > 0.05$) and the Levene test ($p > 0.05$), respectively. The effects of the treatments were assessed by independent t tests and one-way analysis of variance (ANOVA). A Tukey *post-hoc* test (Tukey HSD) was performed to determine the differences between the treatments. The significant and very significant levels were set at $p < 0.05$ and $p < 0.01$, respectively.

Results

Identification and characterization of *GlGT7* and *GlGH16* genes

The available *GT7* and *GH16* genes in red algae, brown algae, and diatoms were identified (Table 1). In Rhodophyta, *C. crispus*, *Gp. chorda*, *Gracilaria domingensis*, and *P. umbilicalis* have 2-5 *GT7* genes. However, only one *GT7* gene was found in *Ectocarpus siliculosus* and *Phaeodactylum tricornutum*. For *GH16*, the red macroalga *G. domingensis* has the most genes (12), followed by *Gp. chorda* (6) and *Gp. lemaneiformis* (4), whereas no *GH16* gene was found in *Aureococcus anophagefferens*, *E. siliculosus*, or *P. tricornutum*. However, in the genomes of red microalgae, such as *Cyanidioschyzon merolae* strain 10D and *Porphyridium purpureum*, neither *GT7* nor *GH16* genes were found.

In *Gp. lemaneiformis*, a total of five *GlGT7* members were annotated in the genome (Table 2). The ORF lengths of *GlGT7* genes varied from 1,181 to 1,682 bp, the MWs of *GlGT7s* were between 44.77 and 64.04 kDa, and the predicted pI values of the *GlGT7*s were between 5.47 and 9.34. Four *GlGH16* sequences identified in *Gp. lemaneiformis* encoded 265 amino acids (aa) to 485 aa, with MWs of 31.91 to 54.92 kDa and pIs between 4.39 and 9.18. Given that red algae typically have a small genome, except for *GlGH16-4*, which has one intron, no other *GlGT7* and *GlGH16* genes have introns.

Promoter response elements

To explore the expression regulatory patterns of the *GlGT7* and *GlGH16* genes, a total of 19 kinds of promoter elements, including light-, stress-, and hormone-responsive elements, were predicted

TABLE 1 Information about the *GT7* and *GH16* genes in Rhodophyta and Ochrophyta.

Species	Taxonomy	Alga type	Genome side (Mb)	GT7	GH16	Resource	Sulfated poly-saccharide	Reference
Chondrus crispus	Rhodophyta	Macroalga	122.2	4	2	Genome	Carrageenan	Collén et al., 2013
Cyanidioschyzon merolae strain 10D	Rhodophyta	Microalga	16.42	0	0	Genome	–	Matsuzaki et al., 2004
Gracilariopsis chorda	Rhodophyta	Macroalga	92.18	5	6	Genome	Agar	Lee et al., 2018
Gracilariopsis lemaneiformis	Rhodophyta	Macroalga	88.98	5	4	Genome	Agar	Sun et al., 2018
Gracilaria domingensis	Rhodophyta	Macroalga	78	3	12	Genome	Agar	Nakamura-Gouvea and Alvis-Lima 2022
Porphyridium purpureum	Rhodophyta	Microalga	19.67	0	0	Genome	Exopolysaccharide	Bhattacharya et al., 2013
Porphyra umbilicalis	Rhodophyta	Macroalga	87.89	2	2	Genome	Porphyran	Brawley et al., 2017
Aureococcus anophagefferens	Ochrophyta	Microalga	60.90	0	0	Genome	Exopolysaccharide	Gann et al., 2021
Ectocarpus siliculosus	Ochrophyta	Macroalga	195.81	1	0	Genome	Homofucan	Michel et al., 2010
Phaeodactylum tricornutum	Ochrophyta	Microalga	27.45	1	0	Genome	Fucoidan	Bowler et al., 2008

(Tables 3-1, 3-2). Multiple light-responsive elements were found in the promoter regions of the five *GlGT7* and four *GlGH16* genes, indicating that the expression of *GlGT7* and *GlGH16* may be regulated by light. Moreover, all the genes contained at least one stress-responsive element, such as those that respond to low temperature, drought, defense, or stress. Among the hormone-responsive elements, elements that respond to ABA and MeJA were the most frequently predicted ones. Regarding the other elements, MYB and MYB-related sites were the only elements related to transcription factors predicted in the *GlGT7s* and *GlGH16s*.

Light and hormone signaling responses of *GlGT7* and *GlGH16*

To further determine what factors regulate the expression of the *GlGT7* and *GlGH16* genes, we selected the most frequently predicted factors, such as light and hormone elements. Under high light conditions (Figure 1A), the expression levels of five *GlGT7* genes were decreased, indicating that the expression of *GlGT7* members could be inhibited by high light, which was consistent with the results that the agar contents significantly decreased (Supplementary Figure S1A). However, diverse expression modes were observed for *GlGH16*s, namely, *GlGH16-2* was downregulated by high light, whereas *GlGH16-4* expression was enhanced 2.00-fold, and the expression of the other two *GlGH16* genes was not significantly altered by high light.

After ABA treatment, the expression levels of *GlGT7-1* and *GlGT7-5* were significantly increased, especially for *GlGT7-1*, with a 3.17-fold expression increase. In addition, *GlGH16-1*, *GlGH16-2*, and *GlGH16-3* were upregulated at least threefold by ABA treatment relative to that of the CK (Figure 1B).

In the presence of MeJA, the expression of *GlGT7-1*, *GlGT7-4*, and *GlGT7-5* was enhanced and *GlGT7-1* displayed a maximal expression

TABLE 2 Features of the *GlGT7* and *GlGH16* genes in *Gracilariopsis lemaneiformis*.

Name	Gene ID	Genomic location	ORF	Intron	AA (aa)	MW (kDa)	pI
GlGT7-1	evm.model.Contig2913.19	66598-68049 (+)	1451	0	483	55.68	6.92
GlGT7-2	evm.model.Contig3878.15	48199-49380 (+)	1181	0	393	44.77	5.47
GlGT7-3	evm.model.Contig3055.2	6977-8245 (+)	1268	0	422	48.71	5.99
GlGT7-4	evm.model.Contig3953.2	5089-6498 (+)	1409	0	469	53.46	9.34
GlGT7-5	evm.model.Contig4364.37	119796-121478 (-)	1682	0	560	64.04	6.45
GlGH16-1	evm.model.Contig216.1	3177-4043(-)	867	0	288	33.89	6.26
GlGH16-2	evm.model.Contig36591.1	9200-9997(+)	798	0	265	31.91	9.18
GlGH16-3	evm.model.Contig3125.10	75872-77329(+)	1458	0	485	54.92	6.06
GlGH16-4	evm.model.Contig29754.1	5951-7044(+)	1014	1	337	38.64	4.39

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TABLE 3-1 Predicted promoter elements of *GlGT7* genes.

Predicted promoter element		*GlGT7-1*	*GlGT7-2*	*GlGT7-3*	*GlGT7-4*	*GlGT7-5*
Light	Light-responsive element	12	9	12	11	7
Stress	Defense and stress responsiveness	1	0	0	2	0
	Drought inducibility	2	0	3	2	1
	Low-temperature responsiveness	1	2	1	0	2
Hormone	Abscisic acid responsiveness	4	2	0	5	5
	Auxin-responsive element	2	2	2	3	0
	Gibberellin-responsive element	0	2	1	1	3
	MeJA-responsiveness	1	7	4	5	2
	Salicylic acid responsiveness	0	0	0	0	0
Others	Anaerobic induction	1	0	0	0	1
	Anoxic specific inducibility	1	1	0	0	0
	Cell-cycle regulation	0	0	2	0	0
	Circadian control	0	1	0	1	0
	Meristem expression	0	1	0	0	1
	MYB-binding site	4	5	6	3	4
	MYB	8	7	9	5	8
	MYB recognition site	0	1	0	3	1
	MYBHv1 binding site	0	1	0	3	1
	Zein metabolism regulation	0	0	1	1	1

TABLE 3-2 Predicted promoter elements of GlGH16 genes.

Predicted promoter element		*GlGH16-1*	*GlGH16-2*	*GlGH16-3*	*GlGH16-4*
Light	Light-responsive element	16	20	8	13
Stress	Defense and stress responsiveness	1	1	0	1
Hormone	Drought-inducibility	2	0	2	2
	Low-temperature responsiveness	3	0	1	2
	Abscisic acid responsiveness	5	5	5	17
	Auxin-responsive element	0	2	0	1
	Gibberellin-responsive element	1	0	0	3
	MeJA-responsiveness	8	4	4	5
	Salicylic acid responsiveness	0	1	0	0
Others	Anaerobic induction	2	0	0	4
	Anoxic specific inducibility	2	0	0	0
	Cell-cycle regulation	0	1	0	0
	Circadian control	0	1	0	0
	Meristem expression	2	2	2	0
	MYB-binding site	0	1	2	4
	MYB	6	6	8	6

(Continued)

TABLE 3-2 Continued

Predicted promoter element		*GlGH16-1*	*GlGH16-2*	*GlGH16-3*	*GlGH16-4*
	MYB recognition site	3	0	2	1
	MYBHv1 binding site	3	0	0	1
	Zein metabolism regulation	0	0	0	0

fold change of 2.00, whereas the other two *GlGT7* genes demonstrated no significant changes in expression (Figure 1C). The majority of the *GlGH16* genes did not significantly change after the addition of MeJA, except that *GlGH16-3* expression was significantly downregulated.

Evolutionary relationships among GT7 and GH16 members

To determine the evolutionary relationships among the GT7 members from different species, an unrooted phylogenetic tree was constructed. A total of 63 GT7 sequences from red and brown seaweeds, marine bacteria, *Homo sapiens*, *Drosophila melanogaster*, *Gallus gallus*, and *Mus musculus* were analyzed in the phylogenetic tree (Figure 2). According to the sequence homology data, these GT7 sequences could be divided into two main clusters: clades I and II. Marine organisms such as *Styela clava*, the photosynthetic Ochrophyta *A. anophagefferens*, and red seaweeds clustered in clade I, and the majority of GT7 proteins of *H. sapiens and M. musculus* clustered in clade II. Interestingly, *E. siliculosus*, *P. tricornutum*, and *D. melanogaster* clustered in clade II. All the GT7 sequences contained the highly conserved motif 1, which encodes the catalytic pocket residue WGGEDDD (Figure 3).

A total of 50 sequences were retrieved for constructing the phylogenetic tree of the GH16 family members (Figure 4). In clade II, *Zea mays* harbors conserved motifs 4, 5, and 8, which are completely different from those of other species, representing a separate evolutionary direction. A close relationship between red seaweeds and marine bacteria was found (clade I). In clade I, the majority of GH16 members contained the common sequence motif 6-EIDVFE (Figure 3).

Interaction network of the GT7 and GH16 families

No genomic information of *Gp. lemaneiformis* is present in the STRING database; however, *Gp. lemaneiformis* and *Gp. chorda* are closely evolutionarily related. In this study, to reveal the functional role of the GT7 and GH16 families, protein–protein interactions (PPIs) were predicted using the STRING database information about *Gp. chorda*. The GT7 (annotated as chondroitin sulfate synthase) interaction network contained three nodes, and the interactions among chondroitin synthase and its functional partners had a confidence score of 0.501 (no significance). Three carbohydrate STs (A0A2V3J539, A0A2V3IMF1, and A0A2V3J7C1) were the only proteins predicted to be incorporated into the interaction network (Figure 5A).

GH16 members (annotated as β-porphyranases) in *Gp. chorda* were chosen to construct the protein interaction network (Figure 5B). The interaction network contained two nodes and interactions among β-porphyranases and syntaxin family proteins with a confidence score of 0.407 (no significance).

Validation of GlGT7-interacting proteins

The full-length sequence of *GlGT7* was cloned into a pGBKT7 bait vector, and the sequence of *GlST* was cloned into a pGADT7 prey vector. Before validation, the toxicity and autoactivation of these recombinants were tested in Y2H yeast. The clones displayed no autoactivation on SD/-Leu/-Trp/-His/-Ade media (Supplementary Figure S2). After cotransformation of pGBKT7-GlGT7s and pGADT7-GlSTs, no clone was found to be positive on SD/-Leu/-

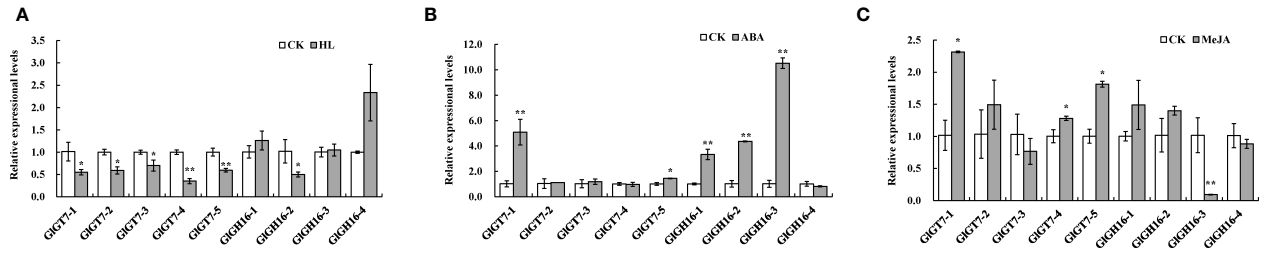

FIGURE 1
Expressions of the *GlGT7* and *GlGH16* genes under high light, ABA, and MeJA treatments. **(A)**, high light treatment (HL); **(B)**, ABA treatment (ABA); **(C)**, MeJA treatment (MeJA); CK, the control treatment. * and ** represent significant ($p < 0.05$) and very significant ($p < 0.01$) differences between the treatments.

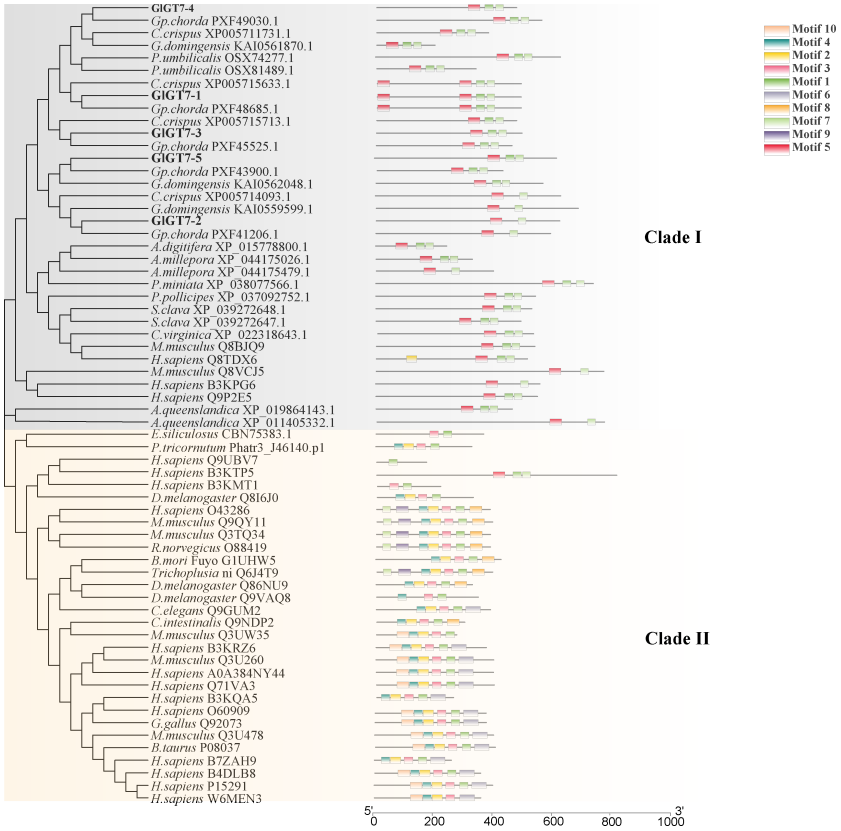

FIGURE 2
Phylogenetic tree and conserved motifs of GT7 members constructed based on the protein sequences using the maximum likelihood method. The bootstrap analysis was carried out with 1,000 replicates.

Trp/-His/-Ade/x-α-gal/-AbA media, indicating that there are no interactions between GlGT7s and GlSTs (Supplementary Figure S3).

Relations of agar and *GlGT7*/*GlGH16* expression levels among three cultivars of *Gp. lemaneiformis*

Under the experimental conditions used in this study, the agar contents of *Gp. lemaneiformis* 981 and Lulong No. 1 were 113% and 37% higher, respectively, than that of the wild-type seaweed (Figure 6A). Agar contents were positively correlated with the expression variations of *GlGT7s* and negatively related to those of *GlGH16s* (Table 4). The relative expression levels of four GlGT7 genes in cultivar 981 were all significantly increased compared with those in the other two cultivars; this was especially true for *GlGT7-4*, whose expression increased 6.02-fold relative to that of the wild-type thalli. In the cultivar Lulong No. 1, only *GlGT7-2* and *GlGT7-4* displayed expression levels higher than those of the wild-type thalli. In contrast to the *GlGT7s*, the *GlGH16* genes showed the response opposite to those of the agar; the *GlGH16* expression levels in cultivar 981 and Lulong No. 1 were all decreased compared with those in the wild-type thalli (Figure 6B).

Relations of agar and *GlGT7*/*GlGH16* transcription under abiotic stresses

To explore the relations between agar and the expression profiles of the *GlGT7* and *GlGH16* genes, agar accumulation and gene expression levels were assessed under heat and low nitrogen stresses. In this study, the agar contents did not significantly change, whereas the expression levels of several *GlGT7* genes fluctuated under moderate heat stress (Figure 7). *GlGT7-1*, *GlGT7-2*, and *GlGT7-5* were downregulated in response to heat stress compared with the CK, whereas *GlGT7-3* and *GlGT7-4* were upregulated—notably the expression of *GlGT7-4* increased 8.70-fold. Four *GlGH16* genes were all upregulated in response to heat stress; this was especially true for *GlGH16-2* and *GlGH16-3*, whose expression significantly increased 4.14- and 2.64-fold, respectively.

Compared with the unchanged agar content under heat stress, the agar content increased 115% under low nitrogen

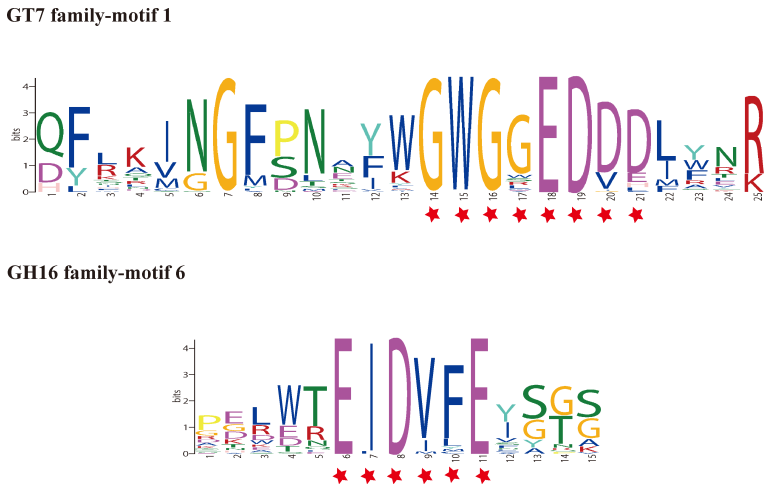

FIGURE 3
Conserved motifs of the GT7 and GH16 families corresponding to the proteins. The red asterisks indicate the catalytic sites.

conditions (Figure 7A). The expression changes of the *GlGT7* and *GlGH16* genes showed distinct expression change patterns under low nitrogen stress (Figure 7B). Low nitrogen stress stimulated the expression of four out of five *GlGT7* genes, especially *GlGT7-4*, whose expression increased 5.90-fold, whereas all four GlGH16 genes were significantly downregulated by low nitrogen stress.

Pearson correlation analysis was performed on the agar variation data and expression profile data of *GlGT7s* and *GlGH16s* under different treatments (abiotic stresses and for the

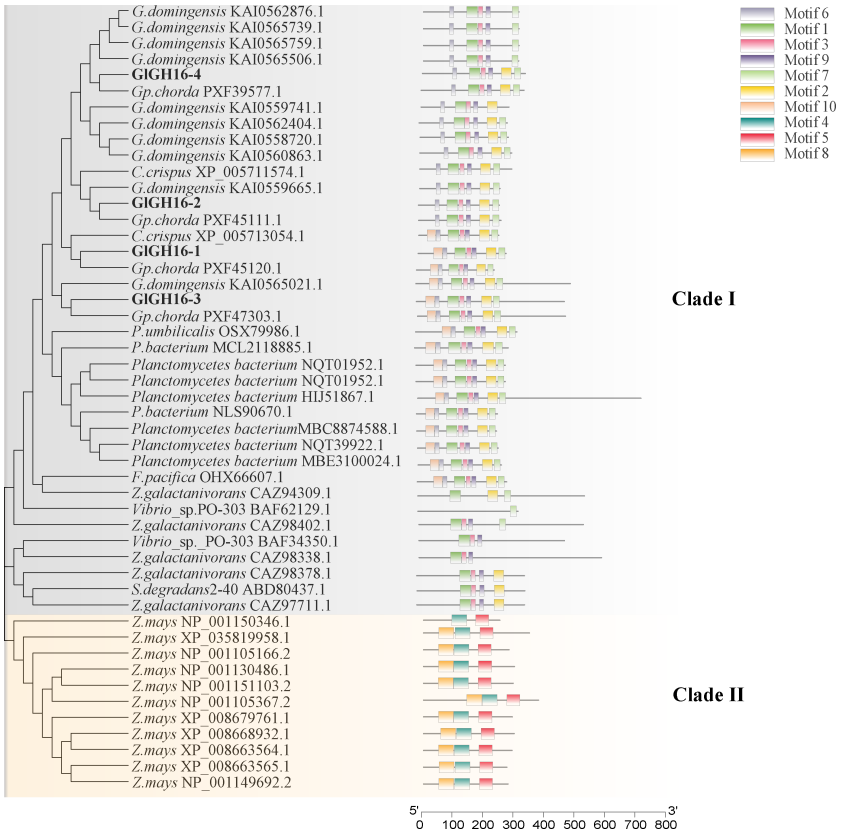

FIGURE 4
Phylogenetic tree and conserved motifs of GH16 members constructed based on the protein sequences using the maximum likelihood method. The bootstrap analysis was carried out with 1,000 replicates.

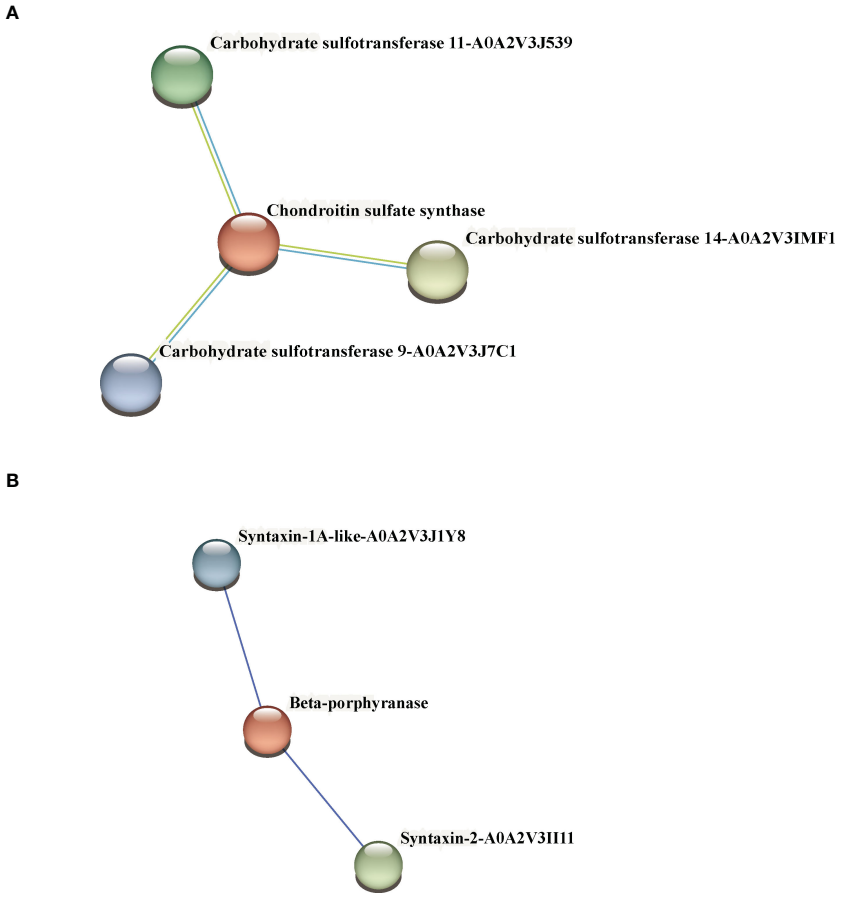

FIGURE 5
Protein–protein interactions among the GT7 **(A)** and GH16 **(B)** members with other proteins in the STRING database. The nodes with various colors represent the proteins that interact with the GT7 and GH16 members. The thickness of the lines represents the confidence scores of the protein–protein interactions.

different cultivars of *Gp. lemaneiformis*). The expression profiles of *GlGT7s* were positively correlated with agar contents; this was especially true for *GlGT7-2* and *GlGT7-5*, whose correlation coefficients were 0.720 and 0.903, respectively. *GlGH16s* were negatively correlated with agar content, and this was especially the case for *GlGH16-4*, which had a correlation coefficient of -0.811.

Cell morphology and fluorescence observations under abiotic stresses

After cultivation under heat and low nitrogen stresses, the thalli were observed with RCA_{120}-fluorescein, which can label β-1,4-galactosidic bonds (Figure 8). The thalli are cylindrical, and the algal cells are mainly concentrated around the circle. Stress

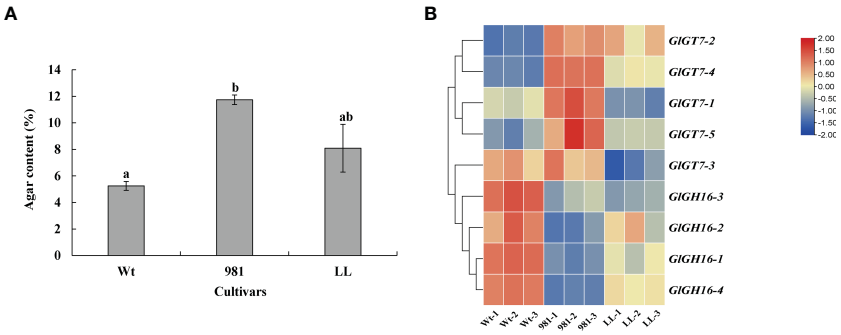

FIGURE 6
Agar contents **(A)** and expression levels of *GlGT7s* and *GlGH16s* **(B)** in the three cultivars of *Gracilariopsis lemaneiformis*. Wt, wild-type; 981, cultivar 981; LL, cultivar Lulong No. 1. The different lowercase letters represent significant differences.

TABLE 4 Pearson correlations between expression profiles of *GlGT7* and *GlGH16* genes and agar contents in different treatments.

	GlGT7-1	*GlGT7-2*	*GlGT7-3*	*GlGT7-4*	*GlGT7-5*	*GlGH16-1*	*GlGH16-2*	*GlGH16-3*	*GlGH16-4*
Agar	0.632	0.720*	0.233	0.625	0.886**	-0.450	-0.014	-0.142	-0.811**
Sig.	0.068	0.029	0.546	0.072	0.001	0.224	0.972	0.715	0.008

* and ** represent the significant and very significant correlation between gene expression profile and agar content, respectively.

treatments had no significant effect on the morphology of the thalli. However, β-1,4-galactan, the main structural component of agar chains, accumulated to significant levels after the thalli were exposed to heat or low nitrogen stresses.

Discussion

Evolution and function of GT7 members

The majority of agar is extracted from agarophytes, in which *Gracilaria*/*Gracilariopsis* contribute up to 60%–80% of the agar produced owing to the abundant agar yields and excellent agar properties. Some studies have explored the correlations between agar yield and the expression of genes, such as those that encode galactose-1-phosphate uridylyltransferase (Li et al., 2010), UDP-glucose pyrophosphorylase (Chang et al., 2014), phosphoglucomutase and phosphomannomutase (Tang et al., 2015), mannose-6-phosphate isomerase and GDP-mannose-3′,5′-epimerase (Hu et al., 2018), and UDP-galactose-4-epimerase (Lim et al., 2019). However, limited research has focused on the influence of GTs on sulfated polysaccharide synthesis. Ficko-Blean et al. (2015) suggested that at least two classes of GTs were required for the formation of α-1,3- and β-1,4-galactopyranosyl linkages in agar polysaccharides. In the present study, we identified five *GT7* genes in the *Gp. lemaneiformis* genome that may be involved in agar biosynthesis. Compact genes are typical for this group, as there is no intron in any of the *GlGT7* genes (Table 2), which is consistent with previous observations in other red algae (Brawley et al., 2017; Chen H. M. et al., 2022). Collén et al. (2013) proposed a possible evolutionary model of red algae: the ancestor of red algae was presumed to have a genome that was rich in introns, and genome reduction may occur in extreme environments, followed by genome expansion through the activity of transposable elements.

In our study, no GT7 sequences were found in red microalgae (Table 1), suggesting that the acquisition of these genes could have occurred after divergence from the red algae Cyanidiales (Lee and Ho, 2022). According to the phylogenetic tree (Figure 2), GlGT7 proteins clustered with GT7 proteins from other red algae, followed by those of marine organisms, such as *S. clava* and sea urchins, which are considered to have entered the eukaryotic lineage through secondary endosymbiosis (Keeling, 2010). Interestingly, the GT7 sequences from brown algae, diatoms, and animals clustered together as candidate genes involved in fucoidan biosynthesis; these genes are present in mammals, plants, and most bacteria. This may indicate the ancestral pathway of fucoidans (Han et al., 2019).

The putative chondroitin sulfate synthase was speculated to participate in carrageenan biosynthesis, as this enzyme has β-1,4-GalT activity, which is necessary for the biosynthesis of carrageenan and agar (Ho, 2020). Here, the catalytic pocket residue WGGEDDD was present in all GT7 proteins (Figure 3). This domain is conserved across most β-1,4-GalT-T7 proteins (Ramakrishnan and Qasba, 2010), demonstrating that the GT7 gene products in red seaweeds have the capacity for β-1,4-galactan transfer. To elucidate the possible regulatory mechanism of agar biosynthesis, using chondroitin sulfate synthase of *Gp. chorda* as the target

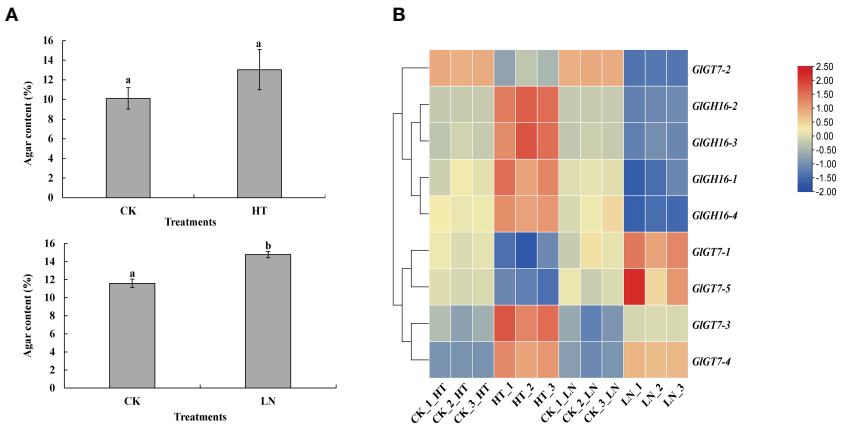

FIGURE 7
Agar content **(A)** and expression levels of *GlGT7s* and *GlGH16s* **(B)** under heat and nitrogen stresses. CK, the control treatment; HT, high-temperature treatment; LN, low-nitrogen treatment. The different lowercase letters represent significant differences.

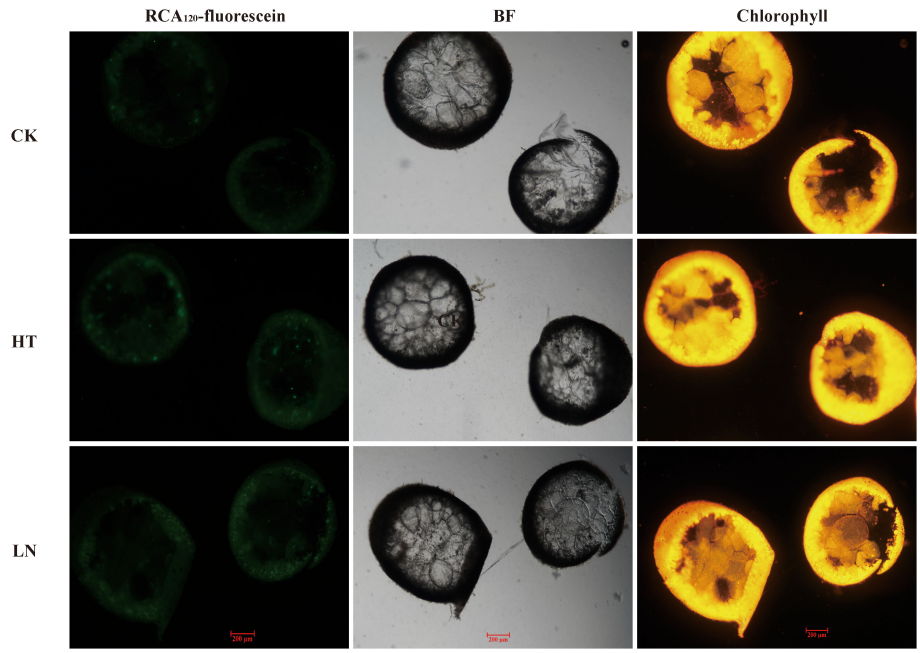

FIGURE 8
Cell morphology and fluorescence observations under abiotic stresses. RCA_{120}-fluorescein and chlorophyll were excited at 488 and 561 nm, and their corresponding emitted fluorescence was detected at 530/30 and 695/40 nm, respectively. BF, bright field; CK, the control treatment; HT, high-temperature treatment; LN, low-nitrogen treatment.

protein, we conducted online protein–protein interaction predictions (Figure 5A). Here, ST was predicted to interact with GT7 proteins. The enzymes such as ST, galactosyltransferase, and sulfurylases are the main classes responsible for sulfated polysaccharide formation (Garcia-Jimenez et al. 2020). ST can transfer the sulfate group from adenosine-3′-phosphate-5′-phosphate sulfate (PAPS) to an agar chain (Lee et al., 2017a). The physicochemical properties and biological activities of sulfated polysaccharides are affected by the pattern and number of sulfate moieties (Ho, 2015). Different life stages and living niches can lead to differential carrageenan qualities, among which ST plays an essential role (Helbert, 2017). The interaction between chondroitin sulfate synthase and ST provides a possible biosynthetic mechanism underlying agar chain production.

Due to the importance of sulfation in agar composition, to validate the possible biosynthetic mechanism of the agar chain, we conducted Y2H assays to validate the interactions between GlGT7s and GlSTs. Although GalTs are in the Golgi apparatus, at a location close to STs, they facilitate fast assembly of these nucleotide sugars into polysaccharide chains (Collén et al., 2013), and the STRING database revealed interactions between GT7s and STs in *Macaca mulatta*, *Latimeria chalumnae*, and *Ornithorhynchus anatinus*. In this study, one-to-one verifications revealed no interactions between these proteins (Supplementary Figure S3), which was consistent with that carbohydrate STs may independently transfer sulfate groups from PAPS to growing agar chains (Lipinska et al., 2020). However, due to the uncertainty of the assembled genome and the lack of experimental research on the agar synthesis mechanism, whether ST and GT function synergistically in the synthesis of agar chains remains to be further explored.

Evolution and function of GH16 members

Sulfated polysaccharides in red seaweeds provide important food and industrial resources. Furthermore, many marine bacteria produce specific GHs to break down these bioactive compounds for carbon use (Hehemann et al., 2012). As agar is the main component of the cell wall in agarophyte seaweeds, its remodeling is vital to regulate agar yield and quality, resulting in the changes in the growth and physiology of seaweeds.

To explore the function of GH16s in algae, *GH16* genes from the genomes of different species were retrieved (Table 1). Sequences of the proteins annotated as β-agarase, β-porphyranase, and carrageenase in CAZy were downloaded. In higher plants, no agarase-related sequences were retrieved from the *Arabidopsis thaliana* genome; however, sequences of 18 genes belonging to the GH16 family were identified at The Arabidopsis Information Resource (TAIR, (https://www.arabidopsis.org/browse/genefamily/index.jsp). In *Z. mays*, 11 GH16 sequences were found, but no similar conserved motifs that matched the sequences of red seaweeds and marine bacteria were detected, which proved the absence of agarase in higher plants. In brown algae, no GH16 sequences related to β-agarase, β-porphyranase, and carrageenase were found; in addition, XTH in *E. siliculosus* is absent, implying that brown algae have evolved different molecular mechanisms for cell wall stress relaxation and expansion (Michel et al., 2010). In red algae, four *GH16* genes in *Gp. lemaneiformis* were identified, and their structures are compact, with either no introns or only one intron within *GlGH16* genes.

Red seaweeds and marine bacteria have similar conserved motif compositions, indicating their possibility of origin and horizontal

gene transfer (HGT). Furthermore, the classic catalytic site EIDVFE is conserved in all red seaweeds, and this module is the classic site in AgaC-type proteins in some marine bacteria (Figure 3), suggesting that the GH16 sequences behave as does β-agarase acting on agar (Naretto et al., 2019). This type of GH is now widely present in marine bacteria and effectively breaks down the structure of G-L6S, the typical backbone of agar in agarophyte (Jiang et al., 2020; Rhein-Knudsen and Meyer, 2021). Because of the essential function of these agarases from bacteria, these GH16 sequences from agarophytes may play a role in cleaving and remodeling agar. Additionally, the protein interaction prediction results demonstrated that the majority of proteins interacting with GH16s are syntaxin proteins (Figure 5B); however, the interaction network between GH16 sequences and predicted proteins needs to be further verified.

Responses of *GT7*/*GH16* to light and phytohormone signals

Promoters are crucial elements that regulate gene expression, and their regulatory effect is the result of multilevel and multifactor interactions. Here, notwithstanding that the most predicted promoter elements responded to light, high light suppressed the expression of *GT7s*, which was consistent with the decrease in agar content (Supplementary Figure S1A). However, the reports about the effect of high light on agar accumulation were few. Several reports can be found about the influence of low light to agar production, e.g., low light conditions decreased the agar content (Lee et al., 2017b); optimum illumination decreased the agar content, whereas low light resulted in agar accumulation in *Gelidium coulteri* (Macler, 1986). Here, the expression levels of four *GlGH16* genes varied in response to high light, and the correlation of *GlGH16s* with the cell wall or agar production was unclear, although GH16s, just like XTH (family GH16), are putatively involved in wall remodeling and expansion (Henrissat et al., 2001; Cosgrove, 2005).

Plant hormones such as auxins, cytokinins, and ethylene are broadly defined in terms of their roles in growth and development, and some are considered to be "stress" hormones, such as brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), and ABA (Santner et al., 2009). Hormone signaling pathways play vital roles in cell wall formation to help resist stress (Wang et al., 2019). Gibberellin levels or signaling can alter cellulose and lignin accumulation in plant secondary cell walls (Wang et al., 2017). The downregulation of genes involved in BR synthesis leads to a reduction in cellulose and lignin contents (Hossain et al., 2012). Cellulose production in *A. thaliana* is regulated *via* BR signaling, with cellulose synthases (CESAs) modulated at both the transcriptional and posttranscriptional levels. The transcription factor BES1 is activated through BR signaling and can directly bind to CANNTG-E motifs in the promoters of CESA genes (Yennawar et al., 2006). Overexpression of *XTH1* can be regulated by hormones including ABA and auxins, resulting in a high cell wall density and thus enhancing tolerance to salt stress (Han et al., 2017). In this study, ABA and MeJA fluctuated the transcription of *GlGT7s* and *GlGH16s* (Figures 1B, C), and yet the agar production almost unchanged after the hormone treatments (Supplementary Figures S1B, C). This result might be partially caused by the treatment time or other reasons, and the potential role of GlGT7s and GlGH16s in agar metabolism after hormone application still needs further exploration.

Agar regulatory mechanisms of the three cultivars in response to abiotic stress

Agarophytes with higher agar yield and superior gelling properties are desired due to the growing demand for agar in the global market. The synthesis of sulfated polysaccharides (mainly agar, carrageenan, and alginate) can be altered by environmental changes and geographical variations (Lee et al., 2016; Véliz et al., 2019). Seasons and life cycle stages of red macroalgae differ from the contents and chemical structures of agar and carrageenan to adapt to the environment (Imbs et al., 2009; Ciancia et al., 2021). Agar can accumulate to resist stress conditions, including acidification (Lee et al., 2019), osmotic stress (Hu et al., 2018), high or low light (Silva-Brito et al., 2021), nutrient deficiency (Rocha et al., 2019), and temperature extremes (Tang et al., 2015). To understand the regulation of agar yield, we investigated the expression profiles of putative *GT7* agar synthesis-related genes and *GH16* cell wall-remodeling genes under high-temperature and low nitrogen stresses. Temperature is one of the most important factors affecting the growth and physiology of *Gp. lemaneiformis*. Agar yield increased with increasing water temperature (Figure 7A). Martin et al. (2013) found that the highest agar yield of *G. gracilis* occurred in summer. Friedlander (1991) reported that both the agar yield and the gel strength of *G. conferta* cultured in tanks were positively correlated with water temperature. However, in the present study, the agar content did not differ compared with that of the CK, which may be due to the differences in culture conditions.

Nitrogen can influence agar yield (Chen Q. L. et al., 2022). The agar contents of *Gracilaria sordida* was shown to be negatively correlated with the nitrogen level of the environment (Pickering et al., 1990). In our study, the agar content of *Gp. lemaneiformis* increased in response to low nitrogen stress (Figure 7A). Combining our expression profile data, we found that the *GlGT7* and *GlGH16* genes co-participated in moderating agar production. Among these *GlGT7* genes, *GlGT7-4* is highly correlated with agar variation. In the three cultivars of *Gp. lemaneiformis*, the agar content of cultivar 981 was higher than that of wild-type thalli, and this phenomenon is in accordance with the increasing expression of *GlGT7s* and the decreasing expression of *GlGH16s* (Figure 7B).

Agar chains are mainly composed of β-1,4- and α-1,3-galactose, which account for approximately 20%–30% of the algal dry biomass. RCA_{120} lectin recognizes carbohydrate chains with non-reducing terminal β-1,4-galactose specific to sulfated galactose (Wang et al., 2011). In this study, the fluorescence intensity of RCA_{120}-fluorescein was enhanced under heat or nitrogen stresses, the phenomenon of which is in accordance with the results of agar content and *GlGT7* expression (Figure 8). This result further verified the correlations between *GlGT7s* and agar accumulation.

Conclusion

This study first reports the identification of the most promising candidates for agar polymerization and degradation, and systematic bioinformatic analysis of GT7 and GH16 in *Gp. lemaneiformis*. Altogether, five *GlGT7* genes and four *GlGH16* genes were identified through whole-genome searching. The compact features, conserved catalytic pocket residues, and evolutionary relationships of the GT7 and GH16 sequences suggested that they function as β-1,4-galactosyltransferases and agarases, respectively. The protein interaction network revealed that GlGT7 can function cooperatively with ST in agar biosynthesis, although the Y2H assays did not support this. *GlGT7* and *GlGH16* gene expressions were sensitive to heat and low nitrogen stress. The fluorescence staining results indicated that β-1,4-galactose accumulated under heat and nitrogen stresses, which was consistent with the expressional variation of the *GlGT7s*. The positive relations of *GlGT7s* and the negative relations of *GlGH16s* with agar yield were confirmed by Pearson correlation analysis. Especially, *GlGT7-2*, *GlGT7-5*, and *GlGH16-4* could be used as molecular markers for indicating agar yield. This study therefore opens up more opportunities for others to confirm their involvement in the agar metabolism.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

Conceptualization, XS and QC; data curation, QC and XY; formal analysis, QC; funding acquisition, NX and XS; investigation, CH and QC; methodology, SL and QC; project administration, NX software, QC, XY and SL; writing—original draft, QC; writing—review and editing, SL, CH, NX, and XS. All authors have reviewed and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1156490/full#supplementary-material

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