Ethylene-regulation of sexual reproduction in the marine red alga *Pyropia yezoensis* (Rhodophyta)

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Abstract

Plant growth regulators (PGRs) play a pivotal role in vascular plants, regulating growth, development, and stress responses; however, the role of PGRs in algae remains largely unexplored. Here the role of ethylene, a simple plant growth regulator, was demonstrated in sexual reproduction of the marine red alga *Pyropia yezoensis*. Application of the ethylene precursor 1-aminocylopropane-1-carboxylic acid (ACC) promoted the formation of spermatia and zygotospores in the gametophytes as well as ethylene production, whereas the growth rate was repressed in comparison to gametophytes not treated with ACC. In addition, gametophytes treated with ACC and mature gametophytes showed enhanced tolerance to oxidative stress. Gene expression profiles revealed upregulation of genes involved in cell division and stress response in gametophytes treated with ACC and in mature gametophytes. These results indicate that ethylene plays an important role in the regulation of gamete formation and protection against stress-induced damage during the sexual reproductive stage. Considered together, these findings demonstrate that ethylene is involved in regulating the switching from a vegetative to a sexual reproductive phase in *P. yezoensis*.

Key index words: ethylene; oxidative stress; *Pyropia*; red algae; sexual reproduction

Abbreviations: ACC, 1-aminocylopropane-1-carboxylic acid; PGR, Plant growth regulator; H_2O_2, hydrogen peroxide; RT-PCR, reverse transcription polymerase chain reaction

Introduction

Plant growth regulators (PGRs) are signal molecules and act as major regulators of plant growth and development. Advances of research PGRs in higher plants for many years provide benefits for many agricultural crops to increase the yield, or to improve the quality of the commercial product (Csukasi et 2009). In contrast, although work with marine algae extracts and bioassays has suggested the presence of PGRs that are common in higher plants, such as auxin, cytokinin, and gibberellin (Bradley 1991), the physiological function of their PGRs remained poorly understood.

Ethylene, a gaseous plant growth regulator, controls many aspects of the higher plant life cycle, including sex reproduction and fruit ripening as well as response to abiotic and biotic stresses (Lin et al. 2009). Ethylene is synthesized by the conversion of methionine to 1-aminocylopropane-1-carboxylic acid (ACC) via ACC synthase (ACS), which is generally considered the rate limiting step during ethylene production. In the subsequent step, ACC is catalyzed by ACC oxidase (ACO) in a reaction that converts ACC to ethylene (Yang and Hoffman, 1984). The extensive studies on ethylene in higher plants contribute to the improvement of various agronomic traits such as the promotion of flowering in pineapple and the hastening of ripening in tomato and melons (Saltveit, 1999).

There are a few reports of endogenous ethylene in marine algae, suggesting that red and green algae generally have higher the production rates of ethylene than those of brown algae (Watanabe and Kondo 1976; Broadgate et al. 2004), and the ACS activity was additionally correlated with ethylene production in a green macroalga (Plettner et al. 2005) and a red macroalga (Garcia-Jimenez and Robaina 2012). In addition, exogenous ACC increased the activity of ACO in the green macroalga *Ulva* (Plettner et al. 2005). For physiological functions of ethylene, effect of exogenous ethylene on the loss of chlorophyll *a* (Chl *a*) was documented in *Ulva* (Plettner et al. 2005), and treatment with ethylene affected cap formation in the unicellular green alga *Acetabularia* depending on the timing of application (Vanden Driessche et al. 1997). Moreover, the thalli of the red macroalga *Pterocladiella capillacea* exposed to ethylene showed the promotion of tetra-sporogenesis (Garcia-Jimenez and Robaina 2012). However, in contrast to higher plants, our knowledge regarding the physiological role of ethylene on marine algae remains limited like any other PGRs.

The marine red alga *Pyropia yezoensis* (Ueda) (formerly *Porphyra*; Sutherland et al. 2011), a genus of Bangiophyceae, is one of the most important marine crops. The heteromorphic life history of Bangiophyceae comprises a blade gametophyte and

filamentous sporophyte. *P. yezoensis* is a monoecious species that produces both male gametes (spermatium) and female gametes (carpogonium) on the same gametophyte. After fertilization, successive cell divisions produce exact clones of the zygote termed as zygotospores that grow into a sporophyte (Blouin et al. 2011). An understanding of the regulatory mechanisms involved in sexual reproduction is a prerequisite for efficient breeding to improve existing plants and create new varieties. However, although influences of light and temperature on the sexual maturation of gametophytes have been reported in *Pyropia*/*Porphyra* species (Ackland et al. 2006), the mechanisms remain to be elucidate.

Previous studies have shown the presence of ethylene and ACC in *Pyropia*/*Porphyra* species (Watanabe and Kondo 1976; Zhang et al. 1993). These findings suggest that ethylene and ACC in *Pyropia*/*Porphyra* appear to have similar functions in higher plants such as development and environmental stress responses. In the present study, we investigated the effect of ethylene on gametogenesis as well as tolerance to oxidative stress, because the protection against stress-induced damage during gametogenesis is very important to produce diverse offsprings. In addition, we compared the profiles of transcripts on preferential genes during sexual reproduction and ACC-responsive genes by RNA-seq analysis. These findings could provide new insights into the regulation of the sexual life-cycle by PGR of red algae and contribute to development of efficient breeding systems in *Pyropia*/*Porphyra* species.

Materials and methods

Algal materials and ACC treatments

The leafy gametophytes of *P. yezoensis* strain TU-1 were cultured in enriched sea life (ESL) medium under the conditions described previously (Uji et al. 2012). To study the role of ethylene in *P. yezoensis*, immature gametophytes were exposed to ACC that is the immediate precursor of ethylene and a widely used chemical replacement for treatment with ethylene. Immature gametophytes microscopically formed only vegetative cells, which had been grown to ca. 20 mm blade length and were cultured in glass flasks (150 mL volume) with 100 mL medium containing 0, 50, or 500 μM ACC (Sigma-Aldrich Co. LLC., USA.) at 15°C under the photoperiod regime: 10 h light:14 h dark using cool-white fluorescent lamps at 80 μmol photons m^{-2} s^{-1}. Five individual of immature gametophytes were transferred into each flask. After treatment with ACC for 10 days, the ratio of mature thalli to total thalli was determined by counting the number of gametophytes formed clusters of spermatangia under a Leica DM 5000 B microscope,

because carpogonium from *P. yezoensis* are almost indistinguishable from surrounding vegetative cells. The blade length of gametophytes was measured after 10 days of treatment with ACC and elongation rate was calculated as the mean percentage of length increase per day using the following formula: Growth rate = [100(BLt − BL0)/BL0]/t, BL0 = initial blade length, BLt = blade length at 10 days culture, t = culture time. These experiments were repeated five times. In addition, observation of discharge of spermata and zygotospores was performed after treatment with ACC for 2 or 3 weeks under a Leica DM 5000 B microscope.

Evaluation of tolerance to oxidative stress

To examine the effect of ACC on tolerance to oxidative stress, immature gametophytes treated with 0, 50, or 500 μM ACC for 10 days were transferred into a Petri dish with 40 mL ESL medium containing 2 mM hydrogen peroxide (H_2O_2, Kanto Chemical co., inc., Japan) for 1 week. In addition, to compare tolerance to oxidative stress between immature and mature gametophytes, immature gametophytes cultured at 15°C under the photoperiod regime: 10 h light:14 h dark at 80 μmol photons m^{-2} s^{-1} were transferred to a chamber at 15°C under the photoperiod regime: 12 h light:12 h dark at 80 μmol photons m^{-2} s^{-1} for promotion of the maturation. Mature gametophytes formed spermantagia, which had been grown to ca. 50 mm in blade length were similarly exposed to 2 mM H_2O_2 for 1 week. After the stress treatments, algal samples were harvested and used for measurement of the maximum photochemical efficiency of photosystem II (PS II) [F_v/maximum fluorescence yield (F_m)] to evaluate the damage of oxidative stress using a portable chlorophyll fluorometer (Opti-Science inc., USA.). The gametophytes were placed at 15°C in the dark for 15 min prior to measurement. The dark-level fluorescence yield (F_0) and the maximum fluorescence yield (F_m) were measured after a saturation pulse, from which the maximum photochemical efficiency of PS II was obtained.

Quantification of pigment content

To examine the effect of ACC on the degradation of photosynthetic pigments (Chl *a*; phycoerythrin, PE; phycocyanin, PC; and carotenoids, Car), immature gametophytes (fresh weight; ca. 0.02 g) were cultured with ESL medium containing 0, 50, or 500 μM ACC for 2 weeks at 15°C under the photoperiod regime: 10 h light:14 h dark at 80 μmol photons m^{-2} s^{-1}. The content of Chl *a* and Car were measured according to Seely et al. (1972), whereas PE and PC contents were determined according to the method by Beer and Eshel (1985). These experiments were repeated three times.

Assays of ethylene production

To assess whether ethylene production in immature gametophytes was affected by treatments with ACC, we measured ethylene levels at 10 days after treatments with 0, 50, or 500 μM ACC. Immature gametophytes (fresh weight; ca. 0.2 g) were cultured in airtight glass flasks (400 mL volume) with silicone rubber stoppers and 250 mL medium containing 0, 50, or 500 μM ACC at 15°C under the photoperiod regime: 10 h light:14 h dark using cool-white fluorescent lamps at 80 μmol photons m^{-2} s^{-1}. After 10 days of culture, a gas sample (4 mL) from the flask headspace was injected into a gas chromatograph (model GC-2014, Shimadzu, Japan) equipped with an activated alumina column (3.0 mm × 2.0 m) and flame ionization detectors. The gas chromatograph was operated isothermally 70°C with an injection port temperature of 130°C and a detector temperature of 140°C. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. The algal thalli were dried on tissue paper to remove excess water before determining the fresh weight. The content of ethylene was expressed in nL day^{-1} g^{-1} fresh weight. These experiments were repeated three times.

Identification of ACC-responsive genes

The immature gametophytes that did not bear the clusters of spermatangia were treated with 500 μM ACC. After 1, 3, or 7 days of treatment with ACC, they were harvested and immediately frozen with liquid nitrogen and stored at −80°C until extraction of RNA for RNA-seq analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. As control specimens, immature gametophytes treated without ACC were also harvested and frozen using liquid nitrogen. Similarly, the mature gametophytes which produced the clusters of spermatangia were harvested and frozen. Both immature and mature gametophytes were cultured under the same culture 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, USA.) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA.). The cDNA libraries were constructed using 5 μg total RNA of each sample with high purity and integrity, and then sequenced using an Illumina HiSeq 2000 (Eurofins genomics, Japan). Raw reads generated by Illumina HiSeq 2000 were used to obtain clean reads by removing adaptor sequences and low quality bases, following which clean reads were mapped onto a reference *P. yezoensis* genome sequence (Nakamura et al. 2013) using BWA ver. 0.7.10 (Li and Durbin 2009).

To determine the differential expression, the expression level for each transcript was calculated using the fragments per kilobase of exon per million mapped reads (FRKM) method. The absolute value of $\log_2$ (ratio of test sample/control) ≥ 3 were considered as significant differences in gene expression. To validate the results of RNA-seq, quantitative RT-PCR (qRT-PCR) analysis was performed as described by Uji et al. (2012). Expression levels of the selected genes were normalized to that of 18S rRNA, an internal reference gene. The primer sequences used for qRT-PCR are listed in supplement Table 1. Three technical replicates were carried out for the samples. The data shown correspond to mean ± SE for the biological replicates.

Statistical analysis

Data are expressed as the means ± standard deviation (S.D.). The 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 statistically significant for mean difference.

Results

Treatments with ACC for 10 days induced the thalli to produce the clusters of spermatangia at the apical part and resulted in a slight loss of pigmentation as well as a softening of the thallus (Fig. 1a-c). The formation rate of the clusters of spermatangia in gametophytes treated with 500 μM ACC (88.0%) and 50 μM ACC (60.0%), respectively, whereas it was merely 4.0% when gametophytes were not treated with ACC (Table 1). At least five clusters of spermatangia per thallus were formed in ACC treated gametophytes. After 2 weeks, release of the spermatia and zygotospores concomitant with the degradation of cell walls was observed in the gametophytes treated with ACC (Fig. 1d, e). In contrast, the growth rate of gametophytes cultured in medium containing 50 and 500 μM ACC exhibited ca. 10.1% and 6.4%, respectively, whereas that of gametophytes grown without ACC exhibited ca. 29.2% (Table 1). Statistical analysis showed significant difference between ACC-non treatment and ACC treatment in experiments on the formation rate of the clusters of spermatangia and the growth rate of gametophytes. In addition, thalli grown for 3 weeks and supplemented with ACC showed separation of the plasma membrane from the cell wall by cell shrinkage as well as clear discoloration (Fig. 1f).

Consistent with the discoloration, the contents of all photosynthetic pigments, namely

Chl *a*, PE, PC, and Car, in the gametophytes treated with ACC were lower than those in the gametophytes not treated with ACC (Table 2). The thalli treated with 50 μM ACC resulted in ca. 40% loss of Chl *a* and PE in comparison to untreated controls, whereas the thalli treated with 500 μM ACC resulted in ca. 69% loss of Chl *a*. The statistical analysis showed significant difference of PE contents between gametophytes without ACC (control) and with 500 μM ACC treatment.

Next, whether ethylene is involved in the protection against oxidative stress was tested. As shown in Fig. 2, when gametophytes were exposed to the non-stress condition, there was no significant difference in F_v/F_m between gametophytes treated and not treated with ACC. On the other hand, the F_v/F_m value in the gametophytes not supplemented with ACC (0.20) significantly decreased after oxidative stress treatment in comparison to those supplemented with ACC (ca. 0.55), indicating that the gametophytes supplemented with ACC acquired against oxidative stress.

In addition, gametophytes cultured in medium containing ACC for 10 days were used for measurement of the ethylene emission. As shown in Fig. 3 and Fig. S1, ethylene release was not detectable in those not treated with ACC, whereas the induction of ethylene production by treatment with ACC was indicated by the increase of ethylene release from the gametophytes treated with ACC. The increase of ethylene production was significantly higher in the gametophytes treated with 500 μM ACC in comparison to those treated without ACC and the ethylene production in gametophytes treated with 500 μM ACC was three-fold higher than that in gametophytes treated with 50 μM ACC.

Transcriptomes were generated using mRNA extracted from immature gametophytes treated with 500 μM ACC for 0, 1, 3, and 7 days, and mature gametophytes not treated with ACC to compare the expression pattern between ACC-responsive and preferential genes during the sexual reproductive phase (Table 3). The number of mapped reads obtained from the five cDNA libraries from gametophytes treated with ACC for 0 (control), 1, 3, and 7 days and mature gametophytes were 6.1, 6.9, 8.6, 6.2, and 7.0 million reads, respectively. The number of reads mapping to each contig was counted to obtain the gene expression data. Among differentially expressed genes, 196, 201, and 353 genes were significantly upregulated after 1, 3, and 7 days of treatment with ACC, respectively. On the other hand, 28, 64, and 45 genes were significantly downregulated in immature gametophytes treated with ACC for 1, 3, and 7 days, respectively. In mature gametophytes, 371 genes showed significant levels of differential expression genes compared with those in immature gametophytes, among which 347 and 24 genes were up- and downregulated, respectively. The gene

homologous to ACS (contig_16082_g3868) found in the draft genomic database of *P. yezoensis* were not significantly fluctuated in both immature gametophytes treated with ACC and mature gametophytes in comparison to immature gametophytes treated without ACC (Data not shown). Representative upregulated and downregulated genes are shown in Table 4 and 5, respectively.

Subsequently qRT-PCR analysis was performed to validate RNA-seq data. Six genes with significant changes in expression in both gametophytes treated with ACC and mature gametophytes were selected for qRT-PCR analysis. As shown in Fig. 4, 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 and preferential genes during sexual reproduction. In RNA-seq, transcripts involved in stress response were found to be upregulated in both gametophytes treated with ACC and mature gametophytes; for example, the genes encoding catalase, glutaredoxin, dnaJ10, and mitochondrial chaperone BCS1. In addition, genes involved in cell cycle progression and division, such as the cyclin and aurora kinase genes, similarly displayed upregulation in both gametophytes treated with ACC and mature gametophytes. The homologs of the respiratory burst oxidase and Dual oxidases were also induced by ACC treatment. Interestingly, the ASPO2608 gene, which has been identified as an overexpressed gene during asexual reproduction (Kitade et al. 2008), as well as the gene encoding RWP-RK domain-containing protein were upregulated in the gametophytes treated with ACC and mature gametophytes. In contrast, transcripts encoding translation elongation factor, RNA-polymerase sigma factor, and gamma subunit of phycoerythrin (PE γ) as well as superoxide dismutase were downregulated in gametophytes treated with ACC.

Discussion

Although marine algae have been known to produce ethylene and ACC, there are limited reports on the physiological effects of ethylene and ACC in marine algae. Here we showed that ethylene plays an important role in *P. yezoensis* by switching the plants from a vegetative to sexual reproductive phase. In a previous study, ethylene promoted sporogenesis in the red macroalga *P. capillacea* (Garcia-Jimenez and Robaina 2012). Thus, ethylene is responsible for both asexual and sexual reproduction in red algae. For green algae, application of ethylene increased Chl *a* degradation in *Ulva* (Plettner et al.

2005) and cell elongation was promoted by both ethylene and ACC in a filamentous charophyte (Ju et al. 2015). In addition, ethylene involves in programmed cell death in the green alga *Chlamydomonas reinhardtii* (Yordanova et al. 2010). These findings suggest that physiological effects of ethylene and ACC on development and stress response may be conserved between red and green algal lineages.

Application of ACC induced the formation of spermatia and zygotospores in gametophytes of *P. yezoensis*. During sexual reproduction of red macroalgae, the spermatangia produce spermatia by mitosis, following which the fertilized female cells undergo several cell divisions to form zygotospores. The RNA-seq analysis revealed an increase of transcripts of homologue genes to cyclin and aurora kinase in the gametophytes with ACC and mature gametophytes. Cyclins and aurora kinases play a central role in cell division, which establishes specific divisions leading to daughter cells with variable cell fates throughout plant development (Francis 2007; Van Damme et al. 2011). These findings suggest that the cyclin and aurora kinase within *P. yezoensis* may function as key regulators of the formation of sexual reproductive cells.

In addition to the induction of gametogenesis by ACC, gametophytes treated with ACC and mature gametophytes indicated enhancement of tolerance against H_2O_2 stress. Reactive oxygen species (ROS) including H_2O_2, are significantly accumulated under various abiotic stress conditions, which result in oxidative damage and eventually cell death (You and Chan 2015). The protection against stress-induced damage during reproductive stage is very important to the survival and reproduction of the population. For example, the sorus had significantly higher activities of ROS scavenging enzyme such as ascorbate peroxidase, catalase and glutathione reductase than adjacent non-sorus blade sectors in the brown alga (Mizuta and Yasui 2010). In this study, upregulation of potential genes involved in tolerance to oxidative stress were observed in gametophytes treated with ACC and mature gametophytes, particularly those encoding catalase, glutaredoxin, and molecular chaperones, such as dnaJ 10 and BCS1. Catalase is one of the antioxidant enzymes and catalyzes the dismutation of H_2O_2 to water and oxygen (Frugoli et al. 1996). Glutaredoxins are small ubiquitous proteins of the thioredoxin family and have been shown to be essential for oxidative stress tolerance in cyanobacteria and higher plants (Rouhier et al. 2008). Molecular chaperones are key components responsible for the stabilization of proteins and membranes as well as protein refolding under stress conditions (Wang et al. 2004). Thus, upregulation of these genes would contribute to enhance oxidative stress tolerance during sexual reproduction in *P. yezoensis*.

In higher plants, ethylene has been described to be involved in the control of ROS

generation and signaling. For example, ethylene stimulated the upregulation of the catalase genes in tobacco leaves (Golemiec et al. 2014), whereas ethylene signaling in *Tamarix hispida* inhibits the expression of superoxide dismutase and peroxidase genes during abiotic stress, thus leading to enhanced ROS levels due to decrease scavenging ability (Wang et al., 2014). Similarly, the catalase gene from *P. yezoensis* has been shown to be strongly induced by ACC treatment, whereas the gene encoding superoxide dismutase was down regulated in the presence of ACC, suggesting that the regulation of ROS by ethylene may play an important role in sexual reproduction of *P. yezoensis*. In addition to antioxidant enzymes, membrane-bound NADPH oxidases known as respiratory burst oxidase proteins (Nox) and Dual oxidases (Duox) that catalyze the generation of ROS are responsible for control of ROS in a wide variety of organisms (Kawahara et al. 2007). Generated ROS cause cell wall degradation by oxidative scission of cell wall polysaccharides during cell expansion, fruit ripening and organ abscission (Fry 1998). In the present study, the Nox and Duox genes from *P. yezoensis* were upregulated in the gametophytes treated with ACC, and release of the spermatia and zygotospores concomitant with the degradation of cell walls was observed in the gametophytes treated with ACC. These results suggest that ACC-inducible Nox and Duox derived ROS may play an important role in release of gametes and spores, but further investigation are required to elucidate the involvement of ROS regulation in sexual reproduction of *P. yezoensis*.

A homolog of ACS genes has been reported in moss *Physcomitrella patens*, while a homolog of ACO genes are absent in the genomes of the ancient groups of land plants such as *P. patens* and *Selaginella moellendorffii* (Kawai et al. 2014). Similarly, one putative ACS gene was identified in the draft gnomic database of *P. yezoensis*, however gene homologous to ACO were not found in the genomic database of red algae including *P. yezoensis*. RNA-seq analysis revealed that PyACS transcripts were not upregulated during sexual development. In higher plants, ACS is not regulated transcriptionally but also post-translationally such as phosphorylation (Tatsuki and Mori 2001). Thus, PyACS may be regulated post-translationally.

Ethylene-response genes in higher plants are regulated by ethylene-responsive factor (ERF) that belongs to the AP2/EREBP-type transcription factors (Merchante et al. 2013); however, the homologues to ERFs are not present in *P. yezoensis* genomic draft sequence data. On the other hand, upregulation of the homologue gene to the RWP-RK domain-containing protein was found in the gametophytes treated with ACC and mature gametophytes. The RWP-RK domain-containing protein largely controls sexual differentiation during gametogenesis in green algae (Nozaki et al. 2006). Thus, the

function of RWP-RK may play a critical role in gametogenesis in both red and green algal lineages. ACC was additionally shown to increase the transcript of the ASPO2608 gene, which has been isolated as an overexpressed gene during the formation and release of asexual spores, archeospores, in *P. yezoensis* gametophytes (Kitade et al. 2008). These results suggest that the mechanisms of sexual and asexual reproduction may share genetic components in *P. yezoensis*. One future task will be to analyze the ACC-responsive genes using genetic transformation that has recently been developed in this species (Hirata et al. 2014, Uji et al. 2014), which leads to the elucidation of the mechanism of not only sexual but also asexual reproduction.

In conclusion, we have shown that exogenous ACC stimulates the production of ethylene, formation of sexual cells, and protection against oxidative stress in gametophytes of *P. yezoensis*. We additionally identified the ACC-responsive genes involved in stress tolerance and cell division. These findings could provide new insights into the regulation of the sexual life-cycle in red algae and clarify the interesting field of plant growth regulator relationships to sexual reproduction in algae.

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Figure legends

Fig. 1 Involvement of 1-aminocylopropane-1-carboxylic acid (ACC) in the formation of spermatangia and zygotospores in gametophytes of *Pyropia yezoensis*. Magnified view of gametophytes during 10 days of culture in medium without ACC, which did not bear the cluster of spermatangia (a). Magnified view of gametophytes during 10 days of culture in medium with 500 μM ACC, which bore the cluster of spermatangia (arrows) (b, c). Gametophytes during 14 days of culture in medium with 500 μM ACC released a number of spermata (arrowheads) (d) and zygotospores (e). Separation of the plasma membrane from the cell wall by cell shrinkage concomitant with a loss of pigmentation in gametophytes during 3 weeks of culture in medium with 500 μM ACC (f). Gametophytes used for all experiments were cultured at 15℃ under 80 μmol photons m^{-2} s^{-1} with a photocycle of 10 h light and 14 h dark. Scale bar = 100 μm (a-c), Scale bar = 50 μm (d-f).

Fig. 2 Involvement of 1-aminocylopropane-1-carboxylic acid (ACC) in tolerance to oxidative stress in gametophytes of *Pyropia yezoensis*

Maximum photochemical efficiency (F_v/F_m) of gametophytes subjected to 0 mM (non-stress) and 2 mM H_2O_2 (oxidative stress) after treatment with 0, 50, or 500 μM ACC are indicated by white and gray boxes, respectively. Columns and vertical bars represent the mean and SD, respectively (n = 5). Asterisk indicates significant difference at $P < 0.05$ between control and treatment.

Fig. 3 Effect of 1-aminocylopropane-1-carboxylic acid (ACC) on the production of ethylene in the gametophytes of *Pyropia yezoensis*

The ethylene production is expressed as means ± SD (nL day^{-1} g^{-1} fresh wt.) of three independent experiments (n = 3). Asterisk indicates significant differences at $P<0.05$ between control and treatment.

Fig. 4 Comparison of gene expression data obtained by RNA-seq and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

qRT-PCR analysis was performed in six selected 1-aminocylopropane-1-carboxylic acid (ACC)-inducible genes in gametophytes of *Pyropia yezoensis* (white boxes). The levels of gene expression obtained from RNA-seq data are indicated by gray boxes (RNAseq). RNA samples were prepared from immature gametophytes 1, 3, or 7 days after treatment with 500 μM ACC or mature gametophytes not treated with ACC. Results are presented as relative expression compared with that in immature gametophytes not treated with ACC. Data of qRT-PCR are presented as means and SD (n = 3). The primers used for qRT-PCR analysis are shown in Table S1.

Fig. S1 GC-mass chromatogram of the production of ethylene in *P. yezoensis*

Immature gametophytes were cultured in glass flasks containing 0 (control), 50, or 500 μM ACC.

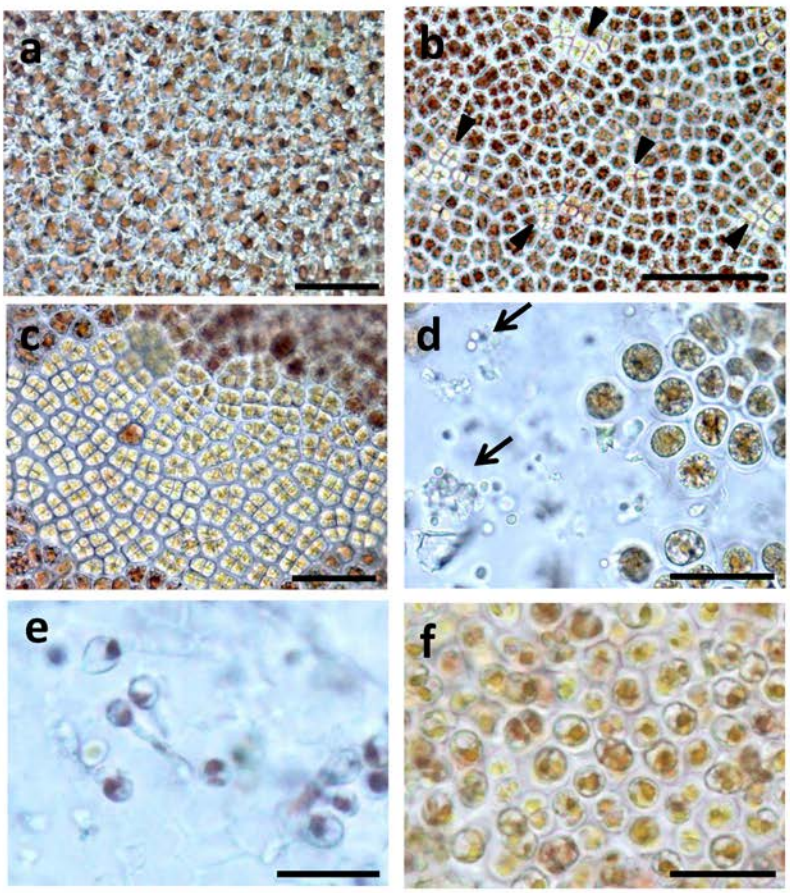

Fig. 1

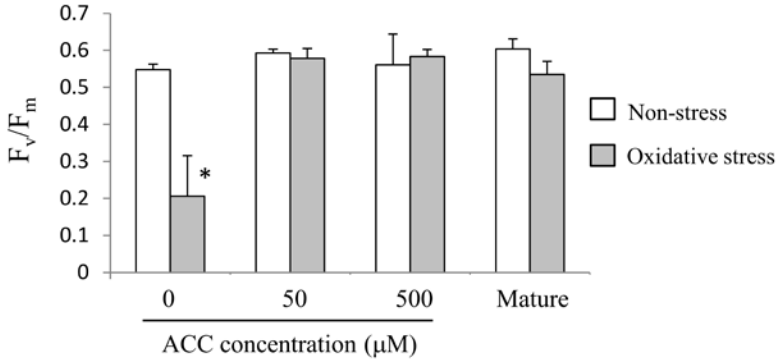
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
F_v/F_m
*
0
50
500
Mature
ACC concentration (μM)
Non-stress
Oxidative stress

Fig. 2

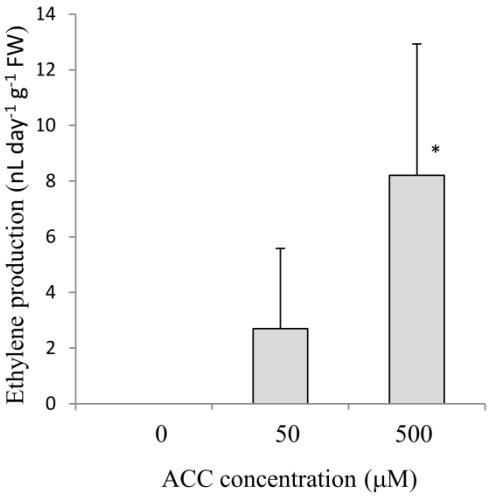
Ethylene production (nL day-1 g-1 FW)
14
12
10
8
6
4
2
0
*
0
50
500
ACC concentration (µM)

Fig. 3

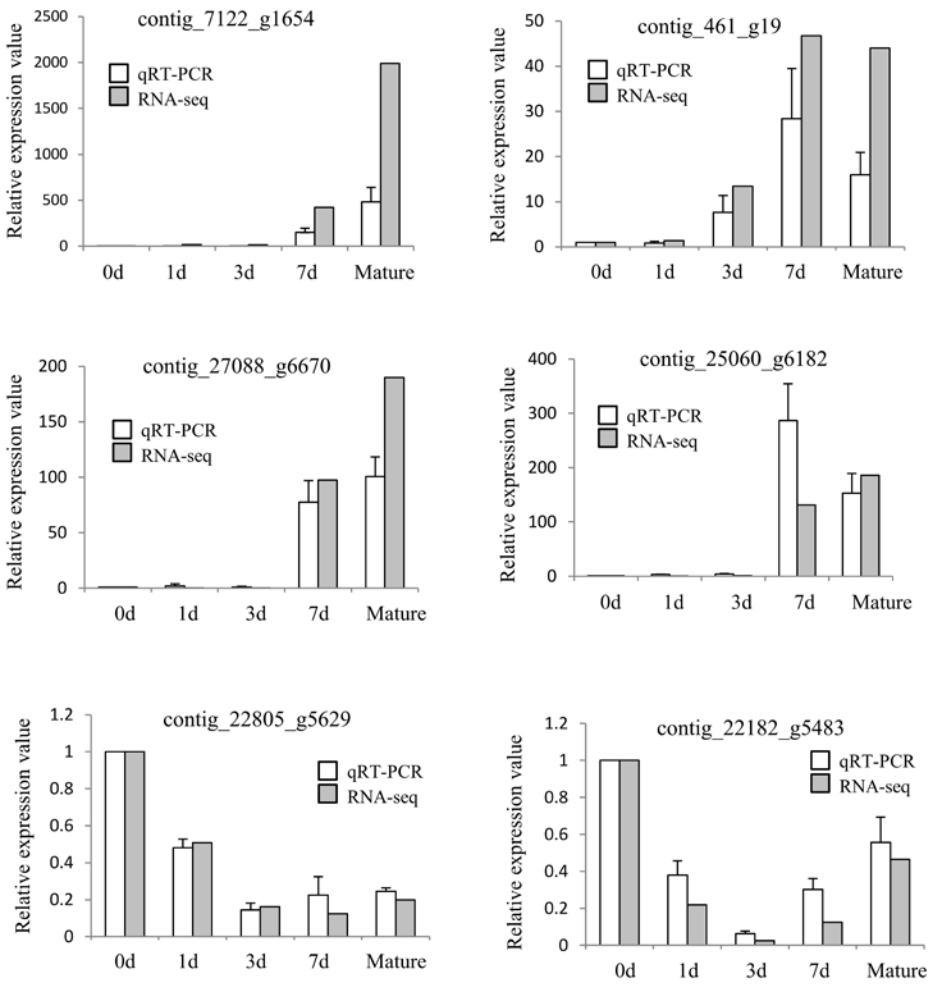
contig_7122_g1654
Relative expression value
2500
2000
1500
1000
500
0
qRT-PCR
RNA-seq
0d
1d
3d
7d
Mature
contig_461_g19
50
40
30
20
10
0
contig_27088_g6670
200
150
100
50
contig_25060_g6182
400
300
200
contig_22805_g5629
1.2
1
0.8
0.6
0.4
0.2
contig_22182_g5483

Fig. 4

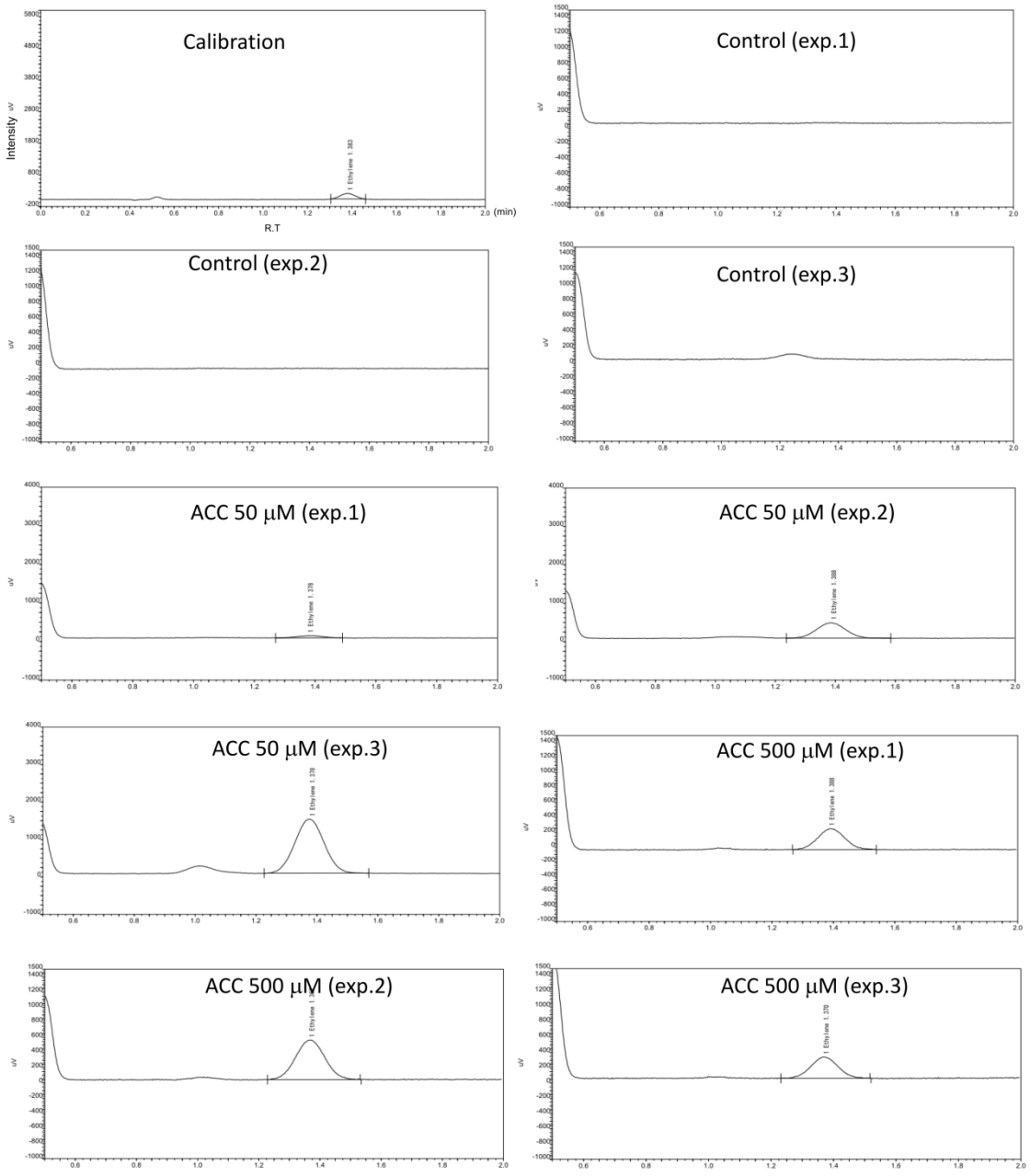
Calibration
Intensity uV
R.T
(min)
Control (exp.1)
Control (exp.2)
Control (exp.3)
ACC 50 μM (exp.1)
ACC 50 μM (exp.2)
ACC 50 μM (exp.3)
ACC 500 μM (exp.1)
ACC 500 μM (exp.2)
ACC 500 μM (exp.3)

Fig. S1

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

ACC concentration (μM)	% of thallus with cluster of spermatangia	Growth rate (mean ± SD% d^{-1})
0	4.0 ±8.9	29.2±6.0
50	60.0 ±20.0*	10.1±3.9*
500	88.0 ±10.9 *	6.4±2.7*

Data are expressed as means ± SD of five independent experiments with 5 thalli each condition. Asterisk indicates significant differences at $P<0.05$ between control and treatment.

Table 2. Effect of ACC on degradation of photosynthetic pigments in the gametophytes of *P. yezoensis*

ACC concentration (μM)	Chl *a*	PE	PC	Car
0	1.13±0.87	4.25±0.61	1.09±0.76	0.06±0.04
50	0.71±0.40	2.40±1.45	0.30±0.08	0.05±0.03
500	0.41±0.27	1.11±0.81*	0.10±0.07	0.02±0.01

Data are expressed as means ± SD of three independent experiments (mg g^{-1} fresh wt.).

Contents of chlorophyll *a* (Chl *a*), phycoerythrin (PE), phycocyanin (PC), and carotenoids (Car) were measured using gametophytes cultured in the medium with 0, 50, or 500 μM ACC. Asterisk indicates significant differences at $P<0.05$ between control and treatment.

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

Sample name	Mapped reads (M)	Up-regulated genes	Down-regulated genes
IMA (Control)	6.1	—	—
IMA+ACC1d	6.9	196	28
IMA+ACC3d	8.6	201	64
IMA+ACC7d	6.2	353	45
MA	7.0	347	24

IMA, immature gametophytes. MA, mature gametophytes

Table 4. Comparative analysis of up-regulated genes between gametophytes treated with ACC and mature gametophytes.

Conting ID	Gene annotation	Fold Change			
		1d	3d	7d	Mature
contig_7122_g1654	catalase [*Pyropia yezoensis*]	4.07	3.72	8.72	10.96
contig_39761_g9180	beta-Ig-H3/fasciclin [*Dinoroseobacter shibae*]	5.73	7.39	7.22	6.50
contig_25060_g6182	ASPO1527 [*Pyropia yezoensis*]	-2.50	-0.49	7.04	7.54
contig_33412_g8069	aurora kinase [*Apis dorsata*]	-0.44	5.47	6.93	5.69
contig_27088_g6670	ASPO2608 [*Pyropia yezoensis*]	-3.26	-4.58	6.61	7.57
contig_606_g32	high light inducible protein [*Pyropia yezoensis*]	1.90	2.98	6.32	6.11
contig_15997_g3840	chaperone protein dnaJ 10 [*Zea mays*]	1.41	1.83	6.21	7.42
contig_18376_g4510	Dual oxidase 1 [*Aegilops tauschii*]	5.67	6.18	5.68	2.81
contig_461_g19	RWP-RK domain-containing protein [*Galdieria sulphuraria*]	0.46	3.75	5.55	5.46
contig_11756_g2797	mitochondrial chaperone BCS1 [*Rhizopus microsporus*]	3.75	3.90	5.00	4.76
contig_10512_g2520	Expansin [*Ostreococcus tauri*]	0.00	0.91	4.97	4.37
contig_28569_g7027	glutaredoxin [*Auricularia subglabra*]	0.31	2.66	4.89	4.08
contig_16363_g3970	U-type cyclin [*Amborella trichopoda*]	-0.16	3.91	4.59	3.65
contig_36762_g8710	respiratory burst oxidase [*Pyropia yezoensis*]	2.15	3.65	3.46	3.32

Table 5. Comparative analysis of down-regulated genes between gametophytes treated with ACC and mature gametophytes.

Conting ID	Gene annotation	Fold Change			
		1d	3d	7d	Mature
contig_42781_g9644	RNA-polymerase sigma factor [*Guillardia theta*]	-2.13	-3.82	-5.60	-1.75
contig_17672_g4335	polyprotein [*Pyropia yezoensis*]	-2.46	-3.42	-4.52	-3.13
contig_27075_g6664	superoxide dismutase[*Pyropia haitanensis*]	-0.53	-2.78	-3.20	-1.69
contig_11106_g2658	alginate lyase [*Pyropia yezoensis*]	-0.21	-2.45	-3.11	-2.81
contig_2865_g583	translation elongation factor P [*Microcoleus sp.*]	-0.76	-1.46	-3.06	-1.15
contig_22805_g5629	Myb-like DNA-binding protein[*Chondrus crispus*]	-0.98	-2.62	-3.00	-2.33
contig_22182_g5483	Gamma subunit of phycoerythrin [*Chondrus crispus*]	-2.20	-5.31	-3.00	-1.10

Table S1. The list of primers used for gene expression analysis by quantitative Real Time PCR

Primer name	Sequence (5'-3')
contig_7122_g1654-F	GAGGTCTTTGTGCGCTTTTC
contig_7122_g1654-R	AGATGCCTCCGCCATACTT
contig_25060_g6182-F	CAAAGGTGCCATCATTTCG
contig_25060_g6182-R	CGAGTCACGCAGAAAGTTCA
contig_27088_g6670-F	GATTGTGAGGGAGGCAAGAA
contig_27088_g6670-R	AGCACCAGAAAAGGAGCAGA
contig_461_g19-F	ATGGGTATCTGCGTCACCATC
contig_461_g19-R	CAGGTCGCTGGACTTCAAGTT
contig_22805_g5629-F	CGGAAGCCCTACACCATTAC
contig_22805_g5629-R	TACTCGCCCGTCTTGTTCTT
contig_22182_g5483-F	GTTGCCTACTCCCTGGACAA
contig_22182_g5483-R	GCAGCACCCTGGAGATAGAA