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Transcriptomic analysis under ethylene precursor treatment uncovers the regulation of gene expression linked to sexual reproduction in the dioecious red alga *Pyropia pseudolinearis* (Ueda).

Ryo Yanagisawa[1], Naoki Sekine[1], Hiroyuki Mizuta[1], Toshiki Uji[1]

[1] Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

Corresponding author:
Toshiki Uji
Division of Marine Life Science, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan
Tel/Fax: +81-138-40-8864
E-mail: t-uji@fish.hokudai.ac.jp

Running title: Transcriptomic analysis of sexual reproduction in dioecious *Pyropia*

Abstract

The marine red alga *Pyropia*, a genus of the class Bangiophyceae, includes dioecious and monoecious species; however, the molecular mechanisms underlying control of their sexual reproduction are still poorly understood. In the present study, we demonstrated that application of the ethylene precursor, 1-aminocylopropane-1-carboxylic acid (ACC), promoted the formation of spermatangia and parthenosporangia in male and female gametophytes, respectively, of the dioecious species *Pyropia pseudolinearis*. In addition, we determined expression profiles of ACC-responsive genes in the gametophytes during sexual reproduction using RNA-Seq and quantitative real-time PCR (qRT-PCR). Genes involved in the regulation of cell division and cell wall organization, such as high-mobility group (*PpHMG*) and glycosyltransferase family (*PpGT14*), were found to be up-regulated in male and female gametophytes treated with ACC. In addition, the relatively rapid ACC-response of the vesicular-trafficking-related genes, flotillin (*PpFLOT*), charged multivesicular body protein 5 (*PpCHMP5*) and peptidase family S8 (*PpS8*) was shown to occur during male and female sexual reproduction. Expression levels of these six genes in the monoecious species *P. yezoensis*, which are homologs to ACC-responsive genes in *P. pseudolinearis*, also increased in gametophytes treated with ACC. These findings could provide new insights into the ACC-regulation of the sexual life cycle in *Pyropia* species.

Keywords *Pyropia* · Red algae · Reproduction · 1-aminocylopropane-1-carboxylic acid · Plant growth regulator

Introduction

The red algae groups, are members of the Archaeplastida supergroup, together with glaucophytes and the Viridiplantae (green algae and land plants), and exhibits different types of sexual life cycle (Hawkes 1990). One of the multicellular red algae, the bangiophytes (members of the class Bangiophyceae), contain important marine crops such as the food "nori", including *Pyropia* and *Porphyra* (laver), and produce non-flagellated male gametes (spermatia) and female gametes (carpogonia) on the gametophytes. Fertilization occurs with the female gametes still retained on the gametophytes and successive cell divisions produce clones of the zygote called carpospores that grow into a filamentous sporophyte.

The bulk of our knowledge on the sexual reproduction of the red algae indicates that external factors such as light (intensity, photoperiod), temperature, and nutrients, regulate their sexual reproduction (Liu et al. 2017). Recent evidence also supports the involvement of plant growth regulators (PGRs) in the sexual reproduction of florideophytes which belong to second group of the multicellular red algae (Garcia-Jimenez and Robaina 2015). For example, polyamines, particularly spermine, favor the maturation of cystocarps that produces carpospores after fertilization and the liberation of spores in *Grateloupia imbricata* and *Hydropuntia cornea* (Guzman-Urióstegui et al. 2002, 2012; Sacramento et al. 2004). In *G. imbricata*, application of exogenous methyl jasmonate to thalli increases the number of cystocarps concomitant with upregulation of the expression of the *GiODC* gene encoding ornithine decarboxylase, which is a key enzyme in the metabolism of polyamines (Garcia-Jimenez et al. 2016; 2017). In addition, ethylene treatment regulated the expression of gene that encodes amine oxidase, which is involved in polyamine degradation during carposporogenesis in *G. imbricata* (Garcia-Jimenez et al. 2018). However, there is a paucity of knowledge about the involvement of PGRs in the regulation of gene expression during sexual reproduction in the bangiophytes.

Pyropia (formerly *Porphyra*) generally exhibits three patterns of sexual gamete formation. Monoecious species, such as *P. yezoensis* and *P. tenera*, produce gametes in mixed male and female mosaic regions on the same gametophyte (Kim 2011), while other monoecious species, such as *P. katadae*, have monoecious blades that are longitudinally divided into male and female halves with a clear line of demarcation between the two sides (Neefus et al. 2008). On the other hand, dioecious species such as *P. pseudolinearis* and *P. dentata*, develop separate male and female gametophytes (Kim 2011). The mechanisms regulating multiple patterns of gamete formation appear to

represent ancestral features of eukaryotic sexual reproduction, because the fossil records (1200 Mya) of *Bangiomorpha*, which closely resemble the extant Bangiales, provide the oldest evidence of sexually reproducing eukaryotes (Butterfield 2000).

In a previous study, application of the ethylene precursor 1-aminocylopropane-1-carboxylic acid (ACC) promoted the formation of spermatia and carpospores in the gametophytes of monoecious *P. yezoensis* and enhanced tolerance to oxidative stress (Uji et al. 2016). Gene expression profiles revealed up-regulation of genes involved in cell division and stress response in gametophytes treated with ACC. These results indicate that ACC plays an important role in the regulation of gamete formation and protection against stress-induced damage during sexual reproduction in monoecious *Pyropia*. In contrast, the molecular base underlying sexual reproduction in dioecious *Pyropia* remains unclear.

The purpose of this study was to examine the effect of ACC on promotion of the formation of reproductive cells in both male and female gametophytes of the dioecious *Pyropia* species, *P. pseudolinearis*, and to identify ACC-responsive genes in the gametophytes during sexual reproduction. In addition, we compared the transcript profiles of ACC-responsive genes between monoecious and dioecious *Pyropia*. These findings could provide new insights into the PGR-regulation of the sexual life cycle in red algae.

Materials and Methods

Algal materials

Vegetative male and female gametophytes of *P. pseudolinearis* are virtually indistinguishable by their appearance until gametophytes form the appropriate sexual organ, which hinders investigations of the time-dependent change in gene expression from a vegetative to sexual reproductive phase of separate male and female gametophytes. To overcome this, vegetative male and female gametophytes were produced via parthenosporophytes isolated from mature male and female gametophytes as described below. Mature gametophytes of *P. pseudolinearis* were collected from the coast of Hakodate (Hokkaido Prefecture, Japan) in December 2015. The female gametophytes bearing carposporangia were cleaned with sterilized seawater and paper towels to remove contaminants attached to the thalli. After washing, the thalli were cultured in glass flasks (150 mL volume) containing 100 mL medium of sterile vitamin-free Provasoli's enriched seawater (PES; Provasoli, 1968) to which 5.0 mg

germanium dioxide (GeO_2) and 0.1 g of the antibiotics penicillin, ampicillin, and streptomycin per L PES, had been added to inhibit the growth of diatoms and bacteria at 15 °C under a photoperiod regime of 12 h light:12 h dark with cool-white fluorescent lamps at 40 μmol photons m^{-2} s^{-1}. After one month, sporophytes (conchocelis) were isolated from the female gametophytes via carpospores released from carposporangia. Vegetative gametophytes of unidentified sex, which had been grown to 10-20 mm blade length after being obtained from sporophytes with conchosporangia via conchospores, were cultured separately to prevent fertilization with male and female gametes, under the same conditions described above. After three months of cultivation, parthenosporophytes were isolated from mature male and female gametophytes. Gametophytes from parthenosporophytes were examined to confirm that they were the same sex as the mother plants.

ACC treatments

To study the role of ACC in *P. pseudolinearis*, vegetative male and female gametophytes forming only vegetative cells (ca. 20 mm blade length) were cultured in glass flasks with 100 mL PES medium containing 0, 50, or 500 μM ACC (Sigma-Aldrich Co. LLC., St Louis, MO, USA.) under the same conditions described above. Five individual vegetative male or female gametophytes were transferred into each flask, keeping the sexes separate. After treatment with ACC for 10 d, the ratio of mature thalli to total thalli was determined by counting the number of gametophytes forming spermatangia in male gametophytes or parthenosporangia without fertilization in female gametophytes under a light microscope (Leica DM 5000 B). In addition, the thallus areas occupied by spermatangia or parthenosporangia were measured by using Image J (https://imagej.nih.gov/ij) and expressed as % of the total thallus area to define maturation degree, because *P. pseudolinearis* thalli produce sexual organs from the apical region to the basal region. The blade length of gametophytes was measured after 10 d treatment with ACC, and growth rate was calculated as the mean percentage of length increase per day using the following formula: growth rate = $[100(BLt - BL_0)/BL_0]/t$, where BL_0 = initial blade length, BLt = blade length at 10 d culture, and t = culture time. These experiments were repeated five times.

Identification of ACC-responsive genes

The vegetative male and female gametophytes (ca. 20 mm blade length), which did not bear spermatangia and parthenosporangia, were treated with 500 μM ACC. After 0, 3, 7, or 14 d treatment with ACC, the gametophytes were harvested and immediately frozen

with liquid nitrogen and stored at −80 °C until extraction of RNA for RNA-seq analysis. Vegetative mixed male and female gametophytes were cultured under the same conditions as described above. RNA extraction was performed as described by Uji et al. (2012), and the quantity and integrity of RNA samples were assessed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA libraries were constructed using 5 μg total RNA of high purity and integrity for each sample and then sequenced using an Illumina HiSeq 2500 by Eurofins Genomics, Tokyo, Japan. Raw reads generated by the Illumina HiSeq 2500 were used to obtain clean reads by removing adaptor sequences and low quality reads.

After quality filtering, *de novo* transcriptome assembly and differential expression analysis were performed using the CLC Genomics Workbench (CLC bio Inc., Kobe, Japan). To estimate the expression pattern of each transcript in different samples, high-quality reads from each sample were mapped on the final transcriptome assembly using CLC Genomics Workbench. The read counts was normalized by calculating number of reads per kilobase per million (RPKM) for each transcript in individual sample. The RPKMs ratio between control and treated samples was converted to fold-changes in the expression of each unigene. An absolute value of $\log_2$ (fold-changes of test sample/control) ≥ 2.5 was considered to indicate a significant difference in gene expression. To validate the results of RNA-seq and to compare the ACC-responsive genes between male and female gametophytes, quantitative real-time PCR (qRT-PCR) analysis was performed as described by Uji et al. (2012). The vegetative male and female gametophytes produced via parthenosporophytes for each sex (ca. 20 mm blade length) were separately cultured in the presence with 500 μM ACC. After 0, 3, 7, or 14 d treatment with ACC, they were harvested and immediately frozen with liquid nitrogen and stored at −80 °C until extraction of RNA for qRT-PCR. Total RNA (0.5 μg per reaction) was used for first-strand cDNA synthesis with a PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio). Real-time PCR was performed with an ABI Prism 7300 sequence detection system (Applied Biosystems/Life Technologies) and LightCycler® 480 System (Roche Diagnostics) under the following conditions: 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 31 s at 60 °C. For qRT-PCR analysis, the cDNA was diluted 10-fold and 1.0-3.0 μl of the diluted cDNA was used by SYBR® Premix Ex Taq™ GC (TaKaRa Bio) following the manufacturer's instructions. The *PpElf1* and *PpAct1* genes were used as an internal control to normalize the amount of mRNA in each reaction. The mRNA amounts of ACC-responsive genes were calculated based on a standard curve. The standard curve for each primer set was prepared by

plotting serial cDNA dilution (1:10–1:10^5) against CT (threshold cycle). The primer sequences used for qRT-PCR are listed in supplement Table 2. Three technical replicates were carried out for each sample (i.e. treatment). The data are represented as the mean ± SD for the biological duplicates for each treatment. To compare the transcript profiles of ACC-responsive genes between *P. yezoensis* and *P. pseudolinearis* gametophytes, sequences of six genes homologous to identified ACC-responsive genes in *P. pseudolinearis* were retrieved from the *P. yezoensis* genome sequence data (Nakamura et al. 2013). The conditions of culture and qRT-PCR in *P. yezoensis* were the same as described for *P. pseudolinearis*. The information on *P. yezoensis* genes and primer sequences used for qRT-PCR are listed in supplementary Tables 3 and 4.

Statistical analysis

Data are expressed as the means ± standard deviation (SD). Statistical analysis was performed using Student's *t* test to elucidate the difference between control (without ACC treatment) and ACC treatment. $P < 0.05$ was considered to be statistically significant for a mean difference.

Results

Effect of exogenous ACC on male and female gametophytes of *P. pseudolinearis*

To test whether ACC promoted sexual reproduction in male and female gametophytes of *P. pseudolinearis*, we firstly obtained vegetative male and female gametophytes from parthenosporophytes. After three months in isolation culture of vegetative gametophytes of unidentified sex, one gametophyte (male) bore spermatangia from the upper to middle parts and liberated spermatia, but the basal part of this gametophyte remained vegetative from which the cells developed into partheno-sporophytes directly. The other gametophyte (female), unfertilized by spermatia, bore parthenosporangia, which appeared to be similar to carposporangia, and liberated parthenospores from the entire gametophyte. The parthenospores also developed into parthenosporophytes after settlement onto the culture medium substrate. Both parthenosporophytes produced conchospores *via* conchosporangia, and liberated conchospores that developed into gametophytes. As a result of the sex determination, all gametophytes (100 individuals) from the male parthenosporophytes produced spermatia, and all gametophytes (100 individuals) from the female parthenosporophytes produced parthenospores where no fertilization occurred. These results indicated that the sex of gametophytes from

parthenosporophytes was the same as the sex of the mother plants. In the following experiments, the gametophytes that formed spermatangia or parthenosporangia were regarded as mature male or female gametophytes, respectively.

Seven days after treatment of vegetative gametophytes with ACC, the thalli at the apex and the margins of the upper regions were observed at the initial stages of spermatangium and parthenosporangium formation in the male and female gametophytes, respectively, which actively underwent cell division (Fig.1). The initial stages of spermatangia and parthenosporangia showed clear and slight discoloration, respectively. The formation rate of spermatangia in male gametophytes after 10 d treatment with 50 μM ACC or 500 μM ACC were 64.0% or 80.0%, respectively, compared to 20.0% in ACC-non treatment (Table 1). The formation rate of parthenosporangia in females was highest in the gametophytes after 10 d treatment with 500 μM ACC (68.0%), whereas no formation of parthenosporangia occurred in gametophytes treated without ACC (Table 2). The growth rate of male gametophytes cultured in medium containing 50 and 500 μM ACC was ca. 3.6%/d and 2.7%/d, respectively, whereas that of gametophytes grown without ACC exhibited a growth rate of ca. 11.5%/d (Table 1). The growth rate of female gametophytes cultured in medium containing 50 or 500 μM ACC was ca. 6.3%/d or 5.4%/d, respectively, whereas that of gametophytes grown without ACC was ca. 12.9%/d (Table 2). In addition, the thallus area occupied by spermatangia was significantly higher in the male gametophytes incubated with 500 μM ACC compared to those with 50 μM ACC, while the area occupied by parthenosporangia was not significantly different between female gametophytes treated with 50 or 500 μM ACC (Table 1 and 2). After 2 wk treatment with ACC, softening and loosening of cell walls of sexual organs at the apical regions of both male and female gametophytes was observed, concomitant with the release of the spermatia and parthenospores (Fig.1).

Identification of ACC-responsive genes in male and female gametophytes of *P. pseudolinearis*

The cDNA libraries were constructed from vegetative mixed male and female gametophytes, which were treated with 500 μM ACC for 0, 3, 7, and 14 d. The libraries were sequenced using the Illumina HiSeq 2500. In total, 50.61 million raw reads were generated from the four samples. The number of raw reads per sample ranged from 11.50 to 13.51 million, with an average of 12.65 million reads per sample and the number of clean reads in each sample ranged from 10.55 to 12.09 million reads. Using

the CLC Genomics Workbench, all clean reads were assembled into 22,523 unigenes, with an N50 length (the shortest sequence length at 50% of the genome) of 1069 bp (Table S1). A total of 11,485 unigenes (50.99%) were annotated in the Swiss-Prot protein sequence database (https://www.uniprot.org/). The number of mapped reads obtained from the four cDNA libraries from gametophytes treated with ACC for 0 (control), 3, 7, and 14 d were 6.1, 7.8, 6.9, and 6.7 million reads, respectively. The number of reads mapping to each contig was counted to obtain the gene expression data. Among differentially expressed genes, 398, 983, and 802 genes were significantly up-regulated (relative to the control) after 3, 7, and 14 d of treatment with ACC, respectively. On the other hand, 90, 87, and 92 genes were significantly down-regulated in vegetative gametophytes treated with ACC for 3, 7, and 14 d, respectively. Summary of transcriptome analysis and representative ACC-up-regulated genes are shown in Table 3 and 4, respectively.

Subsequently qRT-PCR analysis was performed to validate the RNA-seq data. Six genes with significant changes in expression between gametophytes treated with ACC for 0 (control), and 14 d were selected for qRT-PCR analysis. As shown in Fig. 2, the tested genes exhibited the same level of expression profile in both RNA-seq and qRT-PCR analysis, indicating that the expression dataset obtained by RNA-seq was reliable for the expression pattern of ACC-responsive genes. In RNA-seq, transcripts involved in the regulation of cell division, such as the genes involving cell division cycle protein 20 (*PpCDC20*), Type II DNA topoisomerase (*PpTOPII*), high-mobility group (*PpHMG*), and histone H4 (*PpH4*) genes, were found to be up-regulated in gametophytes treated with ACC. In addition, genes involved in cell wall organization, such as expansin (*PpEXPA*) and the glycosyltransferase family (*PpGT14* and *PpGT7*), also displayed up-regulation in gametophytes treated with ACC. Genes encoding proteases such as peptidase family S8 (*PpS8*), the vesicular-trafficking-related proteins such as vesicle-associated membrane protein (*PpVAMP*), flotillin (*PpFLOT*) and charged-multivesicular-body protein 5 (*PpCHMP5*), were also identified as ACC-responsive genes. In contrast, RNA-seq analysis revealed that transcripts of the gene encoding ACC synthase (*PpACS*) were not upregulated during sexual development.

Next, to compare the expression pattern of ACC-responsive genes between male and female gametophytes, real-time PCR was performed on vegetative gametophytes whose sex had been determined. The transcript level of genes related to cell division (*PpTOPII*, *PpCDC20*, *PpHMG*, *PpH4*) increased in both male and female gametophytes after 3 d ACC treatment (Figs. 3 and 4). The maximum expression levels

of these genes in male and female gametophytes were 4.9- to 61-fold (after 14 d ACC treatment) and 173- to 1357-fold (after 7 d ACC treatment) higher than the baseline (0 d ACC treatment). Transcription of the vesicular-trafficking-related genes *PpFLOT* and *PpCHMP5* was highly induced in both gametophytes within 3 d of exposure to ACC. The increase in the transcripts of *PpGT14*, *PpS8* and *PpEXPA* was also observed in both male and female gametophytes (Figs. 3 and 4).

The expression level of ACC-responsive genes was compared between the upper and lower parts of the male gametophytes, because male gametophytes after 14 d ACC treatment mainly formed spermatangia in the upper parts. The expression level of ACC-responsive genes in the upper parts was significantly higher than that in the lower parts, except for the vesicular-trafficking-related genes, *PpFLOT* and *PpCHMP5* (Fig. 5).

To determine whether dioecious *Pyropia* species exhibited transcriptomic regulation during sexual reproduction similar to that of monoecious *Pyropia* species, we investigated ACC-responsive genes in *P. yezoensis* gametophytes. The six tested genes, which were homologs to ACC-responsive genes in *P. pseudolinearis*, were also found to be up-regulated in *P. yezoensis* gametophytes treated with ACC (Fig. 6), an effect also associated with promotion of sexual reproduction. The transcription of genes encoding vesicular-trafficking-related proteins (*PyFLOT* and *PyCHMP5*) peaked at 3 d culture in the presence of ACC, following which they decreased, whereas, the transcription of cell-division-related genes (*PyHMG* and *PyCDC20*) and expansin (*PyEXPA*) peaked at 14 d exposure to ACC.

Discussion

In the present study, application of ACC induced the formation of spermatangia and parthenosporangia in male and female gametophytes of *P. pseudolinearis*, respectively. This finding is the first report that this plant growth regulator plays a key role in the control of both male and female sexual reproduction in gametophytes of dioecious red algae. In previous studies, polyamines were reported to accelerate the maturation of cystocarps in more advanced red algae, classified as the florideophytes (Guzman-Urióstegui et al. 2002, 2012; Sacramento et al. 2004), whereas treatment with polyamines had no effect on sexual reproduction in *Pyropia* species (data not shown). It is interesting that the regulator of sexual reproduction in bangiophytes is ACC rather than polyamines, since ACC and polyamines are derived from the same precursor, S-adenosylmethionine (Miyazaki and Yang 1987). Comparison of the molecular

mechanisms on the actions of these two plant growth regulators in bangiophytes and florideophytes could help to understand how sexual reproduction evolved in red macroalgae.

During sexual reproduction in *P. pseudolinearis* and *P. yezoensis*, packets of 64-128 spermatia are produced from vegetative cells of the gametophyte, while 16-32 carpospores are formed in the carpogonium by a series of mitotic cell divisions. The current study showed that transcripts of *PpH4*, *PpTOPII* and *PpCDC20*, three genes involved in cell division, accumulated in *P. pseudolinearis* gametophytes during spermatogenesis and parthenosporegenesis, processes which involve increased rate of mitotic cell divisions. Cell proliferation is regulated by histones that are universally conserved nuclear proteins and affect structural changes of chromosomes during mitosis, which can be classified into five subtypes: H1, H2A, H2B, H3 and H4 (Jasencakova et al. 2000). The expression of histone genes is correlated with cell proliferation and DNA replication (Meshi et al. 2000). For example, H4 transcripts increase in proliferating tissues such as shoot apical meristems during plant development (Brandstadter et al. 1994). In addition to chromatin dynamics, chromosome segregation during cell division is a highly regulated process that is critical for the production of normal cells, because chromosome segregation errors result in aneuploid cells with variable numbers of chromosomes (Potapova and Gorbsky 2017). TOPII are nuclear enzymes that can decatenate intertwined DNA molecules, and play a major role in chromosome condensation and segregation during mitosis at the metaphase/anaphase transition and at meiosis I in yeast and mammals (Cortes et al. 2003). Previous studies have shown that the activity of TOPII was correlated with mitotic activity in plant tissues (Xie and Lam 1994), and that TOPOII was involved in mammalian spermatogenesis (Har-Vardi et al. 2007). In addition to TOPII, CDC20 serves as a key cofactor for the E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) and also plays a pivotal role in normal chromosome segregation, ensuring the regulation of mitotic progression and mitotic exit (Kapanidou et al. 2017). Knock-down of *CDC20.1* and *CDC20.2* genes from *Arabidopsis* simultaneously by RNA interference (RNAi) resulted in severe delay in plant development and male sterility (Kevei et al. 2011). In *P. pseudolinearis*, the up-regulation of *PpH4*, *PpTOPII* and *PpCDC20* during sexual reproduction show that these genes play important roles in the tight control of cell cycle progress and cell proliferation to ensure production of spermatia and spores (carpospores/parthenospores).

The high-mobility group (HMG)-box domain was originally identified as the domain that mediated the DNA binding of the chromatin-associated HMG proteins of

the HMGB family (Stros et al. 2007). Among the HMGB family, 3xHMG-box proteins, containing three copies of the HMG-box domain, are conserved proteins common to lower and higher plants but absent from other organisms (Antosch et al. 2012). The *Arabidopsis* genome encodes two family members, termed 3xHMG-box1 and 3xHMG-box2. The transcription of *3xHMG-box1/2* genes is coupled with cell proliferation and the association of 3xHMG-box2 with mitotic chromosomes suggests that they play a role in the organization of mitotic chromosomes, while transcription of the *3xHMG-box1* is detected specifically at the 45S rDNA loci (Antosch et al. 2015; Pedersen et al. 2011). Interestingly, we found genes encoding 3xHMG-box proteins in red algae genomes such as those of *P. umbilicalis* (a bangiophyte) (Brawley et al. 2017) and *Gracilariopsis chorda* (a florideophyte) (Lee et al. 2018). The up-regulation of the genes for red algae 3xHMG-box proteins in the mature gametophytes of *P. pseudolinearis* and *P. yezoensis* suggests that red algae 3xHMG-box proteins may play an important role in the regulation of mitotic chromosomes, in common with the Viridiplantae.

In red algae, the spermatial attachment to the female trichogyne, the receptive protuberance of the carpogonium, is mediated by spermatial appendages and female specific lectin (Shim et al. 2012). Kim and Kim (1999) have shown that receptors of wheat germ agglutinin (WGA) bound to N-acetylglucosamine (GlcNAc) were localized in the spermatia, especially in the narrow region connecting the spermatial body and appendages, and moved towards the contracting area with the trichogyne, suggesting that WGA receptors may be necessary for gamete membrane fusion in the red alga *Aglaothamnion oosumiense*. In addition, WGA bound to the mucilage of carpospores in *Pyropia spiralis* is responsible for carpospore settlement to the substratum and the protection of the spores until cell wall deposition (Ouriques et al. 2012). In the present work, transcripts of *PpGT14* encoding to a homolog of N-acetylglucosaminyltransferase, which can transfer GlcNAc residue from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) to the acceptor substrate, was found to have high expression in both the male and female mature gametophytes. These results suggest that PpGT14 may be essential for the synthesis of WGA receptors in spermatial appendages and spore mucilage in mature gametophytes of *P. pseudolinearis*.

In the final reproduction stage of bangiophytes, the wall components around spermatia and carpospores dissolve and the outer wall around the packet breaks, releasing mature sexual cells. In this study, we observed the softening and loosening of cell walls in regions of from the mature gametophytes during the release of spermatia and parthenospores, as well as the high expression level of *PpEXPA* encoding a

homolog to expansin in the mature gametophytes. Expansins are cell-wall-loosening proteins proposed to weaken the non-covalent binding between wall polysaccharides and to make glucans on the surface of the microfibril more accessible to cell-wall-degrading enzymes (Cosgrove 2000). In addition to *PpEXPA*, expression of *PpS8* significantly increased in mature gametophytes. Peptidase family S8, also known as the subtilisin or subtilase family, is the second-largest family of serine proteases (Rawlings et al. 2014). Taylor et al. (1997) indicated the association of male reproductive organs with the serine protease, LIM9, in lily and suggested that one possible role for LIM9 might be in the formation of gaps in the microsporocyte primary cell wall and the loosening of the extracellular matrix. The increase in expansin and peptidase activity may be necessary for loosening of cell walls to liberate sexual cells in *P. pseudolinearis*.

Histochemical and lectin blot analysis of cell walls demonstrated that alterations in the composition of the outer wall occur only during sexual reproduction in both male and female gametophytes in *Bangia atropurpurea* (Cole et al. 1985) and *P. spiralis*, both bangiophytes (Ouriques et al. 2012). The correct composition and structure of the cell wall and plasma membrane are necessary for proper regulation of endocytosis and exocytosis (Battey et al. 1999; Toyooka et al. 2009). Flotillins are membrane-associated proteins that are thought to function in lipid raft-mediated signaling and clathrin-independent endocytosis (Otto and Nichols 2011). In addition, several publications have suggested involvement of flotillins in the release of exosomes (de Gassart et al. 2003; Phuyal et al. 2014), which correspond to the internal vesicles of multivesicular bodies (MVBs), and which are released into the extracellular space by fusion of MVBs with the plasma membrane (Lee et al. 2012). Exosome biogenesis and release are involved in the endosomal-sorting complex required for transport (ESCRT complex), which consists of different protein complexes, ESCRT-0, -I, -II, -III, and the associated AAA ATPase Vps4 complex (Hessvik and Llorente 2018). Of the ESCRT complexes, ESCRT-III, in conjunction with Vps4, appears to be the ‘machine’ that drives internal vesicle formation, while yeast ESCRT-III consists of the four core subunits, Vps32/Snf7, Vps2, Vps20, Vps24, and two ESCRT-III-associated subunits, Vps46/Did2 and Mos10/Vps60 (Peel et al. 2011). CHMP5 is a mammalian homolog of the yeast Vps60 and was identified as an essential component of the positive-selection machinery required for T-cell development (Adoro et al. 2017).

During sporogenesis in red algae, the presence of multivesicular body-like structures has been reported in *Ceramium rubrum* and *Gigartina teedii* (Chamberlain and Evans 1973; Tsekos and Schnepf 1991). MVBs probably contribute to the

formation of the cell wall through involvement with enzyme transport, related to modifications of the cell wall composition in land plants and green plants (Marchant and Robards 1968). During the formation of packets of spermatia in *Bangia*, many vesicles are produced by the inflated endoplasmic reticulum near the plasma membrane and small vesicles fuse to form large vesicles, termed spermatial vesicles, containing a fibrous polysaccharide (Cole and Sheath 1980). In the present study, we found that the abundance of transcripts of genes involved in cell division and cell wall organization peaked at 14 d ACC treatment in male gametophytes, whereas the expression levels of *PpFLOT* and *PpCHMP5* peaked at 3 d (i.e. before formation of spermatangia). The relatively early ACC-response of *PpFLOT* and *PpCHMP5* was also shown in female gametophytes. These findings suggest that early activity of endosomal trafficking may play a role in production of MVB-like structures and spermatial vesicles to organize the cell wall composition of gametes and spores in *P. pseudolinearis*. However, further investigations are required to characterize the function of PpFLOT and PpCHMP5 in the sexual reproduction of *P. pseudolinearis*.

A previous study of *P. yezoensis* had shown that gametophytes treated with ACC exhibited enhanced tolerance to oxidative stress, as well as overexpression of antioxidant- and chaperone-related genes (Uji et al. 2016). On the other hand, ACC treatment had little effect on the improvement of tolerance to hydrogen peroxide in *P. pseudolinearis* gametophytes (data not shown). Consistent with these results, ACC treatment did not significantly affect the expression of antioxidant- and chaperone-related genes in the *P. pseudolinearis* gametophytes. In contrast, the six genes tested, which are homologs to ACC-responsive genes in *P. pseudolinearis*, such as vesicular-trafficking and cell-division-related genes as well as cell-wall-related genes, were found to be up-regulated in *P. yezoensis* gametophytes treated with ACC. These results suggest that the regulatory mechanisms involving gametogenesis and sporogenesis may be similar with respect to sexual reproduction in dioecious and monoecious *Pyropia*.

In conclusion, we showed that exogenous application of ACC promoted the formation of spermatangia and parthenosporangia in male and female gametophytes of dioecious species of *Pyropia*. We also demonstrated the up-regulation of vesicular-trafficking and cell-division-related genes as well as cell-wall-related genes during sexual reproduction in *P. pseudolinearis*. This study provides insights that extend our understanding of the molecular mechanisms underlying transition from a vegetative to a sexual reproductive phase in *Pyropia* species.

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Figure legends

Fig. 1 Involvement of 1-aminocyclopropane-1-carboxylic acid (ACC) in the formation of spermatangia and parthenosporangia in male and female gametophytes of *P. pseudolinearis*. Vegetative cells of male gametophytes cultured in medium without ACC (a). The initial stage of spermatogenesis in male gametophytes after 7 d culture in

medium containing 500 μM ACC (b). Male gametophyte bear many spermatia in spermatangia after 14 d culture in medium containing 500 μM ACC (c). Vegetative cells of female gametophytes cultured in medium without ACC (d). The initial stage of parthenosporogenesis in female gametophytes during 7 d culture in medium containing 500 μM ACC. Arrows indicate the initial stage of parthenosporangia (e). Female gametophytes bear parthenospores in the parthenosporangia after 14 d culture in medium containing 500 μM ACC (f). Male and female gametophytes used for all experiments were cultured at 15 °C under 40 μmol photons m^{-2} s^{-1} with a light cycle of 12 h light and 12 h dark. Scale bar = 50 μm.

Fig. 2 Comparison of gene expression data obtained by RNA-seq and quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR analysis was performed on six selected 1-aminocylopropane-1-carboxylic acid (ACC)-inducible genes in gametophytes of *P. pseudolinearis* (white boxes). The levels of gene expression obtained from RNA-seq data are indicated by gray boxes (RNA-seq). The left and right side of the vertical axis indicate the expression level obtained from RNA-seq and that from qRT-PCR data, respectively. RNA samples were prepared from vegetative mixed male and female gametophytes collected after 0, 3, 7, or 14 d treatment with 500 μM ACC. Results are presented as relative expression compared with that in vegetative gametophytes not treated with ACC. Data from qRT-PCR are presented as means ± SD (n = 3). The primers used for qRT-PCR analysis are shown in Table S1.

Fig. 3 Expression analysis of ACC-responsive genes in male gametophytes of *P. pseudolinaris*. The vegetative male gametophytes were treated with 500 μM ACC. Results are presented as relative expression compared with that in vegetative gametophytes without exposure to ACC (0 d). Data from qRT-PCR are presented as mean ± SD (n=3). Asterisk indicates significantly different means ($P < 0.05$; Student's *t*-test) compared to vegetative gametophytes without ACC (0 d). The primers used for qRT-PCR analysis are shown in Table S1.

Fig. 4 Expression analysis of ACC-responsive genes in female gametophytes of *P. pseudolinaris*. The vegetative female gametophytes were treated with 500 μM ACC. Results are presented as relative expression compared with that in vegetative gametophytes without ACC (0 d). Data from qRT-PCR are presented as mean ± SD (n=3). Asterisk indicates significantly different means ($P < 0.05$; Student's *t*-test) compared to vegetative gametophytes without ACC (0 d).

Fig. 5 Comparison of ACC-responsive genes between the lower and upper parts of mature male gametophytes. The male gametophytes were harvested after 14 d exposure to 500 μM ACC treatment and total RNA was separately extracted from lower (LP) and upper parts (UP). Results are presented as relative expression compared with that in the lower parts. Data from qRT-PCR are presented as mean ± SD (n=3). Asterisk indicates significant difference at $P < 0.05$ between the lower and upper parts (Student's *t*-test).

Fig. 6 Expression analysis of ACC-responsive genes in gametophytes of *P. yezoensis*. The vegetative gametophytes were treated with 500 μM ACC. Results are presented as relative expression compared with that in vegetative gametophytes without ACC (0 d). Data from qRT-PCR are presented as mean ± SD (n=3). Asterisk indicates significantly different means ($P < 0.05$; Student's *t*-test) compared to vegetative gametophytes without ACC (0 d). The primers used for qRT-PCR analysis are shown in Table S3.

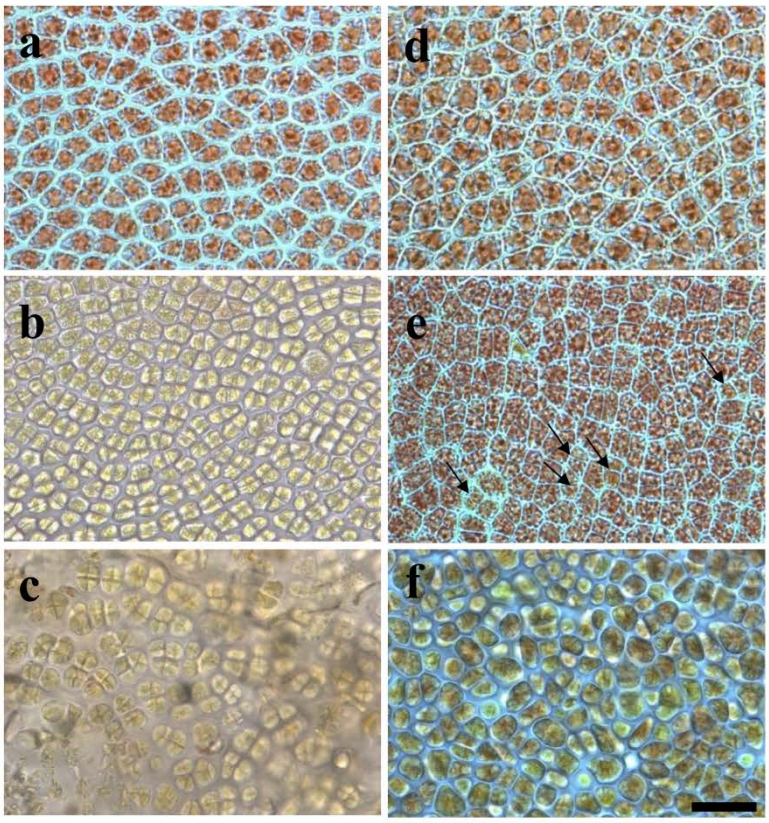


Fig. 1

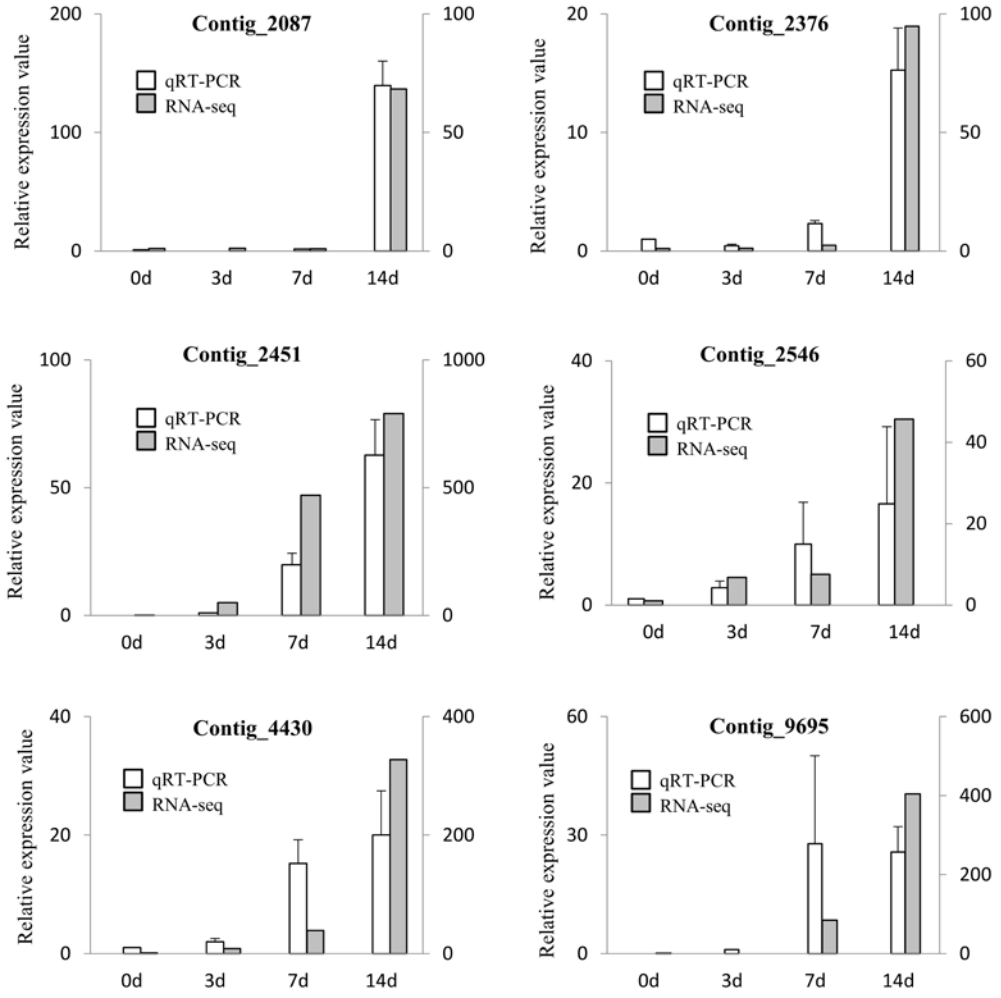

Contig_2087
Contig_2376
Contig_2451
Contig_2546
Contig_4430
Contig_9695
qRT-PCR
RNA-seq
Relative expression value
0d
3d
7d
14d

Fig. 2

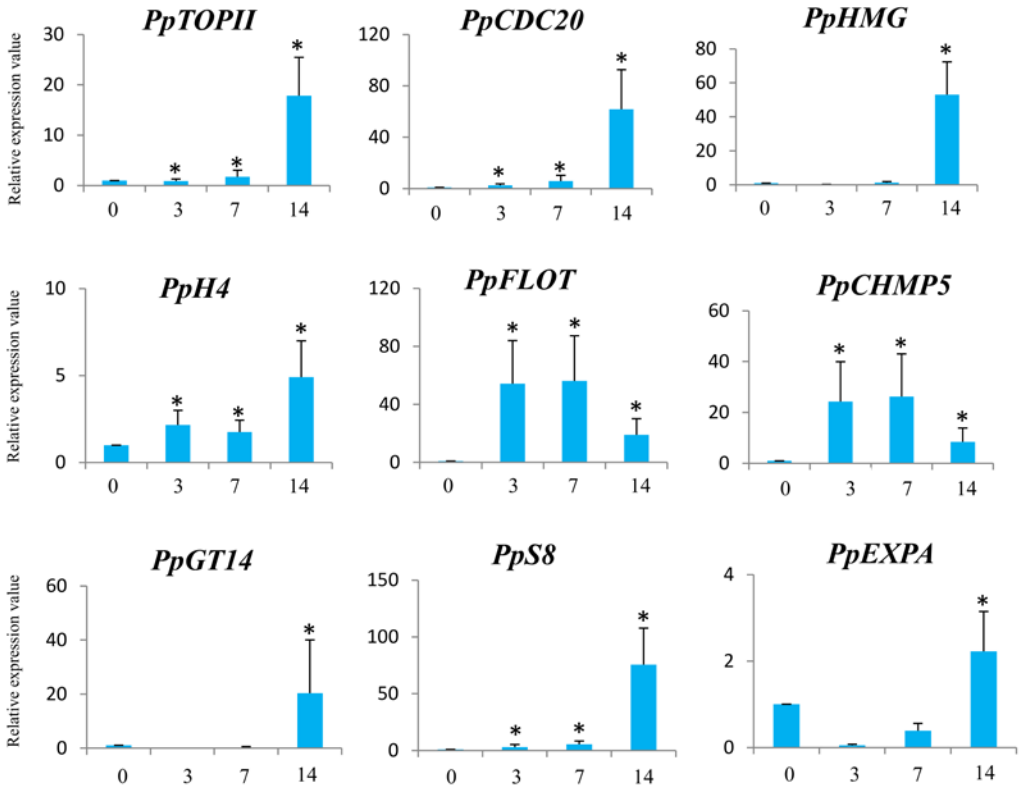

PpTOPII
PpCDC20
PpHMG
PpH4
PpFLOT
PpCHMP5
PpGT14
PpS8
PpEXPA
Relative expression value
0
3
7
14

Fig. 3

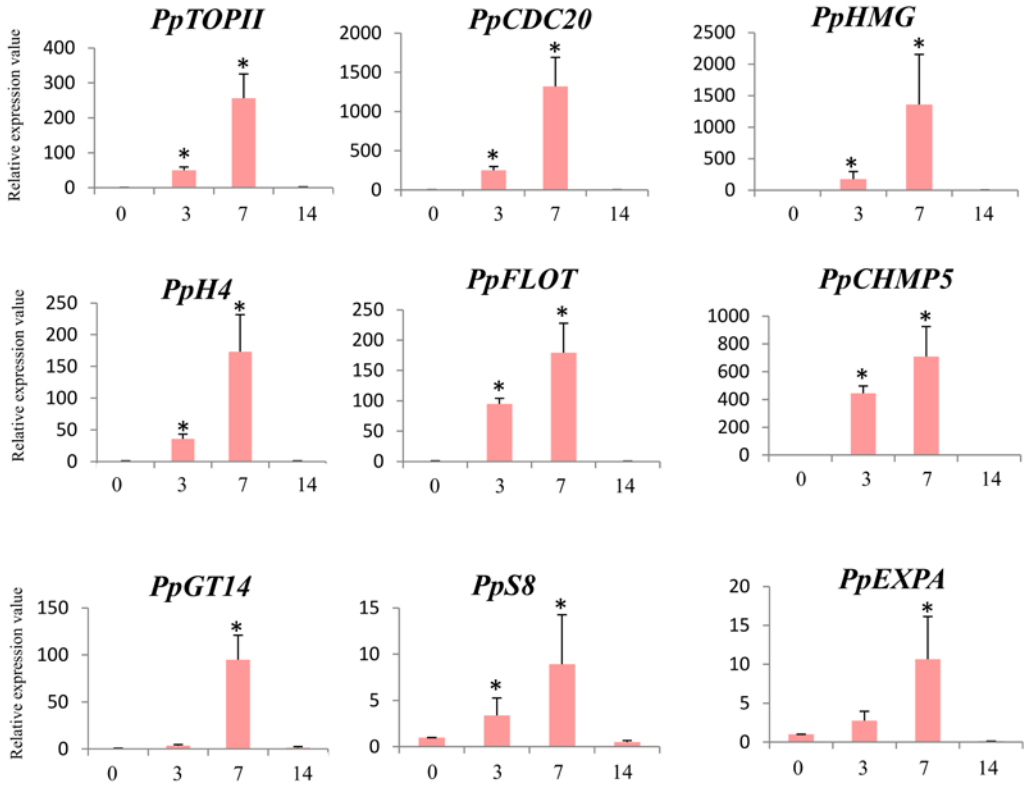

PpTOPII
PpCDC20
PpHMG
PpH4
PpFLOT
PpCHMP5
PpGT14
PpS8
PpEXPA
Relative expression value
0
3
7
14

Fig. 4

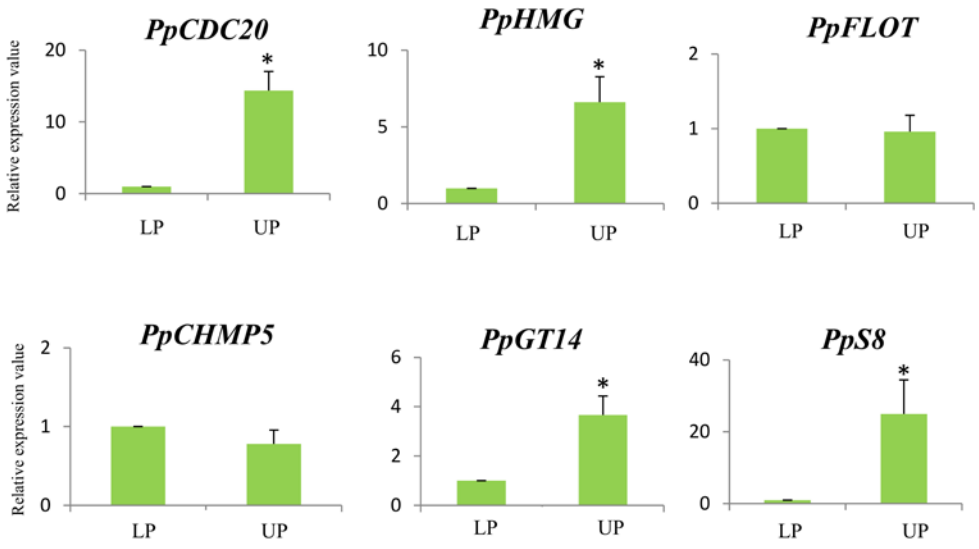

PpCDC20
PpHMG
PpFLOT
PpCHMP5
PpGT14
PpS8
Relative expression value
Relative expression value
20
10
0
10
5
0
2
1
0
2
1
0
6
4
2
0
40
20
0
LP
UP
*

Fig. 5

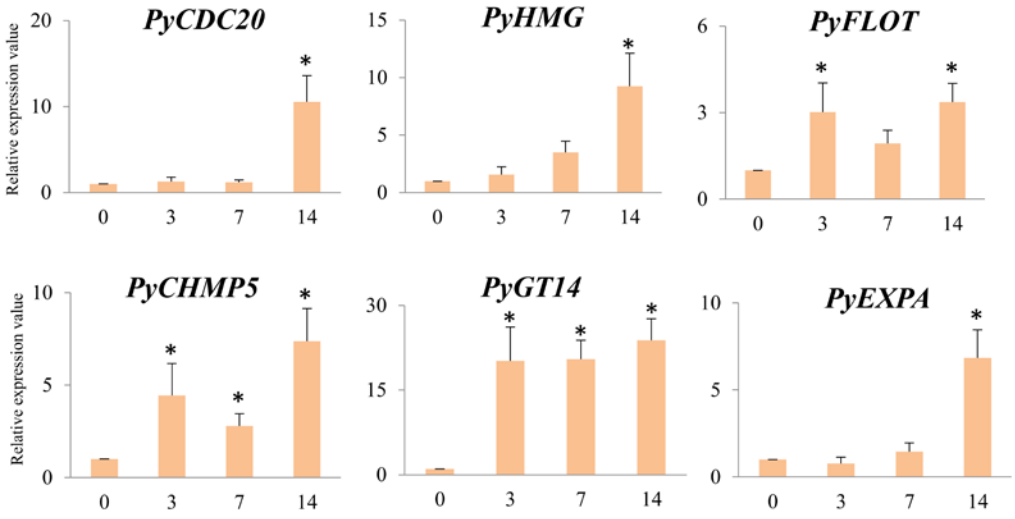

PyCDC20
PyHMG
PyFLOT
PyCHMP5
PyGT14
PyEXPA
Relative expression value
Relative expression value
0
3
7
14

Fig. 6

Table 1. Effect of 1-aminocylopropane-1-carboxylic acid (ACC) on spermatangenesis and growth rate in the male gametophytes of *P. pseudolinearis*

ACC concentration (μM)	% of thallus with spermatangia	% of spermatangia area	Growth rate (mean ± SD% d^{-1})
0	20.0 ±21.9	N.D.	11.5±3.1
50	64.0 ±36.6	1.8 ± 0.8	3.6±1.4 *
500	80.0 ±17.8*	22.6±7.1†	2.7±2.1 *

Data are expressed as means ± SD of five independent experiments with 5 thalli each condition. *Significant differences at $P<0.05$ between control and treatment. †Significant differences at $P<0.05$ between 50 μM ACC and 500 μM ACC treatment. The percent of spermatangia area of non-ACC treated gametophytes, which the number of thallus forming spermatangia was low, expressed as N.D.

Table 2. Effect of 1-aminocylopropane-1-carboxylic acid (ACC) on parthenosporangia and growth rate in the female gametophytes of *P. pseudolinearis*

ACC concentration (μM)	% of thallus with parthenosporangia	% of parthenosporangia area	Growth rate (mean ± SD% d^{-1})
0	0 ±0	N.D.	12.9±4.4
50	12.0 ±24.0	18.5 ±13.5	6.3±2.3
500	68.0 ±27.1*	31.6 ±11.2	5.4±1.9*

Data are expressed as means ± SD of five independent experiments with 5 thalli each condition. *Significant differences at $P<0.05$ between control and treatment. The percent of parthenosporangia area of non-ACC treated gametophytes, which the number of thallus forming parthenosporangia was low, expressed as N.D.

Table 3. Summary of transcriptome analysis in the gametophytes of *P. pseudolinearis*

Sample name	Mapped reads (M)	Up-regulated genes	Down-regulated genes
ACC 0d (Control)	6.1	—	—
ACC 3d	7.8	398	90
ACC 7d	6.9	983	87
ACC14d	6.7	802	92

Table 4 The list of upregulated genes in the *P. pseudolinearis* gametophytes treated with ACC

Conting ID	Abbreviation	Functional categories	Description	Fold Change		
				3d	7d	14d
Contig_5773	GNL	Metabolic process	Gluconolactonase	-0.56	0.18	10.93
Contig_7382	GT14	Glycosyltransferase	Glycosyltransferase family GT14	0.17	7.01	9.26
Contig_4430	HMG	Cell division	High mobility group	3.08	5.29	8.36
Contig_44	FLOT	Vesicular trafficking	Flotillin	6.24	6.14	6.26
Contig_867	OPLA	Metabolic process	5-oxoprolinase	5.48	5.88	5.51
Contig_3800	CP	Protease	Cathepsin B-like cysteine protease	4.00	6.46	5.10
Contig_3647	EXPA	Cell wall organization	Expansin	2.38	3.59	4.43
Contig_342	CHMP5	Vesicular trafficking	Charged multivesicular body protein 5	3.60	3.79	4.43
Contig_4489	TRYP	Protease	Trypsin	1.48	1.48	4.25
Contig_6253	VAMP	Vesicular trafficking	Vesicle associated membrane protein	-0.05	1.67	4.23
Contig_1250	S8	Protease	S8 family peptidase	0.65	0.38	4.23
Contig_3763	GT7	Glycosyltransferase	Glycosyltransferase family GT7	-0.41	2.59	4.03
Contig_5883	CDC20	Cell division	Cell division cycle protein 20	-0.77	0.55	3.88
Contig_4816	TOPII	Cell division	Type II DNA topoisomerase	1.29	1.12	3.74
Contig_1831	H4	Cell division	Histone H4	0.48	1.24	3.13

Table S1. Summary of *de novo* assembly of *P. pseudolinearis*

Description	Numbers
Number of unigene	22,523
Total bases of unigene (bp)	22,931,749
Unigene mean lengths (bp)	1018
N50	1069
Max length (bp)	11,702
Number of annotated unigene	11,485

Table S2. The list of primers used for gene expression analysis in *P. pseudolinearis*

Primer name	Sequence (5'-3')
PpElf1-F1	AACTTCCACAGGGCAATGTC
PpElf1-R1	TCATCATGGGGAAGGAGAAG
PpAct1-F1	GTACAGGTCCTTGCGGATGT
PpAct1-R1	CGAACAAGACCCTGGAGAAG
Pp-2087-F1	GGTGCTACGACGACAAGGAC
Pp-2087-R1	CAGTCCCAGTAGTCCCCAAA
Pp-2376-F1	GCCGAGTCGTACTTCTCCAG
Pp-2376-R1	GTGGAGATTGAGGAGGTGGA
Pp-2451-F1	CTCGGTACGGCCAATGTATC
Pp-2451-R1	GCATGACTTTGGTGTCCTCA
Pp-2546-F1	TCCTATCCCCTCATGCACTC
Pp-2546-R1	AATGAGGGCGTCGACTACTG
PpHMG-4430-F1	GACGCCCTCATTGTACTTGG
PpHMG-4430-R1	CACCCCGACAAGAAGATGAC
Pp-9695-F1	GTGGCTCCACAAAAAGACG
Pp-9695-R1	GACGATGACAACTCGCTGTG
PpGT14-7382-F1	TAGTCGGAACCGGAAATGTT
PpGT14-7382-R1	ACGACCGTCCTCAACACTCT
PpCDC20-5883-F1	GTCGGCAATCCACGAGAC
PpCDC20-5883-R1	TCAACCTCCTCGACTGGAAC
PpEXPA-3647-F1	GCCATTGACGTACACCTCCT
PpEXPA-3647-R1	GGTACGGGGACCTTGACTTT
PpS8-1250-F1	TGGTGGGGTACTTTGTGTCA
PpS8-1250-R1	TTATGGGCGACAGCCTCTAT
PpFLOT-44-F1	GTCGCTCTCGTCCATCTTGT
PpFLOT-44-R1	TCGTCTCGTACACGATCAGC
PpCHMP5-342-F1	GTATCCGCCATATCGTCCAT
PpCHMP5-342-R1	CTGTACAAGAAGCGGGTGCT
PpTOPII-4816-F1	GACAGCTGCGACTTGATCTG
PpTOPII-4816-R1	CGCAGACCTCCTTTGTGAAT
PpH4-1831-F1	AGCCGTACCCGTACAGTGTC
PpH4-1831-R1	TCTATGAGGAGACCCGCAAC

Table S3. The list of tested genes for ACC response in the *P. yezoensis* gametophytes

Conting ID	Abbreviation	Functional categories	Description
Contig_33528	GT14	Glycosyltransferase	Glycosyltransferase family GT14
Contig_41863	HMG	Cell division	High mobility group
Contig_10367	FLOT	Vesicular trafficking	Flotillin
Contig_10512	EXPA	Cell wall organization	Expansin
Contig_13504	CHMP5	Vesicular trafficking	Charged multivesicular body protein 5
Contig_44008	CDC20	Cell division	Cell division cycle protein 20

Table S4. The list of primers used for gene expression analysis in *P. yezoensis*

Primer name	Sequence (5'-3')
PyElf1-F2	CGCACCAAGTCGGACAACTG
PyElf1-R2	ACCACACCAAGAGCGTCCAATC
PyAct1-F2	TCTGCGGAGAAGGAGATTGT
PyAct1-R2	CTTCTCCAGGGTCTTGTTGG
PyGT14-33528-F1	CACCAAGATACCCTCCGAGA
PyGT14-33528-R1	AGAAGCTTCGATGGCAGAAA
PyCHMP5-13504-F1	GGTCAGCAAGACGATGAACA
PyCHMP5-13504-R1	AACTCGTCCATCACCGAATC
PyFLOT-10367-F1	CAGCACTTCCTGGGCTCTAC
PyFLOT-10367-R1	CCCAGGCTCTTCACGTACTC
PyCDC20-44008-F1	CCTGAGATGCTCGACGACTA
PyCDC20-44008-R1	ACGACACGGACGTCACATAG
PyHMG-41863-F1	TGTCGGCGTACCTCCTCTAC
PyHMG-41863-R1	GGCCTTGTACACGACCATCT
PyEXPA-10512-F1	TGACCAAGAAGCACAACTGG
PyEXPA-10512-R1	CACGTCCTTGTACACGTCCT