1 **Characterization of mannitol metabolism genes in *Saccharina***
2 **explains its key role in mannitol biosynthesis and evolutionary**
3 **significance in Laminariales**

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29 Abstract

30 As a unique photosynthetic product in brown algae, mannitol exhibits high synthesis and
 31 accumulation in *Saccharina japonica*. Mannitol acts as a carbon-storage compound and is an
 32 osmoprotectant, imparting increased tolerance to osmotic stress. However, the underlying
 33 biochemical and molecular mechanisms in macroalgae have not been studied. Analysis of
 34 genomic and transcriptomic data has shown that mannitol metabolism in *S. japonica* is a circular
 35 pathway composed of four steps. In this study, one *S. japonica* mannitol-1-phosphate
 36 dehydrogenase (M1PDH2) and two mannitol-1-phosphatase (M1Pase) proteins were
 37 recombinantly expressed to analysis enzyme biochemical properties. RNA sequencing and droplet
 38 digital polymerase chain reaction were used to analyze the gene expression patterns of mannitol
 39 metabolism in different generations, tissues, sexes, and abiotic stresses. Our findings revealed
 40 insights into the mannitol synthesis pathways in brown algae. All genes were constitutively
 41 expressed in all samples, allowing maintenance of basic mannitol anabolism and dynamic
 42 maintenance of the "saccharide pool" *in vivo* as the main storage and antistress mechanism.
 43 Enzyme assays confirmed that the recombinant proteins produced mannitol, with the specific
 44 activity of SjaM1Pase1 being 1.8–4831 times that of other algal enzymes. Combined with the
 45 transcriptional analysis, SjaM1Pase1 was shown to be the dominant gene of mannitol metabolism.
 46 Mannitol metabolism genes in multicellular filamentous (gametophyte) and large parenchyma
 47 thallus (sporophyte) generations had different expression levels and responded differently under
 48 environmental stresses (hyposaline and hyperthermia) in gametophytes and sporophytes. The
 49 considerable variation in enzyme characteristics and expression of mannitol synthesis genes
 50 suggest their important ecophysiological significance in the evolution of complex systems
 51 (filamentous and thallus) and environmental adaptation of Laminariales.

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53 **Key words:** Mannitol metabolism, *Saccharina japonica*, Laminariales, Enzyme activity
 54 verification, RNA-sequencing, Droplet digital PCR

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Introduction

Mannitol is one of the most widely occurring sugar alcohols in nature and is produced by a variety of living organisms, including bacteria, fungi, terrestrial plants, and algae (Iwamoto and Shiraiwa, 2005; Rousvoal *et al.*, 2011). The presence of mannitol has been reported in primary endosymbiotic algae, such as those belonging to Chlorophyta (Dickson and Kirst, 1987; Dittami *et al.*, 2011) and a few species of Rhodophyta (Karsten *et al.*, 1997; Eggert *et al.*, 2006), as well as in secondary endosymbiotic Ochrophyta algae, such as brown algae (Ji *et al.*, 1980; Karsten *et al.*, 1991; Gylle *et al.*, 2009) and diatoms (Hellebust, 1965). As one of the main primary photosynthetic products and storage compounds in Laminariales (Kremer, 1980; Wei *et al.*, 2013; Xia *et al.*, 2016), mannitol can represent up to 15–26% of the dry weight of the organism (Black, 1948; Reed *et al.*, 1985). Moreover, mannitol fulfills key physiological roles, including protection against environmental stress, and can act as an organic osmolyte, compatible solute, antioxidant, or thermal protectant (Iwamoto and Shiraiwa, 2005; Patel and Williamson, 2016; Tonon *et al.*, 2017). Despite the importance of mannitol in the physiology of brown algae, information on its biosynthetic pathway is scarce; the functions of only a few pathway genes have been confirmed, and regulatory mechanisms are poorly understood, warranting further studies.

Saccharina is one of the most important macro-brown algae in the order Laminariales because of its considerably high biomass, dominance, and economic significance (Bartsch *et al.*, 2008). Asian countries have been cultivating *Saccharina* species since the early 1950s (Tseng, 1987), and presently, the annual production (7.7 million tons) of this species is the second highest among all aquaculture species (FAO, 2016). Recently, compounds such as mannitol derived from *Saccharina* have been widely used in health food, medicine, analytical chemistry, and scientific research (Belcher and Nutten, 1960; Saha and Racing, 2011; Varzakas *et al.*, 2012; Wakai *et al.*, 2013). The life history of *Saccharina* comprises several stages, which include single-cell (meiospore), multicellular filamentous (gametophyte, n), and large parenchyma individual (sporophyte, 2n) stages (Bartsch *et al.*, 2008). Its unique heteromorphic alternation of generations makes it quite different from its close relatives in the genus *Ectocarpus*, which lack the parenchyma stage (Cock *et al.*, 2014). Although brown algae are the only secondary endosymbiotic taxa with sophisticated multicellularity (Knoll, 2011; Niklas and Newman, 2013;

87 Cock *et al.*, 2014), the underlying regulatory mechanisms responsible for the structural difference
88 between filamentous brown algae (*Ectocarpus*) and heteromorphic haploid-diploid algae
89 (*Saccharina*) are not well understood. Moreover, Laminariales, like *S. japonica*, are dominantly
90 present in marine ecosystems of cold, temperate, and tropical coastal zones with harsh extremes
91 (Liu and Pang, 2015). Therefore, their major photosynthetic carbohydrates, such as mannitol, may
92 need to evolve distinctive adaptation or acclimation mechanisms.

93 Recently, the availability of the *Ectocarpus siliculosus* genome has paved the way for
94 studying the molecular basis of mannitol biosynthesis in algae (Cock *et al.*, 2010). The
95 biosynthesis involves two enzymatic steps; the first step is the reduction of fructose-6-phosphate
96 (F6P) to mannitol-1-phosphate (M1P) by mannitol-1-P dehydrogenase (M1PDH; EC 1.1.1.17),
97 and the second step is the hydrolysis of the phosphoric ester of M1P to produce mannitol by
98 mannitol-1-phosphatase (M1Pase; EC 3.1.3.22) (Iwamoto and Shiraiwa, 2005). Recent analysis of
99 the distribution of mannitol biosynthesis genes in algae revealed the presence of *M1PDH* and
100 *M1Pase* genes in these species (Tonon, 2017). There are one *M1PDH* gene, two *M1Pase* genes,
101 and one bifunctional *M1PDH/M1Pase* fusion gene (Liberator *et al.*, 1998; Groisillier *et al.*, 2014);
102 the members of Phaeophyceae, including Ectocarpales and Laminariales, possess *M1PDH* and the
103 haloacid dehalogenases (*HAD-M1Pase*). Previous phylogenetic analyses have suggested that these
104 genes were imported into brown algae by horizontal gene transfer from *Actinobacteria* (Michel *et al.*
105 *et al.*, 2010). Later, a more comprehensive assessment across various algal lineages proved that this
106 gene may have been present in nonphotosynthetic eukaryotic host cells involved in endosymbiosis
107 (Tonon, 2017). Native M1PDH and M1Pase activity has previously been characterized in cell-free
108 extracts from red algae, namely *Dixoniella grisea* (Eggert *et al.*, 2006), *Caloglossa continua*
109 (Iwamoto *et al.*, 2001; Iwamoto *et al.*, 2003), and *Caloglossa leprieurii* (Karsten *et al.*, 1997), and
110 from brown algae, namely *Spatoglossum pacificum*, *Dictyota dichotoma*, *Platymonas*
111 *subcordiformis*, and *Laminaria digitata* (Ikawa *et al.*, 1972; Grant and Rees, 1981; Richter and
112 Kirst, 1987); however, the genes encoding these enzymes have not yet been identified. Recent
113 structural and functional genomics research on *E. siliculosus* has analyzed mannitol metabolism,
114 and recombinant *Ectocarpus* M1PDHcat (EsiM1PDHcat; containing only the catalytic domain)
115 and M1Pase2 (EsiM1Pase2) have been characterized (Rousvoal *et al.*, 2011; Groisillier *et al.*,
116 2014; Bonin *et al.*, 2015). Determination of kinetic parameters indicated that EsiM1PDH1cat

117 displays higher catalytic efficiency for F6P reduction compared with M1P oxidation; EsiM1Pase2
118 was shown to hydrolyze the phosphate group from M1P to produce mannitol but was not active on
119 hexose monophosphates, such as glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), and
120 F6P. Gene expression analysis showed that transcription of these three genes from *E. siliculosus*
121 (filamentous brown algae) was under the influence of the diurnal cycle, and *EsM1Pase1* was
122 highly downregulated under hyposaline stress. However, these genes are still not well understood
123 in *S. japonica* (large parenchyma brown algae).

124 In this study, *MIPDH* and *MIPase* genes and their corresponding proteins, which are
125 involved in mannitol synthesis in *S. japonica*, were characterized. Gene expression analyses in
126 different *Saccharina* tissue structures and samples from different stages of life cycle (including the
127 sporophyte and gametophyte generations) and under abiotic stresses were conducted to understand
128 the mechanisms regulating mannitol metabolism genes in Laminariales. The results of this study
129 will expand our understanding of biosynthesis and degradation pathways and regulatory
130 mechanism of carbohydrates and their ecophysiological and evolutionary significance in
131 Laminariales and will provide a basis for artificial synthesis of mannitol *in vitro* and in transgenic
132 plants for conferring salt tolerance.

133

134 **Materials and methods**

135 *Algal sample collection*

136 Preserved *S. japonica* haploid gametophytes (male and female gametophytes) were available as
137 laboratory cultures and obtained from Laboratory of Genetics and Breeding of Marine Organisms.
138 Fresh samples of the *Saccharina* sporophytes (rhizoids, stipe, blade tip, blade pleat, blade base,
139 and blade fascia) were collected from east China (Rongcheng, Shandong Province, 37°8'53"N,
140 122°34'33"E). To detect the influences of abiotic factors, the female gametophytes and blade base
141 of sporophytes were cultured under different temperatures (control: 8°C; hyperthermia: 18°C),
142 salinities (control: 30‰; hyposaline: 12‰), and circadian rhythms (control: 30 $\mu\text{mol photons/m}^2\cdot\text{s}$
143 for 12 h; darkness: no irradiance for 12 h). These samples were used for RNA sequencing and
144 digital polymerase chain reaction (PCR) analysis.

145

146 *Sequence analysis*

147 Based on the analysis of the *S. japonica* genome (NCBI accession number JXRI000000000.1, and
148 resequencing genome data, Tao Liu, unpublished data) and transcriptome database (OneKP
149 accession number OGZM), the unigenes related to *MIPase* were verified using the BLASTX
150 algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignment was performed
151 with ClustalX (Thompson *et al.*, 1997). Sequence identities were calculated using the Clustal
152 Omega tool (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

153

154 *Purification of recombinant proteins expressed in Escherichia coli*

155 Genes were synthesized (Shanghai Xuguan Biotechnological Development Co. LTD) to construct
156 recombinant plasmids. *S. japonica MIPDH1* (*SjaMIPDH1*), *MIPDH2* (*SjaMIPDH2*), and
157 *MIPase1* (*SjaMIPase1*) were cloned in pET32a and *S. japonica MIPase2* (*SjaMIPase2*) was
158 cloned in pGEX-6p-1. These recombinant plasmids were transformed in *E. coli* BL21 (DE3) cells,
159 and the integrity of their sequences was verified by sequencing. Isopropyl
160 β -D-1-thiogalactopyranoside was added at concentrations of 0.5 mM to induce overexpression of
161 the target proteins, and the bacterial cultures were incubated for 16 h at 20°C. His-Binding-Resin
162 and GST-Binding-Resin were used according to the manufacturer's instructions
163 (www.yuekebio.com). The proteins were stored at -80°C.

164

165 *Assays for enzyme kinetics*

166 The *SjaMIPDH* and *SjaMIPase* activities of the purified enzymes were detected using previously
167 described methods (Groisillier *et al.*, 2014; Bonin *et al.*, 2015). For enzymatic characterization,
168 four sugar and polyol phosphoesters, which were considered potential substrates, were tested;
169 these substrates were MIP, F6P, G1P, and G6P (Sigma, St. Louis, MO, USA). The effects of pH
170 on the enzymatic activities of the purified proteins were determined in the range from 5.0 to 9.0
171 for *SjaMIPDH* and 5.5 to 10.5 for *SjaMIPase*. The effects of temperature on these enzymes were
172 determined over a range from 10°C to 60°C. The influence of NaCl was assessed over a final
173 concentration range from 0 to 1000 mM in the reaction mixture. Four replicates were analyzed for
174 each condition to ensure the consistency of the experimental results. In each case, boiled purified
175 recombinant enzyme was used as a negative control.

176

177 *RNA sequencing and Droplet digital PCR*

178 Total RNA was extracted using an improved CTAB method (Gareth *et al.*, 2006). Three
179 micrograms of RNA per sample was used as input material for the RNA sample preparation.
180 Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina
181 (NEB, USA) following the manufacturer's recommendations, and index codes were added to
182 attribute sequences to each sample. The clustering of the index-coded samples was performed on a
183 cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according
184 to the manufacturer's instructions. After cluster generation, the library preparations were
185 sequenced on an Illumina HiSeq platform, and 125-/150-bp paired-end reads were generated.
186 HTSeq v0.6.1 was used to count the read numbers mapped to each gene. The fragments per
187 kilobase of transcript per million mapped reads (FPKM) of each gene was then calculated based
188 on the length of the gene and the reads count mapped to this gene. Droplet digital PCR analysis
189 was conducted according to the previously described methods (Chi, *et al.*, 2017). The results
190 represent mean values of three replicates. All the data were subjected to one-way analysis of
191 variance followed by Student's *t*-tests.

192

193 **Results**

194 *Identification of brown algal mannitol metabolism genes*

195 Genomic sequencing data of *S. japonica* and transcriptomic data of 19 brown algal species
196 belonging in Laminariales, Ectocarpales, Desmarestiales, Dictyotales, Fucales, and Ishigeales
197 were identified by BLASTX analysis for mannitol metabolism genes. Two unigenes were found to
198 be related to *MIPDH*s in most species (named *MIPDH1* and *MIPDH2* according to the naming
199 convention for *E. siliculosus* *MIPDH*s), and only two Ectocarpales possessed the third *MIPDH*
200 like *E. siliculosus* (Table S1). The identity between *MIPDH1* and *MIPDH2* within species was
201 approximately 55.1–58.6%. *MIPase* genes showed more conservation in brown algae. All 19
202 species contained two homologs of *MIPases*, which were named *MIPase1* and *MIPase2*, and
203 their identity within species was 55.6–68.2% (Table S2). Only one *M2DH* was verified in 15
204 Phaeophyceae species, and the identity between different species was approximately 69.9–72.8%

(Table S3). All 19 species contained two homologs of *HKs*, which were named *HK1* and *HK2*, and their identity within species was approximately 61.0–67.4% (Table S4).

SjaM1PDH and *SjaM1Pase* cDNA sequences were deposited in GenBank with accession numbers MF706368, MF706369, MF440344, and MF465902. After aligning brown algal M1PDH amino acid sequences, conserved blocks A to E of PSLDRs (defined by Klimacek *et al.*, 2003) were identified, while M1PDH1 and M1PDH3 had an additional extension N-terminal domain (Figure 1A). The comparison of M1Pases confirmed the conservation of brown algal M1Pases, including the catalytic machinery and the Mg^{2+} cofactor binding site (Figure 1B).

213

214 *Characterization and confirmation of the functions of mannitol biosynthesis genes* 215 *from S. japonica*

216 Native *SjaM1PDH2*, *SjaM1Pase1*, and *SjaM1Pase2* were overexpressed in *E. coli* to characterize
217 the M1PDH and M1Pase activity of *S. japonica*. Although several attempts were made to
218 overexpress *SjaM1PDH1*, no protein was observed, similar to *E. siliculosus M1PDH1*. The
219 specificity of *SjaM1PDH2* was determined by assaying activity in presence of different potential
220 substrates. Reduction of F6P, G6P, and G1P and oxidation of M1P, F6P, G6P, and G1P were tested.
221 These experiments showed that the *SjaM1PDH2* enzyme only had reduction activity in the
222 direction of mannitol synthesis; no oxidation activity was detected (Table 1). In addition, the
223 reduction activity was also detected for other sugar substrates, indicating that *SjaM1PDH2* was
224 not specific for F6P (Table 2). Purified *SjaM1PDH2* had a specific activity of 0.36 $\mu\text{mol}/\text{mg}$
225 protein/min for F6P reduction with NADH at pH 8.0. This activity was in the range of those
226 measured for algal M1PDHs listed in Table 1. The *SjaM1Pase* activities were determined in 100
227 mM Tris-HCl buffer. The specific activity of *SjaM1Pase1* (144.93 $\mu\text{mol}/\text{mg}$ protein/min) was
228 significantly higher (almost 22 times) than that of *SjaM1Pase2* (6.60 $\mu\text{mol}/\text{mg}$ protein/min) in the
229 presence of 1 mM M1P (Table 3). The enzymatic reactions were performed in the presence of F6P,
230 G1P, and G6P to investigate the substrate specificity of *SjaM1Pases*. The phosphatase activity was
231 also detected for all sugar phosphates that were tested for each *Saccharina* protein. The activity of
232 *SjaM1Pase1* for such substrates was always lower than for M1P, as was observed for most brown
233 and red algae M1Pases (Table 4). However, *SjaM1Pase2* exhibited the highest phosphatase
234 activity in the presence of G1P, which was almost 1.1 times that of M1P, and was similar to the

235 substrate specificity of M1Pase from *Dixoniella grisea*. In addition, more than 90% enzymatic
236 activity was detected in SjaM1PDH2 and both SjaM1Pases after storage at 4°C for 72 h,
237 suggesting that the recombinant proteins were quite stable under the purification conditions tested.

238

239 Table 1. Comparison of the biochemical characterization of M1PDH and its activity determined in
240 brown and red algae.

	Specific activity $/V_{max}$ ($\mu\text{mol mg}$ $\text{protein}^{-1} \text{min}^{-1}$)	K_m (mM) M1P	k_{cat} (s^{-1})	pH optimum	Temp optimum (°C)	References
Brown algae						
<i>Saccharina japonica</i> (SjaM1PDH2)	0.36	2.02	0.44	8.0	40	This study
<i>Ectocarpus siliculosus</i> (EsM1PDH1cat)	46.2	0.19	32.3	7.0	30	Bonin <i>et al.</i> , 2015
<i>Spatoglossum pacificum</i>	0.46	0.28	NT	6.5	NT	Ikawa <i>et al.</i> , 1972
<i>Platymonas subcordiformis</i>	NT	5.5	NT	7.5	NT	Richter and Kirst, 1987
Red algae						
<i>Caloglossa continua</i>	228	0.15	NT	7.2	NT	Iwamoto <i>et al.</i> , 2003
<i>Caloglossa leprieurii</i>	0.26	1.4	NT	7.0	NT	Karsten <i>et al.</i> , 1997
<i>Dixoniella grisea</i>	0.06	1.18	NT	NT	NT	Eggert <i>et al.</i> , 2006

241 NT: Not tested.

242

243 Table 2. Summary of M1PDH activity determined in brown and red algae. Percentages of
244 maximum activities were calculated according to values reported in the different publications
245 indicated below.

	Fructose-6P	Glucose-6P	Glucose-1P	References
Brown algae				

<i>Saccharina japonica</i> (SjaM1PDH2)	100	98.7	96.3	This study
<i>Ectocarpus siliculosus</i> (EsM1PDH1cat)	100	0	0	Bonin <i>et al.</i> , 2015
<i>Spatoglossum pacificum</i>	100	1.3	0	Ikawa <i>et al.</i> , 1972
<i>Platymonas subcordiformis</i>	100	0	0	Richter and Kirst, 1987

Red algae

<i>Caloglossa leprieurii</i>	100	15.2	0	Karsten <i>et al.</i> , 1997
<i>Dixoniella grisea</i>	100	0.6	0	Eggert <i>et al.</i> , 2006

246

247 Table 3. Comparison of the biochemical characterization of M1Pase and its activity determined in

248 brown and red algae.

	Specific activity/ V_{max} ($\mu\text{mol mg}^{-1} \text{min}^{-1}$)	K_m (mM) M1P	k_{cat} (s^{-1})	pH optimum	Temp optimum (°C)	References
Brown algae						
<i>Saccharina japonica</i> (SjaM1Pase1)	144.93	0.83	128.02	8.5	50	This study
<i>Saccharina japonica</i> (SjaM1Pase2)	6.60	0.02	8.14	8.5	30	This study
<i>Ectocarpus siliculosus</i> (EsM1Pase2)	0.03	0.67	0.02	7.0	NT	Groisillier <i>et al.</i> , 2014
<i>Dictyota dichotoma</i>	10.22	0.83	NT	7.0	NT	Ikawa <i>et al.</i> , 1972
<i>Spatoglossum pacificum</i>	79.3	NT	NT	7.0	NT	Ikawa <i>et al.</i> , 1972
<i>Laminaria digitata</i>	1.12	NT	NT	NT	NT	Grant and Rees, 1981
Red algae						
<i>Caloglossa continua</i>	62.5	0.41	NT	7.4	NT	Iwamoto <i>et al.</i> , 2001
<i>Caloglossa leprieurii</i>	0.45	1.2	NT	7.2	NT	Karsten <i>et al.</i> , 1997

	<i>Dixoniella grisea</i>	13.14	6.3	NT	7.3	NT	Eggert <i>et al.</i> , 2006
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249 NT: Not tested.

250

251 Table 4. Summary of M1Pase activity determined in brown and red algae. Percentages of
252 maximum activities were calculated according to values reported in the different publications
253 indicated below.

	Mannitol-1P	Glucose-1P	Glucose-6P	Fructose-6P	References
Brown algae					
<i>Saccharina japonica</i> (SjaM1Pase1)	100	27.5	21.3	8.7	This study
<i>Saccharina japonica</i> (SjaM1Pase2)	89.9	100	69.6	34.8	This study
<i>Ectocarpus siliculosus</i> (EsM1Pase2)	100	0	0	0	Groissillier <i>et al.</i> , 2014
<i>Dictyota dichotoma</i>	100	0	0	0	Ikawa <i>et al.</i> , 1972
<i>Spatoglossum pacificum</i>	100	0	0	0	Ikawa <i>et al.</i> , 1972
<i>Laminaria digitata</i>	100	11.3	3.1	0	Grant and Rees, 1981
Red algae					
<i>Caloglossa continua</i>	100	0	0	1.8	Iwamoto <i>et al.</i> , 2001
<i>Caloglossa leprieurii</i>	100	NT	NT	32.4	Karsten <i>et al.</i> , 1997
<i>Dixoniella grisea</i>	18.2	100	5.0	11.6	Eggert <i>et al.</i> , 2006

254 NT: Not tested.

255

256 The activities of the three purified proteins under different conditions of temperature and pH
257 were determined to elucidate the biological characteristics of mannitol synthesis enzymes. Based
258 on these experiments, the optimum temperature for SjaM1PDH2 was found to be 40°C, whereas
259 the activities were 82% and 91% of the maximum activity at 30°C and 50°C. The optimum
260 temperature for SjaM1Pase1 was 50°C, with 95% and 83% of residual activity at 40°C and 60°C,

261 respectively. The optimum temperature for SjaM1Pase2 was much lower (30°C), with the activity
262 being less than 54% of the maximum activity at other temperatures (Figure 2A–C). The optimum
263 pH for SjaM1PDH2 was determined to be 8.0, with 51% and 56% of residual activity at pH 7.0
264 and 9.0, respectively. The optimum pH for both SjaM1Pase1 and SjaM1Pase2 was determined to
265 be 8.5, and 78% of the SjaM1Pase2 activity remained intact at pH values from 7.5 to 9.5, whereas
266 the corresponding activities for SjaM1Pase1 were less than 72% of the maximum activity (Figure
267 2D–F).

268 The purified recombinant SjaM1PDH2 and SjaM1Pase proteins exhibited typical
269 Michaelis-Menten kinetics when assayed with increasing concentrations of their substrates, and
270 apparent V_{max} and K_m values were determined from the Lineweaver-Burk plots (Figure 3). The K_m
271 value for SjaM1PDH2 was 2.02 mM, which was about 10-fold higher than that for
272 EsiM1PDH1cat (0.19 mM), indicating lower substrate binding capacity than EsiM1PDH1cat
273 (Table 1). The substrate binding capacity of two *S. japonica* M1Pases showed large differences:
274 the binding capacity of SjaM1Pase2 ($K_m = 0.02$ mM) was highest (almost 21–315 times) among
275 the M1Pases of other brown and red algae, almost 41 times higher than that of SjaM1Pase1 ($K_m =$
276 0.83 mM). Interestingly, both the SjaM1Pases showed much higher catalytic reaction efficiency
277 and catalytic rates than EsM1Pase2 (Table 3); SjaM1Pase1 had the highest k_{cat} value (almost
278 four-orders of magnitude higher than *Ectocarpus* M1Pase2).

279 The assays conducted to assess the influence of NaCl concentration on the activity of the
280 recombinant *S. japonica* proteins showed a dose-dependent effect on SjaM1Pases (Figure 4A, B).
281 A nearly linear decrease in activity was observed in the presence of NaCl at concentrations
282 ranging from 0 to 1 M. About 60% of the initial activity remained intact in the presence of 1 M
283 NaCl for both the enzymes, which indicated that these SjaM1Pases may resist high NaCl
284 concentrations. However, SjaM1PDH2 activity was not affected by NaCl addition (Figure 4C).

285

286 *Transcriptomic analysis in mannitol metabolism*

287 The expression of mannitol synthesis genes from *S. japonica* was determined by analyzing the
288 transcriptomic data of *S. japonica* (Tao Liu, unpublished data), as well as the transcriptomic data
289 of 19 Phaeophytes published by us in the OneKP database (www.onekp.com). All the
290 Phaeophyceae species expressed two *SjaM1Pase* genes, and more than 80% of Phaeophyceae

291 species expressed two *SjaMIPDH* genes.

292 To analyze the gene expression levels in different generations, tissues, sexes, and
293 environmental conditions of *S. japonica*, RNA-seq and droplet digital PCR experiments were
294 conducted. The regulation of metabolism in *Saccharina* is highly complex and can be divided into
295 four general levels. First, the genes (including different gene family numbers) were all expressed
296 constitutively. The gene expression levels were examined at various generations (female and male
297 gametophytes; sporophytes) and in different tissues (rhizoids, stipe, blade tip, blade pleat, blade
298 base, and blade fascia) of *Saccharina*. All four genes (including seven family numbers) were
299 detected in different samples (Figure 5). The FPKM value range of *Saccharina* transcripts was
300 approximately 1.2–300 (Table 5).

301 Second, most pathway genes encode reversible enzymes, which control the balance between
302 mannitol and F6P and dynamically maintain the “saccharide pool” *in vivo*. As a circular pathway,
303 mannitol metabolism is closely related to alginate, fucoidan, laminarin, and trehalose metabolism
304 through the intermediate product F6P. The first gene that transforms F6P to mannitol is *MIPDH*
305 (*MIPDH1* and *MIPDH2*), and the first gene that transforms into alginate and fucoidan is mannose
306 phosphate isomerase *MPI* (*MPI1* and *MPI2*) (Chi *et al.*, 2017). The expression levels of
307 *SjaMIPDH* and *SjaMPI* were opposite in most tissues (Figure 6). For example, the expression
308 level of *SjaMPI1* was 3.8 times that of *SjaMIPDH1* in blade fascia, whereas the expression level
309 of *SjaMIPDH1* was 2.7 times that of the *SjaMPI1* in stipe.

310 Third, enzyme activity and gene expression analyses were used to determine the dominant
311 genes in mannitol synthesis. The mannitol synthesis pathway includes two genes, *MIPDH* and
312 *MIPase*; the former catalyzes the reversible reaction, and the latter only catalyzes the positive
313 reaction. Regarding the two *MIPase* family members, the expression of *SjaMIPase1* in different
314 tissues was significantly higher than that of *SjaMIPase2* (the former was 2.0–19.4 times that of
315 the latter; Figure 7). The expressional levels of *SjaMIPase2* in different tissues were not
316 significantly different, but were adversely affected by *SjaMIPase1* under hyperthermia (18°C)
317 stress. *SjaMIPase2* was obviously upregulated 2.0 times, whereas *SjaMIPase1* was
318 downregulated 2.0 times.

319 Finally, the overall gene expression profiles differed between the gametophyte and
320 sporophyte generations. The average gene expression levels in the gametophyte and sporophyte

generations were further compared. Most genes (*SjaM1PDH1*, *SjaM1Pase1*, *SjaM2DH*, *SjaHK1*, and *SjaHK2*) were expressed at significantly higher levels in sporophytes than in gametophytes, with increases of 2.8-, 7.3-, 3.1-, 5.6-, and 4.7-fold, respectively (Figure 8A). However, the expression levels of *SjaM1PDH2* and *SjaM1Pase2* did not differ significantly between the two generations. In addition, there were no significant differences in the expression of all pathway genes in female and male gametophytes (Figure 8B). To complete these observations, further analysis was focused on changes in the expression of the genes using algal samples subjected to abiotic stress. Interestingly, these genes in different generations had the opposite response mechanisms to environmental stress. Under hyposaline conditions, the transcript levels of all genes were upregulated, exhibiting increases of 1.2–16.2-fold in gametophytes. In contrast, the expression levels of all genes were decreased 1.9–3.7-fold in sporophytes (Table 5). Furthermore, these variations followed a similar trend under hyperthermic stress. The transcript levels of all genes were upregulated (1.2–12.5 times) in gametophytes, whereas most genes (except *SjaM1PDH2* and *SjaM1Pase2*) were downregulated (1.4–3.0 times) in sporophytes (Table 5).

Table 5. The upregulated and downregulated ratios of the gene FPKM doses under hyposaline (12‰) and hyperthermia (18°C) conditions.

Genes	Sporophytes		Gametophytes	
	Control/ Hyposaline	Control/ Hyperthermia	Hyposaline/ Control	Hyperthermia/ Control
<i>SjaM1PDH1</i>	2.9	2.0	3.7	1.9
<i>SjaM1PDH2</i>	3.7	0.2	1.2	7.9
<i>SjaM1Pase1</i>	1.9	3.0	1.9	2.7
<i>SjaM1Pase2</i>	3.5	0.5	2.0	1.2
<i>SjaM2DH</i>	2.7	1.7	16.2	12.5
<i>SjaHK1</i>	3.6	2.9	4.6	1.3
<i>SjaHK2</i>	3.5	1.4	6.6	2.3

Discussion

Mannitol metabolism genes were constitutively expressed in brown algae, which satisfied the requirement for mannitol synthesis and accumulation for physiological

342 *metabolism*

343 Mannitol is the fundamental carbon-storage molecule and osmotic regulator in brown algae, and
 344 mannitol metabolism is one of the main traits that makes brown algae unique compared with other
 345 eukaryotic algae (Shao *et al.*, 2014). As a basal metabolic pathway, mannitol metabolism has
 346 fewer pathway steps (a total of four steps) and relatively few gene family members (1–2 copies of
 347 each gene in most brown algae). This mechanism differs obviously from some special elements,
 348 such as halogen metabolism (about 89 gene family members in *S. japonica*, unpublished data),
 349 and because of the single product (mannitol), this pathway does not contain complex synthesis and
 350 modification genes, such as glycosyltransferase, sulfurtransferase, and mannuronate
 351 C5-epimerases genes, as observed in alginate and fucoidan metabolism (which contain dozens to
 352 more than 100 genes) (Chi *et al.*, 2017). Therefore, analysis of mannitol metabolism is critical for
 353 the study of pathway regulation and environmental adaptation of brown algae.

354 Of the four pathway genes, *MIPDH*, *MIPase*, and *HK* genes have a “backup” gene (only
 355 *M2DH* has one copy). Moreover, about 80% of brown algal species in our analysis also harbor
 356 similar gene numbers. There are three *MIPDH* unigenes in *E. siliculosus*, but only two copies are
 357 found in the *S. japonica* genome. The transcriptomic data of 19 Phaeophyceae species also
 358 identified two *MIPDH* copies expressed in brown algae, except Ectocarpales. To confirm the
 359 *MIPDH* gene duplicates in brown algae, genome sequencing data of *Undaria pinnatifida* and
 360 *Costaria costata* (both belonging to Laminariales) were analyzed; these organisms were both
 361 found to possess *MIPDH1* and *MIPDH2*. The third unigene of *MIPDH* in Ectocarpales may be
 362 explained by the duplication of an *MIPDH1* sequence (Tonon *et al.*, 2017). Unlike in other
 363 primary endosymbiotic (e.g., red algae) and secondary endosymbiotic (e.g., Dictyochophyceae)
 364 algae, which have only one gene copy (Tonon *et al.*, 2017), brown algae have two *MIPase* genes
 365 (Table S2). Transcriptome analysis demonstrated that all four genes were constitutively expressed
 366 (at least at the RNA level) in brown algae. In addition, further investigations revealed that these
 367 genes are also constitutively expressed at various generations and in different tissues (Figure 5;
 368 Table S5). Even under dark conditions, all genes in the pathway were detected (the FPKM dose
 369 was 12.0–158.8, Table S5). During the development of *Saccharina*, zygotes divide continuously
 370 from a single cell to form thallus sporophytes, which exhibit consistent increases in length, width,
 371 and thickness. Mannitol is a central compound in plant carbon metabolism and in transportation

and distribution of the organic assimilate. Moreover, mannitol has important physiological functions, such as osmotic regulation, antioxidant, thermal protection, and respiration substrate (Schmitz, *et al.*, 1972; Davison & Reed, 1985). Therefore, these results suggest that brown algae consistently synthesize mannitol for carbon storage and energy.

Specific expression and regulation of the circular metabolism

Saccharina mannitol metabolism is a unique circular metabolic pathway, having few steps (four genes, seven copies), i.e., far less than other metabolic pathways such as alginate (six genes, 133 copies) (Chi *et al.*, 2017), fucoidin (nine genes, 71 copies) (Chi *et al.*, 2017), and trehalose (three genes, 11 copies) (Chi *et al.*, 2015; unpublished data) pathways. As the shared substrate, F6P can be used to synthesize other basic metabolites, such as alginate and fucoidan, and the regulation of mannitol synthesis genes in Laminariales could involve a complex integrated system. For example, the expression level of the first gene in mannitol, alginate, and fucoidan metabolic pathways was found to differ substantially among tissues (Figure 6), indicating that more F6P could be utilized in the synthesis of alginate and fucoidan. This is consistent with the finding that the accumulation of mannitol has an inverse relationship with that of alginate and fucoidan (Kaliaperumal and Kalimuthu, 1976; Ji, 1963). Mannitol metabolism can finely regulate the balance between mannitol and F6P and further affect other related pathways, such as alginate and fucoidan, which function to maintain the “saccharide pool” *in vivo*.

The analysis of enzyme activity and gene expression reveals that MIPase1 is the dominant gene of mannitol synthesis

As the enzyme catalyzing the positive reaction, MIPase may be the rate-limiting gene for mannitol synthesis. Although the two copies of MIPase were found to be expressed constitutively, there were major differences in their enzyme activities and expression patterns.

MIPase enzymes exhibit mannitol biosynthesis activity and have different biochemical properties. In brown algae, only one homolog of the *MIPase* gene (*MIPase2*) was confirmed to show activity in *E. siliculosus* (Groisillier *et al.*, 2014; Bonin *et al.*, 2015). No enzymatic studies have been conducted for *MIPase1*; only their nucleotide sequences were reported from *E. siliculosus*. In this study, SjaMIPase1 and SjaMIPase2 were both confirmed to have MIPase

activity and were assumed to be involved in the mannitol biosynthesis pathway in brown algae (Figure 2). The specific enzyme activity of SjaM1Pase1 was much higher (21.9 times) than that of SjaM1Pase2, and the former enzyme had a higher k_{cat} value (15.7 times) than that of the latter (Table 3). This indicated that SjaM1Pase1 had stronger catalytic ability than SjaM1Pase2. In terms of expression levels, SjaM1Pase1 also showed significantly higher expression than SjaM1Pase2 in all tissues (Figure 7). These results suggested that *SjaM1Pase1* was the dominant gene in mannitol synthesis and was mainly responsible for the synthesis and accumulation of mannitol, whereas *SjaM1Pase2* acted as a backup, having an important role under some conditions. For example, when *SjaM1Pase1* was downregulated under hyperthermic stress in sporophytes, *SjaM1Pase2* exhibited the opposite response mechanism under the same temperature stress.

All genes and copies (family members) of mannitol metabolism in *S. japonica* are involved in mannitol metabolism and show the characteristic of higher expression for the dominant gene. Multiple copies of genes are important for maintaining the normal biochemical metabolism in evolution. Mannitol metabolism gene duplication is an important biological mechanism to maintain the core photosynthetic carbon storage in *S. japonica*. Different copies of genes were still transcribed, and their gene products exhibited activity conducive to avoiding the influence of gene loss, recombination, and mutation on gene integrity and enzyme function. Interestingly, most genes were downregulated under hyperthermic stress in sporophytes, whereas *SjaM1PDH2* and *SjaM1Pase2* were upregulated (about 5.6 and 2.0 times, respectively; Figure 7, Table S5). These findings suggested that there were different mechanisms of regulation for backup genes.

Complex regulation of the expression of mannitol metabolism-related genes revealed the importance of this process in the evolution (filamentous and thallus) and environmental adaptation of brown algae

Laminariales algae have a heteromorphic haploid-diploid life cycle, with a macroscopic thallus sporophyte and microscopic gametophyte among different generations (Bartsch *et al.*, 2008). Most mannitol metabolism genes (except *SjaM1PDH2* and *SjaM1Pase2*) had significantly higher expression in sporophytes (filamentous generation) than in gametophytes (thallus generation; Figure 8A). This is consistent with the results of our mannitol content analysis in *Saccharina*, in which the dry weight of mannitol was found to be lower in gametophytes (male gametophytes

432 with 23.4% and female gametophytes with 24.6%) than in sporophytes (26.3%). These results
433 indicated that the sporophytes may have much higher ability to synthesize mannitol, potentially
434 because the sporophytes (large thallus with tissue differentiation) require more mannitol synthesis
435 and degradation to satisfy normal growth and development and adapt to the changing environment.
436 However, there were no significant differences in gene expression between female and male
437 gametophytes (Figure 8B). This is also consistent with our observation that the mannitol contents
438 in male and female gametophytes were 23.4% and 24.6%, respectively, suggesting that different
439 sexes of gametophytes had similar mechanisms for regulating mannitol metabolism.

440 The mechanisms regulating mannitol metabolism in response to environmental stress are
441 opposite between different generations. Under hyposaline stress, all genes in gametophytes were
442 upregulated, consistent with the results observed in *MIPase2* from *E. siliculosus* (Grosillier *et al.*,
443 the 2014), whereas the sporophyte genes were all downregulated (Figure 9A). Similar results were
444 observed under hyperthermic conditions (Figure 9B). Notably, mannitol is the main organic
445 osmolyte in *Saccharina* and most other brown algae, countering salinity stress and acting as an
446 antioxidant and heat protectant (stabilization of proteins) (Schmitz *et al.*, 1972; Davison and Reed,
447 1985; Iwamoto and Shiraiwa, 2005). The gametophyte stage is the stage that is most vulnerable to
448 external stress in the entire life-history of the organism (Ye *et al.*, 2015). Therefore, during this
449 stage, mannitol metabolism is increased to respond to stress, particularly as an osmotic adjustment
450 substance to quickly respond to hyposaline stress, maintain intracellular osmotic pressure, and
451 protect algae against hyperthermia-induced damage. Distinct regions of carbon sources and carbon
452 sinks exist along the thalli because of their large size and differentiation (Schmitz and Lobban,
453 1976; Buggeln, 1983). *Saccharina* involves transport from source to sink, i.e., from mature blade
454 areas, which produce a surplus of photoassimilates, to the intercalary carbon-requiring meristems
455 (Bartsch *et al.*, 2008). The imported organic compounds in the sink tissues are rapidly metabolized
456 and incorporated into polysaccharides and proteins (Schmitz and Lobban, 1976). The blade base
457 of the sporophytes (meristem) exhibited reduced mannitol metabolism, probably related to
458 reduction of mannitol degradation and incorporation, thereby decreasing transportation from
459 carbon sources to carbon sinks and maintaining mannitol accumulation in mature blades to adapt
460 to stress.

461

462 *Comparative analysis of RNA-seq and droplet digital PCR*

463 The droplet digital PCR method was used to verify the transcriptional sequencing results. The
 464 gene expression in gametophytes was analyzed under stresses (hyposaline and hyperthermia).
 465 Most of the genes were upregulated under these stress conditions, consistent with the results of
 466 RNA-seq. For example, *SjaMIPDH1* increased by 4.0 and 1.3 times under hyposaline and
 467 hyperthermia stresses, respectively, as determined by droplet digital PCR analysis, whereas
 468 RNA-seq analysis showed that *SjaMIPDH1* increased by 3.7 and 1.9 times under the same
 469 conditions (Table S6). RNA-seq and droplet digital PCR analyses yielded similar experimental
 470 results. Notably, droplet digital PCR was relatively inexpensive and had a shorter experimental
 471 time, whereas RNA-seq was beneficial for further analysis of the relationship between mannitol
 472 metabolism and other pathways.

474 *Characterization of mannitol synthesis genes in brown algae may provide more* 475 *effective genes for industrial production of mannitol and for plant genetic breeding*

476 Mannitol is a commercially valuable compound and is now widely used in the food,
 477 pharmaceutical, medical, and chemical industries (Saha and Racine, 2011; Song and Vieille, 2009).
 478 Most of the commercial production of mannitol is carried out by chemical hydrogenation of
 479 fructose, or it is extracted from seaweed (Saha and Racine, 2011; Xia *et al.*, 2016). Because of the
 480 problems associated with chemical production and extraction, microbial production has been the
 481 subject of significant interest in recent years (De Guzman, 2005; Saha and Racine, 2011). The
 482 most widely used *MIPase* gene from a protozoan parasite *Eimeria tenella* (Liberator *et al.*, 1998)
 483 had been expressed in cyanobacterium (Jacobsen and Frigaard, 2014), proteobacteria
 484 (Reshamwala *et al.*, 2014), and firmicutes (Wisselink *et al.*, 2005) and resulted in the
 485 accumulation of mannitol in the cells and in the culture medium. Interestingly, the substrate
 486 binding capacity of *E. tenella* MIPase ($K_m = 0.07$ mM) was lower than that of *SjaMIPase2* ($K_m =$
 487 0.02 mM), and its catalytic efficiency ($k_{cat} = 430$ s⁻¹) was much lower than that of *SjaMIPase1*
 488 ($k_{cat} = 6453.5$ s⁻¹), which indicated that the *MIPase* genes from brown algae may be better
 489 candidates for microbial production. Furthermore, mannitol biosynthesis is one of the most
 490 extensively tested targets for improving salt tolerance in plants by genetic engineering (Iwamoto
 491 and Shiraiwa, 2005). The high salinity tolerance of transgenic plants may due to the accumulation

of mannitol in the cells (Tarczynski *et al.*, 1992, 1993; Karakas *et al.*, 1997). The introduction of algal genes in transgenic plants may confer a greater advantage in terms of salt tolerance (Iwamoto *et al.*, 2001). The analysis of genes in the mannitol synthesis pathway can provide enzymes with higher substrate specificity and specific activity that would be useful for plant breeding research in the future.

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Figure legends

Figure 1. Structure-based sequence alignment of SjaM1PDHs (A) and SjaM1Pases (B). A. Alignment of the M1PDH from *Saccharina japonica* and some other brown algae species. Five conserved blocks as defined by Klimacek *et al.* (2003) for PSLDRs, named A to E, are represented above the conserved consensus sequence. B. Alignment of the crystallized HAD-like protein ATU0790 from *Agrobacterium tumefaciens* strain C58 (PDB code 2FDR) with orthologs of M1Pases from different organisms. Motifs I to IV, defined according to Burroughs *et al.* (2006), are represented above the conserved consensus sequence.

Figure 2. Enzymatic characteristics of recombinant SjaM1PDH2 and SjaM1Pases. A. Influence of temperature on SjaM1PDH2 activity. Enzyme activity at 40°C was set to 100%. $P < 0.05$ compared to 40°C. B. Influence of temperature on SjaM1Pase1 activity. Enzyme activity at 50°C was set to 100%. $P < 0.05$ compared to 50°C. C. Influence of temperature on SjaM1Pase2 activity. Enzyme activity at 30°C was set to 100%. $P < 0.01$ compared to 30°C. D. Influence of different pH on the activity of SjaM1PDH2. Enzyme activity at pH 8.0 was set to 100%. $P < 0.01$ compared to pH 8.0. E. Influence of different pH on the activity of SjaM1Pase1. Enzyme activity at pH 8.5 was set to 100%. $P < 0.01$ compared to pH 8.5. F. Influence of different pH on the

521 activity of SjaM1Pase2. Enzyme activity at pH 8.5 was set to 100%. $P < 0.05$ compared to pH 8.5.

522 The values represent means $\pm$ SD calculated from three assays.

523 Figure 3. Kinetics of enzymatic activity of SjaM1PDH2 (A), SjaM1Pase1 (B) and SjaM1Pase2

524 (C). The values represent means $\pm$ SD calculated from three assays.

525 Figure 4. Influence of NaCl concentration on the activities of recombinant SjaM1Pase1 (A),

526 SjaM1Pase2 (B) and SjaM1PDH2 (C). The values represent means $\pm$ SD calculated from three

527 assays.

528 Figure 5. Expression levels of mannitol metabolism genes in different generations (sporophytes

529 and gametophytes) and tissues (rhizoids, stipe, blade tip, blade pleat, blade base, and blade fascia).

530 All genes were constitutive expressed in different samples.

531 Figure 6. Expression levels of *SjaM1PDHs* and *SjaMPIs* in different tissues (rhizoids, stipe, blade

532 tip, blade pleat, blade base, and blade fascia). The expression levels of *SjaM1PDHs* and *SjaMPIs*

533 were opposite in most tissues.

534 Figure 7. Expression levels of *SjaM1Pases* in different tissues (rhizoids, stipe, blade tip, blade

535 pleat, blade base, and blade fascia) and under hyperthermic stress. The expression of *SjaM1Pase1*

536 was higher than that of *SjaM1Pase2* in all tissues. The expressional level of *SjaM1Pase2* was

537 adversely affected by *SjaM1Pase1* under hyperthermia (18°C) stress.

538 Figure 8. (A) Expression levels of mannitol metabolism genes at different generations

539 (gametophytes and sporophytes). Most of the gene transcript levels of sporophytes were much

540 higher than that of gametophytes ($P < 0.05$). (B) Expression levels of mannitol metabolism genes

541 at different sexse (female gametophytes and male gametophytes). No significant differences were

542 observed between these samples.

543

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Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8