CHANGES IN CELL WALL STRUCTURE DURING RHIZOID FORMATION OF *SILVETIA BABINGTONII* (FUCALES, PHAEOPHYCEAE) ZYGOTES[1].

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Running Title: Rhizoid formation of *Silvetia* zygotes

ABSTRACT

We examined the ultrastructure of the cell wall and immunolocalization of alginates using specific antibodies against M-rich alginates and MG blocks during rhizoid formation in fucoid zygotes, *Silvetia babingtonii*. The thallus region of 24-hour old zygotes had a cell wall made of three layers with different fiber distribution. In the 12-hour old zygotes, three layers in the thallus were observed before rhizoid formation, namely the inner, middle and outer layers. During rhizoid elongation, only the inner layer was apparent close to the rhizoid tip area. Immunoelectron microscopy detected M-rich blocks of alginate on the inner half of the cell wall, irrespective of the number of layers in the thallus and rhizoid regions. The MG blocks were seen to cover a slightly wider area than M-rich alginate blocks. It was suggested that parts of M in mannuronan would be rapidly converted to G, and MG-blocks are generated. Transcriptome analysis was performed using 3 -, 10 -, and 24 h-old zygotes after fertilization to examine the relationship between gene expression and alginate synthesis over time. The expression of two mannuronan C5-epimerase homologs that convert mannuronic acid into guluronic acid in alginates were upregulated or downregulated over the course of the examination.

Keywords: cell wall, cryofixation, immunoelectron microscopy, RNA-seq, *Silvetia babingtonii*, transmission electron microscopy

Abbreviations: AF, after fertilization; BAM, brown algae monoclonal antibody; MC5E, mannuronan C5-epimerase; RFCW, Rhizoid-flank cell wall; G, Guluronic acid; M, Mannuronic acid; RTCW, Rhizoid-tip cell wall; TCW, Thallus cell wall

INTRODUCTION

Many seaweeds grow in the intertidal region where environmental factors, including temperature, wave shock, intensity and quality of light, salinity, and drying, are changeable. The cell wall protects cells from physical shocks and unfavorable factors, such as herbivores. Therefore, seaweeds develop characteristic polysaccharides in the cell wall to enhance stress tolerance (Kloareg and Quatrano 1988, Popper et al. 2011). In brown algae, the main components of the cell wall are alginates, sulfated fucans, and cellulose. The mature sporophyte of laminarialean species contains three times more alginate than cellulose and sulfated fucan, whereas that of fucalean species contains more than twice the alginate compared to cellulose and sulfated fucan (Mabeau and Kloareg 1987, Skriptsova et al. 2012). Alginate consists of 1,4 linked mannuronic acid (M) and guluronic acid (G) residues. M-rich blocks produce flexible gels, while G-rich blocks cause rigidity. Accordingly, the M/G ratio relates to the properties of the cell wall, such as gel strength and stretching. Young sporophytes of *Costaria costata* show a high M/G ratio, and the ratio gradually decreases with thallus growth (Wu et al. 2014). The conversion of M residue into G residue is carried out by different mannuronan C5-epimerases (MC5E) expressed in different developmental stages and environmental conditions (Nyvall et al. 2003, Tonon et al. 2008). The alginate biosynthesis pathway in brown algae is not fully resolved, especially the first production of a polymer of M residues (mannuronan) (Michel et al. 2010). It is predicted that, shortly after synthesis, the alginate should consist of M-rich blocks; therefore, the M-rich region might hold the clues to the understanding of alginate synthesis.

There are some ultrastructural observations of the cell walls of the brown algae. The results showed that three or more cell wall layers were identified in the apical cell of *Sphacelaria rigidula* (Karyophyllis et al. 2000), the meristematic epidermal cells of *Dictyota dichotoma* (Evans and Horrigan 1972), zygotes of fucoid algae (Novotny and Forman 1975, Callow et al. 1978, Bisgrove and Kropf 2001), and the lateral cell wall in gametophytes and sporophytes of *Ectocarpus siliculosus* (Terauchi et al. 2016). The cell wall layers were distinguished by the distribution of the cell wall fibers and the matrix. Terauchi et al. (2016) clarified the quantification of the frequency of the electron-opaque fibrils (alginate fibrils) and their junction based on three-dimensional analysis using rapid freezing fixation and electron microscopy and tomography. The results showed that the alginate fibers were distributed more densely and in a more complex arrangement in the inner layer than in the middle layer.

Tip-growing apical cells of *Sphacelaria* showed a characteristic cell wall structure. In *Sphacelaria rigidula*, the apical dome of the tip cell had a two-layered cell wall. A very thin amorphous layer (L1) covered the cell, and the fibrous layer (L2) existed just beneath the L1 layer. Other parts of the tip cell and the thallus cells were covered with four layers of cell walls, with L3 and L4 added to L1 and L2 (Karyophyllis et al. 2000). In *Sphacelaria furcigera*, bud (branch) formation was observed (Burns et al. 1982a, b). The axial cell wall is composed of four layers (CW1–CW4), but the initial bud region is covered by two newly synthesized layers (CWO and CWI). Burns et al. (1982a) suggested that alginates are abundant in CW2, CW4, and CWI, while sulfated fucans were prominent in CW1, CW3, and CWO. Cell wall structure on the rhizoid tip in fucoid zygotes has been observed (Novotny and Forman 1975, Callow et al. 1978, Bisgrove and Kropf 2001). Deposition of the highly sulfated

105 fucan occurs in the tip growth region (Quatrano and Crayton 1973, Quatrano and Steven
106 1976, Callow et al. 1978, Brawley and Quatrano 1979, Torode et al. 2016). However,
107 the changes occurring in the cell wall structure during rhizoid development remain
108 unclear.
109 Different appearances in the multilayered cell wall could reflect differences in
110 cell wall components, which would produce different physicochemical properties. To
111 examine the distribution of polysaccharides on the cell wall, immunoelectron
112 microscopy using anti-alginate antibody (Chi et al. 1999, Nagasato et al. 2010, Terauchi
113 et al. 2012, Terauchi et al. 2016), an anti-fucoidan antibody (Nagasato et al. 2010), and
114 a binding assay using a conjugation of cellulase or alginate lyase-colloidal gold were
115 applied (Nagasato et al. 2010, Terauchi et al. 2016). At the light microscopic level,
116 metachromatic analysis using toluidine blue and periodic acid-Schiff (PAS) was
117 performed to detect alginate and sulfated fucan (Novotny and Forman 1975, Burns et al.
118 1982a). Recently, monoclonal antibodies that recognize the different structures of
119 alginate and sulfated fucan were produced, and immunofluorescence data on the
120 localization of these epitopes in some brown algal species were collected (Torode et al.
121 2016, Linardić and Braybrook 2017, Rabillé et al. 2019, Linardić et al. 2020).
122 Antibodies recognizing different epitopes showed spatial and temporal differences in
123 localization. By analyzing the localization of these antibodies through immunoelectron
124 microscopy, it was expected that we could obtain more useful information relating to
125 the multilayered cell wall of the brown algae.
126 In brown algae, there is little existing information on how the cell wall changes
127 to generate tip-growth from the thallus cell. To address this question, we observed cell
128 wall structure during rhizoid growth in the fucoid alga *Silvetia babingtonii* zygotes,

using the rapid freezing and freeze substitution technique for transmission electron microscopy (TEM). This method prevents loss of soluble polysaccharides, namely alginate and fucan. In this study, we focused on localization of the alginate by immunoelectron microscopy using antibodies that recognize different epitopes. Moreover, to examine the synthesis regulation of alginate on rhizoid growth. We carried out transcriptomic analysis using mRNA obtained from zygotes before and after germination.

MATERIALS AND METHODS

Materials

Mature *Silvetia babingtonii* (Harvey) E.A. Serrão, T.O. Cho, S.M. Boo & Brawley was collected from September to November 2006-2010 at Charatsunai, and 2018 at Botofurinai, Muroran, Hokkaido, Japan. The collected mature thalli were brought to the laboratory and wiped with gauze after washing with sterile seawater. The zygote preparation procedure was the same as in the previous paper (Abe 1970, Nagasato et al. 2010). They were placed in continuous light (30-40 μmol photons·m^{-2}·s^{-1}) at 18 °C overnight. Then, the thalli were transferred to a cool chamber at 4 °C for 2 h after darkening with aluminum foil. Adding fresh seawater induced the release of eggs and sperm into the medium. The age of the zygotes was counted from 1 h after pouring seawater. Zygotes were cultured in PES medium (Provasoli 1968) containing 40 μg·mL^{-1} chloramphenicol at 18 °C in continuous light.

Electron microscopy and Immunoelectron microscopy

152 Zygotes were cultured on gel support films (ATTO Co., Tokyo, Japan). Before
153 fixation, the films were cut into small triangle shapes less than 1cm. Rapid freezing and
154 freeze substitution procedures were performed according to previous reports (Nagasato
155 and Motomura 2002, Nagasato et al. 2010). Finally, the samples were embedded in a
156 Spurr low-viscosity resin (Polysciences, Warrington, USA). Ultrathin sections were cut
157 using a diamond knife on an ULTRACUT ultramicrotome (Reichert-Jung, Vienna,
158 Austria). Sections were picked up on a formvar-coated one-slot copper grid, stained
159 with EM stainer (Nisshin EM, Tokyo, Japan), an alternative stain for uranyl acetate
160 based on lanthanide salts, and then lead citrate (Reynolds 1963). Observations were
161 performed using a JEM-1011 electron microscope (JEOL, Tokyo, Japan).
162 Localization of the alginate was examined using antibodies against various
163 alginate blocks (Torode et al. 2016). Nickel grids with sections were incubated with
164 blocking solution (2.5% skimmed milk, 5% normal goat serum, and 0.05% NaN_3 in
165 PBS; 137 mM NaCl, 2.7 mM KCl, 4.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 30
166 min at 37 °C and then transferred to anti-alginate antibodies, BAM6 (M-rich epitopes)
167 and BAM7 (MG epitopes) diluted 1:5 with PBS for 1 h at 37 °C. The grids were then
168 washed with PBS three times and incubated for 1 h at 37 °C with a goat anti-rat IgG
169 (whole molecule) conjugated with 10 nm colloidal gold particles (Sigma, St. Louis,
170 Missouri, USA) diluted 1:20 with blocking solution. After washing with PBS and
171 distilled water, sections on the grids were treated with EM stainer in this case.
172
173 *Quantification of colloidal gold particles*
174 To quantify the distribution of alginate in the thallus cell wall (TCW) and
175 rhizoid-tip cell wall (RTCW), the distance between each colloidal gold particle and

176 plasma membrane was measured using ImageJ 64 (NIH, Bethesda, MD, USA).
177 Measurements were made within three replicate rectangle regions of cell wall. Each
178 rectangle stretched from the plasma membrane to the outer edge of the cell wall and had
179 0.75 μm wide sides over the plasma membrane and at the surface of the cell wall (Fig.
180 S1). Three different zygotes were measured (n=9). The thickness of the cell wall
181 differed in each sample; thus, the relative length between the plasma membrane and the
182 edge of the cell wall was set as 0-1. The distance of gold particles was assumed to
183 follow a beta distribution because the distance was continuous and ranged from 0 to 1.
184 Because the number of particles differed between the antibodies, the relative density of
185 distance was used to investigate the distributions. The kernel function and bandwidth
186 obtained the function to express the density. Assessment of the function was confirmed
187 by goodness of fit tests (GFT). Beta regression was used to investigate the effects of
188 antibodies (BAM6 and BAM7), and the cell wall regions (TCW and RTCW) used as
189 explanatory variables, on the distance, used as a response variable. Since interactions
190 between these three explanatory variables were unlikely, the analyses were conducted
191 on the respective explanatory variables. All statistical analyses were performed using R
192 software (version 4.0.2) (R Core Team 2020) with the libraries *fitdistrplus* (Delignette-
193 Muller and Dutang 2015) and betareg (Cribari-Neto and Zeileis 2009).
194
195 *RNA-seq and differential expression analysis*
196 Total RNA was extracted from zygotes at 3, 10, and 24 h after fertilization
197 using cetyltrimethylammonium bromide (CTAB) buffer (Pearson et al. 2006). The
198 extract was purified with a chloroform-isoamyl alcohol solution (24:1 v/v). After
199 precipitation with 3 M LiCl, contaminating DNA was removed with an RNase-free

DNase Set (Qiagen, Hilden, Germany). Total RNA samples with OD260/280 >1.8, OD260/230>2.0, were used for library construction.

The cDNA library construction, sequencing with Illumina Novaseq6000 (150 bp paired-ends), and generating clean reads by filtering raw reads were performed by Filgen Inc. (Nagoya, Japan). The clean reads were assembled using Trinity software (Haas et al. 2013). The assembled contigs were clustered and filtered out using CD-HIT-EST on CD-HIT suite server (Li and Godzik 2006) with 90% similarity, and the longest open reading frame (ORF) sequences were identified by TransDecoder (v5.5.0) (Haas et al. 2013). Sequences with fewer than 100 amino acids were discarded. The longest ORF sequences were annotated using the annotation pipeline on Maser (Management and Analysis System for Enormous Reads) platform (https://cell-innovation.nig.ac.jp) (Kinjo et al. 2018). Clean reads were aligned onto the longest ORF sequences and quantified using Salmon (Patro et al. 2017). Data normalization and identification of differentially expressed genes (DEGs) without replicates were performed using an iterative *deseq* pipeline in the TCC package (Sun et al. 2013). False discovery rates (FDR) ≤ 0.05 were considered as DEGs. The Z-scores based on the transcripts per million (TPM) were calculated using R software (3.6.2; http://www.R-project.org/), and the heatmap of genes for the biosynthetic pathway of alginates was drawn using the gplots (3.0.1.2) package. The Bioconductor package topGO version 2.38.1 (Alexa et al. 2006) were used for Gene Ontology (GO) enrichment analysis in R software. Over-representative GO terms in up-regulated or down-regulated genes in three comparisons (3 h AF vs 10 h AF, 3 h AF vs 24 h AF and 10 h AF vs 24 h AF) were identified by Fisher's exact test in combination with the 'weight' algorithm, using a minimum p-value cut-off of 0.01.

224

225 *Real-Time qPCR analysis*

226 To analyze the transcriptional expression of DEG, RT-qPCR was performed.
227 Synthesize of cDNA as template was performed by PrimeScript™ RT reagent Kit with
228 gDNA Eraser (TaKaRa, Shiga, Japan) using total RNA 50 ng in 20 μl reaction mixture
229 according to the manufacturer's protocol. Specific primers for each contig were
230 designed using Primer-BLAST (Table S1). The reaction mixture contained 5 μL SYBR
231 Premix Ex Taq II (TaKaRa), 0.4 μl specific primers (10 μM), and 1 μl cDNA template
232 in a total of 10 μl. qPCR was performed using Eco Real-Time PCR system (Illumina,
233 San Diego, CA, USA). After 95 °C for 30 s, the cycling parameters were 95 °C for 5 s,
234 and 60 °C for 1 min and were performed for 40 cycles. The melting curve analysis was
235 as follows: 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s. Transcript levels
236 corresponding to the comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1 genes
237 (putative mannuronan C5-epimerase) were compared with the transcript levels of the
238 actin gene (actin; comp21090_c0_seq1.p1). The RT-qPCR was performed in triplicate
239 using three RNA samples from each zygote at the ages of 3, 10, and 24 h. The
240 differences in mean relative expression level between the developmental hours (3, 10,
241 and 24 h) were examined by Tukey's multiple comparison test.

242

243 *Identifying mannuronan C5-epimerase from* S. babingtonii *assembled contigs and*
244 *molecular phylogenetic analysis*

245 A reciprocal blast search against 24,486 proteins of the brown alga *Ectocarpus*
246 *siliculosus* (http://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2) was also
247 conducted to identify genes for enzymes in the biosynthetic pathway of alginates using

BLAST+ version 2.7.1 (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST). To construct the MC5E phylogenetic tree, 36 sequences from *E. siliculosus* (https://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2), one sequence from *Saccharina japonica* (Inoue et al. 2016), and seven sequences from assembled *S. babingtonii* contigs were gathered. Three bacterial AlgG sequences from *Azotobacter vinelandii* (P70805), *Pseudomonas aeruginosa* (Q51371), *and P. fluorescens* (P59828) were added to the alignment file as an outgroup. Phylogenetic analysis of amino acid sequences of MC5E was carried out using web service NGphylogeny.fr (https://ngphylogeny.fr/) (Lemoine et al. 2019). The sequences were aligned using MAFFT with the iterative refinement method and the scoring matrix Blosum62 (Katoh et al. 2002). Ambiguously aligned non-informative regions were removed with BMGE (Criscuolo and Gribaldo 2010). Twenty sequences were removed due to insufficient sequence length or having identical sequences to the others from the phylogenetic analysis. The model test for maximum likelihood (ML) method was carried out by SMS (Lefort et al. 2017), and phylogenetic analysis was performed using PhyML (Guindon et al. 2010). The best-fit model was Blosum62 + G + I + F based on Akaike information criteria (AIC). Bootstrap values (Felsenstein 1985) were obtained from the analyses of 100 pseudoreplicates with Booster (Lemoine et al. 2018). Domain searches against the InterPro server (https://www.ebi.ac.uk/interpro/) were also carried out.

RESULTS

Immediately after fertilization, the *Silvetia* zygotes showed a spherical shape (Fig. 1a). Rhizoid formation was initiated at 12 h after fertilization (AF) (Fig. 1b). The first and second cytokinesis occurred at 20 h and 24 h AF, respectively (Fig. 1c, d). We

observed the ultrastructure and occurrence of alginate in cell walls of the zygotes at 12 h and 24 h AF. Three cell areas were observed in terms of cell wall structures (Fig. 1e): (i) the thallus cell wall (TCW), (ii) the rhizoid-flank cell wall (RFCW), and (iii) the rhizoid-tip cell wall (RTCW).

At 12 h AF, the TCW had an electron-opaque structure with parallel-oriented fibrils (Fig. 2a). In the RFCW, three layers with different contrasts, dark-light-dark, were visible (Fig. 2b). The RTCW was less stained and had irregularly oriented fibrils (Fig. 2c). The cell wall structure in the TCW at 24 h AF (Fig. 3a) was dependent on the cutting angle of sections relative to the plasma membrane. Obliquely cut sections in which the lipid bilayer of plasma membrane is unclear revealed three distinct layers with different opacities and fiber orientations (Fig. 3b). The cell wall was roughly divided into the inner layer (IL) having a rough fibrous net with porous, middle layer (ML) showing the fine fibrous net, and outer (OL) layers with a rough fibrous net. The width of the layers was calculated. Each layer was almost the same width representing 32, 37, and 31% of the whole cell wall in IL, ML, and OL, respectively. The percentage showed the mean value from the result by triplicated counting on each of three zygotes (Table S2). However, it was difficult to define each layer on a perpendicular section of the cell wall to the plasma membrane, because the difference of orientation among the three layers was hard to be clarified by narrow fiber spacing and high electron-opacity (Fig. 3c), as in 12 hour-old zygotes (Fig. 2a). In the RFCW, the outer and middle layers were hardly observed (Fig. 3d, e). First, the outer layer was not apparent at the thallus pole-facing side of the rhizoid tip (the site indicated by a double arrowhead in Fig. 3a) and the middle layer disappeared toward the tip proper. The remaining inner layer became thicker closer to the rhizoid tip (Fig. 3f). While well-structured in other

296 regions, the distribution of fibrils was irregular or wavy closer to the tip. The mucilage
297 material covered the outside of the rhizoid. The amorphous mucilaginous layer was not
298 included in the numbering of the cell wall layer in this study.

299 To locate alginate epitopes in the different cell wall regions, immunoelectron
300 microscopy using anti-alginate antibodies was used at 24 h AF (Fig. 4). Localization of
301 M-rich blocks with BAM6 antibody was evident in the inner half of the TCW, RFCW,
302 and RTCW (Fig. 4a, b, c). Fewer M-rich blocks were recognized in the RFCW (Fig. 4b,
303 Table 1), compared to the other two regions. With BAM7-conjugated gold particles,
304 MG-alginates appeared more uniformly and abundantly distributed in the cell wall than
305 M-rich blocks (Figs. 4d, e, f). The mean value of the number of gold particles using
306 BAM7 was shown in Table 1. The mean value of gold particle number with BAM7 was
307 higher as RTCW>RFCW>TCW. However, Tukey's multiple comparison tests showed
308 no significant difference in the number of gold particles with BAM6 and BAM7 among
309 TCW, RFCW, and RTCW.

310 To make comparisons across different antibodies, the width of the cell wall
311 between the plasma membrane and the edge of the cell wall was divided into ten
312 segments. Distributional patterns of gold particles in the cell wall over the relative
313 distance (0-1) were estimated by the function fitted at 10 segments (GFT, $P < 0.01$ for
314 all cases) (Fig. 5). The density of particles binding to BAM6 peaked at a distance of
315 approximately 0.3 in TCW, and it was flattened through 0.7 while decreasing. And it
316 dropped after 0.7 toward 1.0. The high density segments were at 0.1 to 0.7, which was
317 consistent with the region of the half of the inner layer and whole a middle layer. The
318 high density distribution with BAM7 in TCW was with a range of 0 to 0.7, which was
319 flatter than it with BAM6 in TCW. These results showed that the gold particles binding

320 to BAM7 were distributed closer to the plasma membrane compared with BAM6 in
321 TCW (beta regression, Z=-6.193, P<0.001). In RTCW, the density of particles binding
322 to BAM6 peaked at a distance of approximately 0.2, and became flatter between 0.5 to
323 0.9 after a peak. Compared with BAM6 in TCW, the high density segments were
324 shifted closer to plasma membrane (beta regression, Z=-6.726, P<0.001). In the density
325 distributions of gold particle with BAM7 in RTCW was flat with a range of 0 to 1.0,
326 and a plateau was observed from 0.1 to 0.4. There was no significant difference in the
327 distribution of density of gold particle between BAM7 in RTCW and BAM7 in TCW,
328 or BAM6 in RTCW.
329 To understand the genes involved in early development of the zygotes,
330 transcriptomic analysis was performed at three different developmental stages (3 h AF:
331 time of increased wall thickness, 10 h AF: time of initiation of rhizoid germination, and
332 24 h AF: time when the second cytokinesis finished and the rhizoid elongated) under
333 the current culture condition (Fig. S2). The timeline might change depending on the
334 cultural condition, so the event of characteristic morphological change in the early
335 development of fucoid zygotes was treated in this study. The short read data are
336 summarized in Table S3. *De novo* assembly produced 97,825 contigs. Filtering with
337 CD-HIT-EST reduced the size of the assembly to 78,268 contigs, and the 27,622
338 longest ORF contigs were detected by TransDecoder. A total of 11,087 contigs (40%)
339 were annotated by BLASTX with the UniProt database (Table S4), and 61-63% of reads
340 were mapped to the 25,890 longest ORF contigs. Then, differentially expressed genes
341 (DEGs) were detected for 107 contigs in 3 h AF vs. 10 h AF, 47 contigs in 3 h AF vs.
342 24 h AF, 125 contigs in 10 h AF vs. 24 h AF of the 25,890 contigs (Fig. S3; Tables S5-
343 S7). GO enrichment analysis indicates that sulfotransferase activity (GO:0008146) and

344 extracellular region (GO:0005576) were significant in the regulated genes at 24 h AF
345 (Table S8).
346 Reciprocal best-hit blast against *Ectocarpus siliculosus* reference proteins
347 recovered 39.6% (9,699) proteins in the group of longest ORFs in *S. babingtonii*
348 (Table S9). The BLAST search indicated that some alginate synthetic pathway
349 enzymes, two mannose-6-phosphate isomerase (*MPI*), one phosphomannomutase
350 (*PMM)*, one GDP-mannose 6-dehydrogenase (*GMD*), and seven *MC5E*, were conserved
351 in *S. babingtonii*. The expression patterns of the enzymes' genes are summarized in Fig.
352 S4. The expression levels of *MPI* (comp32248_c1_seq4.p1) and *GMD*
353 (comp32002_c0_seq6.p1) remained stationary throughout the experiment. Five *MC5Es*
354 (comp12459_c0_seq1.p1, comp21318_c0_seq3.p1, comp25876_c0_seq1.p1,
355 comp32408_c2_seq24.p1 and comp32951_c0_seq4.p1) were up-regulated as the
356 developmental stage proceeded, and one (comp33109_c0_seq1.p1) was slightly
357 suppressed in 24 h AF.
358 To clarify the molecular function, domain search and phylogenetic analysis of
359 MC5Es to clarify the molecular function were performed. The catalytic MC5E domain
360 and the signal peptide region were detected in four contigs (Fig. S5). The phylogenetic
361 tree of MC5E*s* shows five clades supported with high to moderate value (42-100%) and
362 seven MC5E*s* homologs of *S. babingtonii* were scattered within these five clades (Fig.
363 S5). Two *MC5E* homologs (comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1)
364 were detected as DEGs in the different developmental stages of the zygotes. RT-qPCR
365 was carried out on two contigs (comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1)
366 (Fig. 6). The expression level of comp32408_c2_seq24.p1stayed lower until 10 h AF

and increased eight times at 24 h AF. Comp33109_c0_seq1.p1 expression level declined to one-fifth at 10 h AF from 3 h AF.

DISCUSSION

Ultrastructural comparisons of cell walls of the rhizoid and thallus parts of fucoid zygotes have been reported (Novotny and Forman 1975, Vreugdenhil et al. 1976, Callow et al. 1978, Bisgrove and Kropf 2001). Previous studies showed that the cell wall of the thallus region consists of two layers, while the rhizoid cell wall had one or two layers (Novotny and Forman 1975, Vreugdenhil et al. 1976, Callow et al. 1978, Bisgrove and Kropf 2001). We observed three layers in the TCW in *Silvetia babingtonii* zygotes at 24 h AF. Occasionally, it was difficult to define the number of layers in the TCW, even in the same zygote. The cutting angle of the section affected the ultrastructural appearance of the cell wall structure (Terauchi et al. 2016). To avoid this, we prepared consecutive serial sections in each zygote and observed the cell wall structure. Bisgrove and Kropf (2001) mentioned a porous area adjacent to the plasma membrane within the thicker inner layer using chemical fixation. Although they did not count it as another layer, we considered that the porous area corresponds to the inner layer observed in the present study using rapid-freezing and freeze substitution (Fig. 3b).

Regarding the rhizoid cell wall, we observed that the two outer and middle layers from the cell wall of the thallus were not apparent along the rhizoid flank (RFCW), and only the inner layer surrounded the rhizoid tip. The inner layer became gradually thicker, and the cell wall fibrils showed an irregular orientation close to the rhizoid tip. This observation is the first to explain how cell wall structure changes

391 during rhizoid elongation in fucoid zygotes at the ultrastructural level. We considered
392 that the cleavage of the two outer layers occurred during rhizoid elongation, not during
393 its initiation. In the RTCW of the zygote at 12 h AF, namely, the stage of initiation of
394 rhizoid elongation, the cell wall layer could not be clearly seen (Fig. 2c); however, the
395 three layers were observed in the RFCW (Fig. 2b). At the beginning of tip growth, the
396 three layers were present; however, the outer two layers might break due to the
397 extension of the cell during further tip outgrowth. On the other hand, the inner layer
398 could be extended by the addition of new cell wall materials and remodeling.
399 Ultrastructural observation in the pollen tube growth of *Lycopersicum peruvianum* and
400 *Lilium longiflorum* showed that a part of the cell wall is broken on the germination
401 (Cresti et al. 1977, Miki-Hirosige and Nakamura 1982). During germination, new cell
402 wall subdivided into pectic outer layer and callosic inner layer is synthesized between
403 the intine and plasma membrane. The apical dome of pollen tube is always covered with
404 the callosic inner layer. The outer pectic layer breaks in lily pollen germination with the
405 intine. Newly synthesized polysaccharide is important for the tip growth in this case. In
406 the zygotes of *S. babingtonii*, the appearance timing of three layers is not known,
407 however, there is the possibility that a newly synthesized inner cell wall layer before
408 rhizoid formation would be essential for its elongation as in the pollen tube growth of
409 lily.
410 While, in the growing root hair of *Arabidopsis thaliana,* breaks of cell wall has
411 not been observed. The growing root hair cell wall shows a single layer, and the mature
412 one is surrounded by two layers with different textures and orientation of
413 microfilaments (MFs) (Akkerman et al. 2012). A similar pattern has been observed in
414 *Equisetum hyemale* (Emons and Wolters-Arts 1983). Expansin, a protein that promotes

415 cell wall loosening without degradation of cell wall polymers, is involved in root hair
416 initiation and elongation in many land plants (Cho and Cosgrove 2002, Cosgrove 2015).
417 The apical dome of the pollen tube consists of an outer layer with pectin and a
418 translucent inner layer with callose and cellulose in *A. thaliana* (Dardelle et al. 2010,
419 Chebli et al. 2012) and *Nicotiana tabacum* (Geitmann et al. 1995, Ferguson et al. 1998).
420 In the apical dome of the outer layer, highly esterified pectin deposits are created by
421 exocytosis, while localization of low-esterified pectin increases from the transition area
422 between the apex and axial region in *A. thaliana* (Chebli et al. 2012). De-esterified
423 pectin is produced by pectin methyl esterase from methyl esterified pectin, and the
424 degree and pattern of methyl-esterification of pectin influence the elasticity of the gel
425 (Willats et al. 2001). In the transition area, remodeling of the cell wall by accumulation
426 of callose and cellulose with de-esterification of pectin occurs, and this affects the
427 aperture of the pollen tube and promotes tip-growth in *A. thaliana* (Chebli et al. 2012).
428 We had expected that elongation of the rhizoid cell wall would be caused by
429 secretion of new cell wall material and remodeling of existing components in the
430 zygotes of *S. babingtonii*. We focused on the spatial distribution of alginate because it is
431 the main polysaccharide of the brown algal cell wall and affects its mechanical
432 properties. Therefore, immunoelectron microscopy using antibodies against the
433 different epitopes, BAM6 and BAM7, was conducted against the TCW and the RTCW.
434 There was no significant difference in the distribution of gold particles of BAM7
435 between the thallus and rhizoid regions. The high density of the gold particle with
436 BAM6 was observed close to the plasma membrane in the RTCW, which differs from
437 the situation in the TCW (Fig. 5). Alginate synthesis pathways in brown algae are not
438 fully understood. However, it is considered that alginate is initially synthesized as a

polymer of mannuronic acid (mannuronan) by alginate synthase, and a part of the mannuronic acid (M) is converted into guluronate acid (G) reside by mannuronan C5-epimerases (MC5Es) (Michel et al. 2010). In other words, the M residue-rich area would be considered to be the relatively newly synthesized alginate. However, this study showed that the distribution of gold particles binding to BAM7 was wider than BAM6 at the sites adjacent to the plasma membrane at TCW and RTCW. From this result, it was expected that parts of M in mannuronan would be rapidly converted to G after polymerization. In this study, the outer layer was labeled as neither BAM6 nor BAM7. In *Ectocarpus siliculosus*, the alginate lyase conjugated with gold particles binds to the entire cell wall but labeling with an anti-alginate antibody is limited to the inner two layers of the three (Terauchi et al. 2016). Therefore, it was considered that the outer layer of *S. babingtonii* zygotes contained alginate with different types of MG blocks, which are recognized by BAM6 and BAM7. We also performed immunoelectron microscopy using the anti-alginates BAM8 to BAM11 antibodies (Torode et al. 2016), however they were unable to label the outer layer of the cell wall in *S. babingtonii* zygotes.

Transcriptomic analysis was performed in 3-, 10-, and 24-hour-old zygotes of *S. babingtonii* and focused on gene expression related to alginate modification. The enrichment of term "sulfotransferase activity" (GO:0008146) and "extracellular region" (GO:0005576) indicate some cell wall components with sulfated fucan might be actively modified during rhizoidal growth. By constructing a *de novo* transcriptome assembly, we found homologous genes required for alginate synthesis and modification (Michel et al. 2010). Previous genomic or transcriptomic studies revealed that there were 31 MC5E genes in *Ectocarpus* (Michel et al. 2010), 105 genes in *Saccharina*

463 *japonica* (Ye et al. 2015), and 38 genes in *Sargassum thunbergii* (Liu et al. 2014). In
464 this study, 19 MC5E genes were identified by BLAST against the UniProt database
465 (Table S3), and the number was smaller than that of other brown algae. Because our
466 RNA-seq data are only from the early developmental stages (3-24 h AF), some MC5E
467 genes in *S. babingtonii* might not be expressed. Seven *S. babingtonii*'s MC5Es included
468 a common MC5E domain, but each MC5E included a variety of domain structures
469 similar to genes in *Ectocarpus* (Fischl et al. 2016). This protein domain diversity in the
470 MC5Es family contributes to the regulation of alginate composition and remodeling in
471 the cell wall. Within the data, two *MC5E* homologs (comp32408_c2_seq24.p1 and
472 comp33109_c0_seq1.p1) showed significantly different gene expression during
473 development of the zygotes. This is consistent with the different transcriptional patterns
474 of MC5E genes related to developmental stage, thallus region, and different phases of
475 an algal life cycle (Nyvall et al. 2003, Tonon et al. 2008, Fischl et al. 2016, Linardić et
476 al. 2020). Some reports show that the transcripts required for rhizoid formation were
477 synthesized prior to 5 h AF (Quatrano 1968, Kropf et al. 1989). From these findings,
478 comp33109_c0_seq1.p1 may support rhizoid germination, while it is also possible that
479 the gene may be related to the expansion of the cell wall and the formation of the
480 multilayer before rhizoid formation. The expression level of comp32408_c2_seq24.p1
481 increased in 24 h AF, suggesting that the gene would involve cell wall remodeling for
482 rhizoid elongation.

483 There are some reports showing cross-linking of polyphenol to alginate in the
484 cell wall in brown algae (Salgado et al. 2009, Deniaud-Bouët et al. 2014). It is
485 considered that phenol involves in the adhesion of the zygotes on the substratum and
486 rigidity of the cell wall. Vanadium-dependent bromoperoxidase is a candidate to

catalyze alginate-phenol complex. RNA-seq data in this study showed the 4 in 12 candidate *vBPO* genes were detected as DEG (Table S4, S7). During early development of *F. vesiculosus* zygotes, vBPO activity increases gradually after fertilization (Lemesheva et al. 2020). These results matched this study data. It is expected that how the alginate-phenol complex affects the property of the cell wall and involved in the morphogenesis becomes clear.

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700 Figure Legends

701 Fig. 1. *Silvetia* zygote development. (a-d) 30 min AF, 12 h AF, 20 h AF, 24 h AF,
702 respectively. Arrows and arrowheads indicate the first and second cytokinetic planes,
703 respectively. (e) Illustration indicating three cell wall regions observed by TEM; the
704 thallus cell wall (diagonal dot line area), the rhizoid-flank cell wall (striped area), and
705 the rhizoid-tip cell wall (plane area). Scale bars=50 μm (a-d).

706

707 Fig. 2. Ultrastructure of (a) TCW, (b) RFCW, and (c) RTCW in zygotes at 12 h AF.
708 Arrows show the outer edge of cell wall. Arrowheads show plasma membrane. IL; the
709 inner layer, ML; the middle layer, OL; the outer layer. Scale bars=500 nm.

710

711 Fig. 3. Zygotes at 24 h AF. (a) View of the *Silvetia* rhizoid. A double arrowhead
712 indicates the point from which the two outer layers start to be reduced. (b) Oblique
713 section of TCW. The cell wall consists of three layers. (c) Perpendicular section of
714 TCW. (d) RFCW. The outer layer is disappearing. (e) RFCW. The middle layer fades
715 away. (f) RTCW has only one layer, which is made of rough fibrils. Thick mucilage
716 covers the rhizoid. Arrows indicate the outer edge of cell wall. Arrowheads show
717 plasma membrane. IL; the inner layer, ML; the middle layer, OL; the outer layer. Scale
718 bars=10 μm (a), 500 nm (b-f).

719

720 Fig. 4. Immunogold localization of alginate within cell wall in the zygotes at 24 h AF.
721 (a), (b), (c) show BAM6 localization. (d), (e), (f) indicate BAM7 localization. (a) (d)
722 TCW, (b) (e) RFCW, (c) (f) RTCW. Arrows show the outer edge of cell wall. Insets

723 show the magnified images of dashed line rectangles. Arrowheads show plasma
724 membrane. Scale bars=500 nm (a-f), 100 nm (insets).
725
726 Fig. 5. The distribution of the relative density of relative distance of gold particles
727 binding to BAM6 and BAM7 in TCW and RTCW at 24 h-old zygotes. The relative
728 distance between plasma membrane and the cell-wall edge ranges from 0 to 1. The sum
729 of density is adjusted to 10. Grey lines show the function obtained by kernel function
730 and bandwidth.
731
732 Fig. 6. Quantitative analysis of putative *Mannuronan C5-epimerase* genes transcript
733 level in 3, 10, and 24 hour-old zygotes. (a) comp32408_c2_seq24.p1, (b)
734 comp33109_c0_seq1.p1. The relative gene expression was set at one at 3 h AF. The
735 error bars indicate SDs of three separate experiments. Tukey's multiple comparison tests
736 were conducted to investigate significant differences between the treatment hours.
737 Asterisks (*) indicate significant differences at $P < 0.01$.
738
739 Fig. S1. A diagram showing the measurement area of gold particles bound to antibodies
740 on the cell wall. Measurements are made within three replicate rectangle regions of
741 TCW, RFCW, and RTCW of the cell wall. Each rectangle stretches from the plasma
742 membrane to the outer edge of the cell wall and has 0.75 μm wide sides over the plasma
743 membrane and at the surface of the cell wall (Fig. S1). Three different zygotes are
744 measured (n=9).
745
746

747 Fig. S2. Three different developmental stages of the zygotes for transcriptomic analysis.
748 (a) 3 h AF, (b) 10 h AF, (c) 24 h AF. Arrows and arrowheads indicate the first and
749 second cytokinetic planes, respectively.
750
751 Fig. S3. MA plots (log expression ratio vs. mean average expression) comparing gene
752 expression during zygote germination. Differential expression genes were shown as
753 magenta points (TCC, FDR < 0.05). (a) 3 h AF vs. 10 h AF, (b) 3 h AF vs. 24 h AF, (c)
754 10 h AF vs. 24 h AF.
755
756 Fig. S4. Schematic representation of the alginic acid biosynthetic pathways with
757 expression levels of putative enzymatic genes during development of the zygotes. The
758 gene expression level was shown as TPM and Z-score. MPI: mannose-6-phosphate
759 isomerase, PMM: phosphomannomutase, GMD: GDP-mannose 6-dehydrogenase,
760 MC5E: mannuronan C5-epimerase. Asterisks indicate differential expression genes.
761
762 Fig. S5. Phylogenetic and putative protein structural analysis of mannuronan C5-
763 epimerases in *S. babingtonii*. Numbers indicate the bootstrap values in the maximum
764 likelihood analysis. Asterisks indicate differential expression genes. Each shape on the
765 schematic protein sequences indicates the catalytic MC5E domain (purple box), a
766 transmembrane domain (cyan oval), a signal peptide region (orange box), and the WSC
767 domains (green box).
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769