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α-aminoisobutyric acid mimics the effect of 1-aminocyclopropane-1-carboxylic acid to promote sexual reproduction in the marine red alga *Pyropia yezoensis* (Rhodophyta)

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Abstract
1-aminocyclopropane-1-carboxylic acid (ACC), a precursor for ethylene, stimulates the switch from a vegetative to a sexual reproductive phase in the marine red alga *Pyropia* species. This study explored the effects of ethylene biosynthesis inhibitors on the sexual reproduction of gametophytes of the red alga *Pyropia yezoensis* to gain a functional understanding of the role of ACC as a plant hormone in red algae. Here we show that two inhibitors of ACC synthesis in higher plants, 2-aminoethoxyvinyl glycine (AVG) and aminooxyacetic acid (AOA), had no effect on the growth and gametogenesis of *P. yezoensis*. In contrast, exogenous application of α-aminoisobutyric acid (AIB), a structural analog of ACC that blocks the conversion of ACC to ethylene in higher plants, induced the formation of spermatangia and carpospores in a similar manner as ACC, without endogenous ACC accumulation. The treatment of AIB failed to inhibit ethylene production in the gametophytes. The present results suggest that AIB mimics the effect of ACC to induce the sexual reproduction and support our previous study that ACC has a role in the regulation of the sexual reproduction independent from ethylene.

Keywords 1-aminocylopropane-1-carboxylic acid · Ethylene · Plant hormone · *Pyropia* · Red algae · Sexual reproduction

Introduction

Plants produce hormones (also known as phytohormones or plant growth regulators) that regulate plant growth, development, and environmental responses (Vanstraelen and Benkova 2012; Xia et al. 2015; Verma et al. 2016). In plant hormone research, the use of a wide variety of small molecules such as agonists, antagonists, and inhibitors of the biosynthetic pathway and transports have significantly enhanced our understanding of the molecular basis of hormone actions in higher plants (Fonseca et al. 2014; Rigal et al. 2014). In macroalgae, treatment with auxin efflux inhibitors such as naphthylphthalamic acid (NPA), elevates indole-3-acetic acid (IAA) accumulation and reduces environmental polarization in response to gravity and light vectors in embryos of *Fucus distichus*, a brown alga (Basu et al. 2002; Sun et al. 2004). Jasmonate (JA) is related to cystocarp development in the red macroalga *Grateloupia imbricata* and the application of phenidone, a specific inhibitor of lipoxygenases (LOX) that catalyzes the first step in the biosynthesis of JA, decreases the number of the cystocarps concomitant with the repression of the JA release (Garcia-Jimenez et al. 2016). In addition, callus induction from leaf explants of the brown alga *Sargassum horneri* was achieved when grown in medium supplemented with uniconazole, a triazole-type inhibitor of cytochrome P450 enzymes for gibberellin biosynthesis, trans-zeatin biosynthesis, and abscisic acid catabolism (Uji et al. 2016b). However, in contrast to higher plants, few reports are available on the use of small molecules involved in plant hormones in macroalgal research, hindering the elucidation of the functional roles of plant hormones.

Ethylene, a simple gaseous plant hormone, is derived from methionine (Met), which is first converted to S-adenosylmethionine (SAM) by SAM synthetase. SAM is then converted into 1-aminocylopropane-1-carboxylic acid (ACC), an ethylene precursor (Yang and Hoffman 1984). This step is catalyzed by ACC synthase (ACS), an aminotransferase that requires pyridoxal 5′-phosphate (PLP) as a cofactor. In the second step, ACC is

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oxidized by an O_2-activating non-heme iron enzyme, ACC oxidase (ACO), giving rise to ethylene, carbon dioxide (CO_2), and hydrogen cyanide. Ethylene production can be manipulated by the inhibition of ACS and ACO activity (Fig. 1). For instance, 2-aminoethoxyvinyl glycine (AVG) and aminooxyacetic acid (AOA) inhibit the activity of ACS and consequently decrease ethylene production by preventing the production of ACC (Amrhein and Wenker 1979; Boller et al. 1979; Yang and Hoffman 1984). Additionally, α-aminoisobutyric acid (AIB) is a structural analog of ACC that blocks ACO activity by acting as a competitive inhibitor of the ACC substrate (Satoh and Esashi 1980; 1982; Serrano et al. 1990). The pharmacological manipulation of ethylene biosynthesis using these inhibitors has increased our understanding of the roles of ethylene in higher plants (Tamimi and Timko 2003; Tian et al. 2014).

The marine red alga *Pyropia* (formerly *Porphyra*), a genus of Bangiophyceae, is a significant marine crop that is harvested to produce the food "nori". The heteromorphic life history of *Pyropia* is comprised of a blade gametophyte and filamentous sporophyte (Fig. 2). This genus forms male (spermatia) and female (carpogonia) gametes during sexual reproduction. After fertilization, successive cell divisions occur to produce clones of the zygote called carpospores that will grow into sporophytes. Our previous research revealed that the exogenous application of ACC promoted gametogenesis and enhanced the antioxidant capacity in accompany with ethylene emission in the monoecious *P. yezoensis* (Uji et al. 2016a). Research on the dioecious species *Pyropia pseudolinearis* revealed that ACC can modulate the expression of genes involved in the regulation of cell division and cell wall organization, which leads to the formation of spermatangia and parthenosporangia in male and female gametophytes, respectively (Yanagisawa et al. 2019). Our recent study suggests that ACC acts as a signaling molecule independent from ethylene in the regulation of sexual reproduction through alterations to the redox state in *P. yezoensis* (Uji et al. 2020).

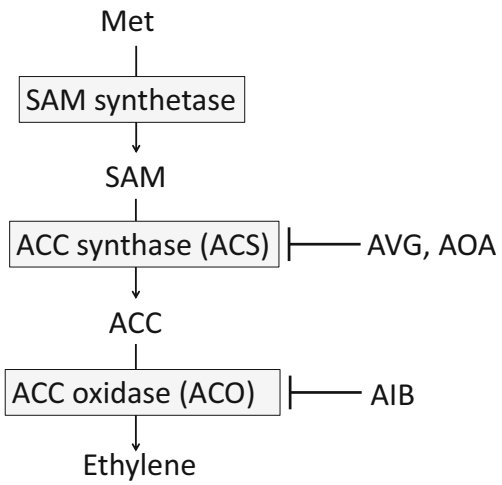

Fig. 1 The ethylene biosynthetic pathway and the inhibitors used in this study. Met, Methionine; SAM, S-adenosyl-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, 2-aminoethoxyvinyl glycine; AOA, aminooxyacetic acid; AIB, α-aminoisobutyric acid

In the present study, we investigated the effects of three ethylene biosynthetic pathway inhibitors on the sexual reproduction of *P. yezoensis* gametophytes to gain a better understanding of ACC plant hormone action in *Pyropia* species. The present results suggest that AIB mimics the effect of ACC to induce the sexual reproduction in *P. yezoensis*.

Materials and methods

Algal cultures

Standard cultivation of gametophytes of *Pyropia yezoensis* strain TU-1 was conducted at 15 °C in glass flasks (150 mL volume) with 100 mL of medium. The medium consisted of autoclaved seawater enriched with sterile vitamin-free Provasoli solution (PES; Provasoli 1968). Light was supplied by cool-white fluorescent lamps at 60 μmol photons m^{-2} s^{-1} irradiance with a photoperiod regime of 10 h light:14 h dark (short day condition) or 14 h light:10 h dark (long day condition).

Chemical treatments

The gametophytes of *P. yezoensis* were treated with the chemical compounds generally used as ethylene biosynthesis inhibitors in higher plants. Five individual immature gametophytes of ca. 20 mm blade length that were microscopically determined to bear only vegetative cells were cultured in glass flasks (150 mL volume) with 100 mL PES medium with either three inhibitors prepared each concentration: 0, 5, 50 μM aminoethoxyvinylglycine (AVG) (Cayman Chemical Company, USA); 50, 500 μM α-aminoisobutyric acid (AIB) (Tokyo Chemical Industry, Japan); 50, 500 μM aminooxyacetic acid (AOA) (FUJIFILM Wako Pure Chemical Corporation, Japan); or 50, 500 μM ACC (Tokyo Chemical Industry). Two pieces of glass (20 mm × 25 mm) were placed on the bottom of the culture flask to capture the formation of carpogonia, the female gametes. The gametophytes were cultured with the chemical reagents for 7 days, then the number of gametophytes that had formed clusters of spermatangia were counted under a Leica DM 5000 B microscope (Leica Microsystems, Japan). In this study, a mature gametophyte was defined as a thallus bearing at least five clusters of spermatangia because carpogonium from *P. yezoensis* are almost indistinguishable from surrounding vegetative cells. In addition, after 14 days the numbers of discharged carpospores attached to the pieces of glass were counted under a microscope as an index of formation of carpogonia. The gametophyte blade lengths were measured after 10 days and growth rate was calculated as the mean percentage of length increase per day using the following formula:

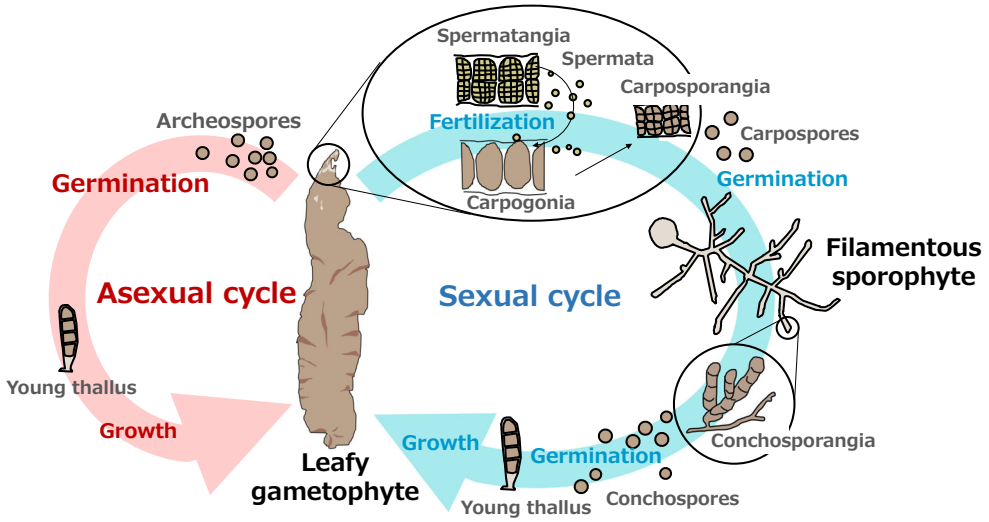

Fig. 2 Life cycle of *Pyropia* species. The macroscopic leafy gametophytes of *Pyropia*, which are harvested to produce the food for nori or laver, bear non-motile male (spermatia) and female (carpogonia) gametes on the thallus during sexual reproduction. Fertilization occurs when the female gametes are still retained on the gametophytes and successive cell divisions produce clones of the zygote (carpospores), which develop into microscopic filamentous sporophytes referred as the conchocelis. The mature sporophytes produce conchospores that are released from conchosporangia and settle on the substratum, where they germinate to form new gametophytes. Some *Pyropia* such as *P. yezoensis* can also reproduce asexually in the gametophytes by asexual spores called archeospores

Growth rate = $[100(BL_t - BL_0)/BL_0]/t$, where BL_0 = initial blade length, BL_t = blade length at days of culture, t = culture time. The experiments under the long day or the short day culture were conducted at three or eight replicates with 5 thalli each condition, respectively. In these experiments, thalli treated without inhibitors were used as a control.

Measurement of ethylene production

To examine whether AIB has an inhibitory effect on ethylene production in gametophytes, we measured ethylene levels after 10 day treatments with AIB. Vegetative gametophytes (fresh weight: FW ca. 0.1 g) were cultured in 20 mL aluminum sealed vials with butyl-gum septa (GL Science, Japan) with 10 mL of medium containing 0, 500 μM ACC, or 500 μM ACC and AIB, at 15 °C under a 10 h light:14 h dark photoperiod regime using cool-white fluorescent lamps at 60 μmol photons $m^{-2}s^{-1}$. After 10 days the samples were analyzed by GC analysis of the volatile compounds. For the static headspace GC analysis, the sample vial was transferred into the HS-20 headspace auto-sampler (Shimadzu, Japan) of the GC apparatus. The headspace gas in the vial was automatically pressurized at 60 °C for 2 min and then immediately injected through a loop into a GC-2014AFSC (Shimadzu) equipped with an HP-1 capillary column (50 m length, 0.32 mm i.d. and 1.05 μm film thickness; Agilent Technologies, USA) and a flame ionization detector. An initial oven temperature of 40 °C for 5 min was followed by heating at 3 °C min^{-1} to 70 °C, then 20 °C min^{-1} to 200 °C, and finally, the temperature was held at 200 °C for 4 min. Both the injection port and the flame ionization detector were set at 250 °C. The content of ethylene was expressed in nmol $day^{-1}g^{-1}$ FW. These experiments were repeated in triplicate and thalli treated with ACC were used as a control.

Measurement of ACC content

ACC concentrations in the gametophytes treated with AIB were determined following the method of Wächter et al. (1999) with minor modifications. The algal materials were harvested, immediately frozen with liquid nitrogen, and stored at − 80 °C until the extraction of ACC. A 1.5 g sample (FW) was ground in liquid nitrogen with a mortar and pestle, followed by homogenization in 7.5 mL 80% methanol with 2 mg L^{-1} dibutylhydroxytoluene. The extract was stored at room temperature for 45 min, then centrifuged for 15 min at 2000 × *g* at 20 °C. The extraction was repeated with 6 mL 80% methanol and the supernatants were evaporated to dryness with a Rotary Evaporator at 40 °C. The dry residue was dissolved in 2 mL distilled water and 4 mL dichloromethane, then centrifuged at 2000 × *g* at 20 °C for 5 min. The conversion of ACC to ethylene was performed following the procedure described by Lizada and Yang (1979). A 1.6 mL aliquot of the upper aqueous phase was mixed with 0.1 mL $HgCl_2$ (20 mM) in a 20 mL aluminum sealed vial with a butyl-gum septum. NaOCl-solution (5% NaOCl and 50% NaOH (2:1, v/v)) was injected through the septum with a syringe. The vials were shaken, incubated for 5 min and then the sample was assayed for ethylene by GC analysis according to the method described above. The ACC content was expressed as nmol g^{-1} FW. These experiments were repeated in triplicate and thalli treated without ACC/AIB were used as a control.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The results of treatments with and without inhibitors of the ethylene biosynthetic pathway were analyzed using Mann–Whitney's U test. For all analyses, $p < 0.05$ or 0.01 were considered statistically significant.

Results

Effects of ethylene inhibitors on growth and gametogenesis

When the gametophytes were cultured with 5, 50 μM AVG or 5, 50 μM AOA under the long day culture condition that promotes the sexual reproduction, there were no significant differences in spermatangia formation and growth rates between thalli treated with and without the inhibitors (Table 1). These results imply that ACS inhibitors generally used in higher plants are ineffective in the gametophytes. Contrary to expectations, gametophytic thalli treated with 50 μM or 500 μM AIB formed significantly more spermatangia clusters and gametophytes cultured with 500 μM AIB exhibited slight growth repression compared to the thalli in the absence of chemical compounds (control) (Table 1).

Comparison of the effects of AIB and ACC on induction of sexual reproduction

The results in the effects of AIB prompted us to compare with the induction level of AIB and ACC on the sexual reproduction in the gametophytes. The gametophytes were cultured with 50, 500 μM ACC or 50, 500 μM AIB under the short day culture condition that represses the sexual reproduction. Treatment with 500 μM AIB promoted the formation of spermatangia on the upper parts of the thalli, whereas 500 μM ACC promoted spermatangia formation on the upper to middle sections (Fig. 3). All of the gametophyte thalli that were cultured with 50, 500 μM ACC or 50, 500 μM AIB formed spermatangia, whereas only 22.5% of thalli formed spermatangia when the gametophytes were cultured without chemical compounds (control) (Fig. 4a). The growth rate of gametophytes cultured in medium containing 50 and 500 μM ACC exhibited 14.6% and 13.7%, respectively, whereas that of gametophytes grown with 50 and 500 μM AIB exhibited 20.2% and 12.2%, respectively (Fig. 4b). The number of discharged carpospores from the gametophytes treated with ACC or AIB was significantly higher than the control, but AIB has less effective than ACC (Fig. 4c). These results indicate that AIB can induce the sexual reproduction in a similar manner as ACC.

Evaluation of AIB as an inhibitor of ACC to ethylene conversion

The ACC contents of thalli treated with AIB were investigated to consider the possibility that AIB promoted the sexual reproduction through endogenous ACC accumulation by blocking the conversion of ACC to ethylene in the gametophytes. As shown in Fig. 5, exogenous application of 50 and 500 μM ACC increased the contents of ACC in vivo, whereas no accumulation of ACC was confirmed in thalli supplemented with 500 μM AIB. These results indicated that AIB can induce the sexual reproduction without endogenous ACC accumulation and raised the possibility that AIB is ineffective inhibitor of ACO. To confirm this, the ethylene production was examined in thalli treated with AIB. Since our previous study revealed small amount of ethylene release in the gametophytes under normal conditions (Uji et al. 2016a), we used thalli increased ACC/ethylene in vivo by supplement with 500 μM ACC as a control. The results showed that there were no significant differences in ethylene production between the thalli treated without AIB and with 0.5 mM AIB (Fig. 6). In addition, no inhibition of ethylene production in the gametophytes treated with the even high doses of AIB (10 mM AIB) (Fig. 6) which the concentration strongly

Table 1 Effect of ethylene biosynthetic pathway inhibitors on spermatangenesis and growth rate in *Pyropia yezoensis* gametophytes under the long day culture condition

Treatments (μM)	% of thallus with cluster of spermatangia	Elongation rate (mean ± SD% day^{-1})
Control (No treatment)	33.3 ± 21.6	17.3 ± 1.2
AVG 5	40.0 ± 37.4	12.5 ± 5.2
AVG 50	33.3 ± 29.4	11.9 ± 1.5
AOA 5	33.3 ± 16.3	15.2 ± 3.0
AOA 50	66.7 ± 8.2	13.5 ± 0.7
AIB 50	100.0 ± 0.0*	12.8 ± 2.1
AIB 500	100.0 ± 0.0*	8.4 ± 5.7

Data are expressed as means ± SD of three independent experiments with 5 thalli each condition

Asterisks indicate significant differences at $p < 0.05$ between control and treatments

Elongation rate = $[100(BL_t - BL_0)/BL_0]/t$, BL_0 = initial blade length, BL_t = blade length at 10 days culture, t = culture time

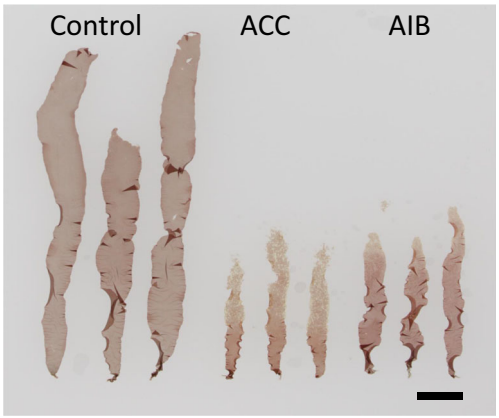

Fig. 3 Effect of α-aminoisobutyric acid (AIB) on sexual reproduction in *Pyropia yezoensis* gametophytes under the short day culture condition. Gametophytes were cultured in media containing 0 or 500 μM ACC, or 500 μM AIB. The thalli treated with ACC and AIB formed spermatangia, which were clear or discolored. Scale bar = 10 mm

inhibits the conversion of ACC to ethylene in higher plants (Satoh and Esashi 1982; Serrano et al. 1990).

Discussion

Our recent study of *P. yezoensis* suggests that ACC acts as a signaling molecule independent of ethylene signaling in the regulation of sexual reproduction through alterations to the redox state (Uji et al. 2020). Interestingly, previous studies also indicate that ACC regulates plant development in the higher plant *Arabidopsis thaliana*, independent of its role as an ethylene precursor (Polko and Kieber 2019; Vanderstraeten et al. 2019). Based on these findings, Vanderstraeten et al. (2019) suggest that AIB may have the capacity to interact with ACC binding proteins (e.g., putative ACC receptors), because AIB is structurally similar to ACC. Supporting this possibility, the present study revealed that AIB promoted the formation of spermatangia and carpospores in gametophytes without endogenous ACC accumulation in a similar manner as ACC. However, the comparative experiment between ACC and AIB treatments revealed that AIB promoted the formation of spermatangia on the upper parts of the thalli, whereas ACC promoted spermatangia formation on the upper to middle sections (Fig. 3). In addition, the number of discharged carpospores in the gametophytes treated with AIB was smaller than that of ACC (Fig. 4).These results suggest that AIB binds on the same target of ACC such as a putative ACC receptor to act ACC signaling, but the binding capacity of AIB may be weaker than that of ACC in *P. yezoensis*. Future work is needed to identify ACC receptor(s) and ACC signaling pathway(s) to uncover the mechanism regulating the sexual reproduction in *Pyropia*.

Recent genetic analyses of mutants that affect biosynthesis or plant hormone responses have revealed a number of signaling

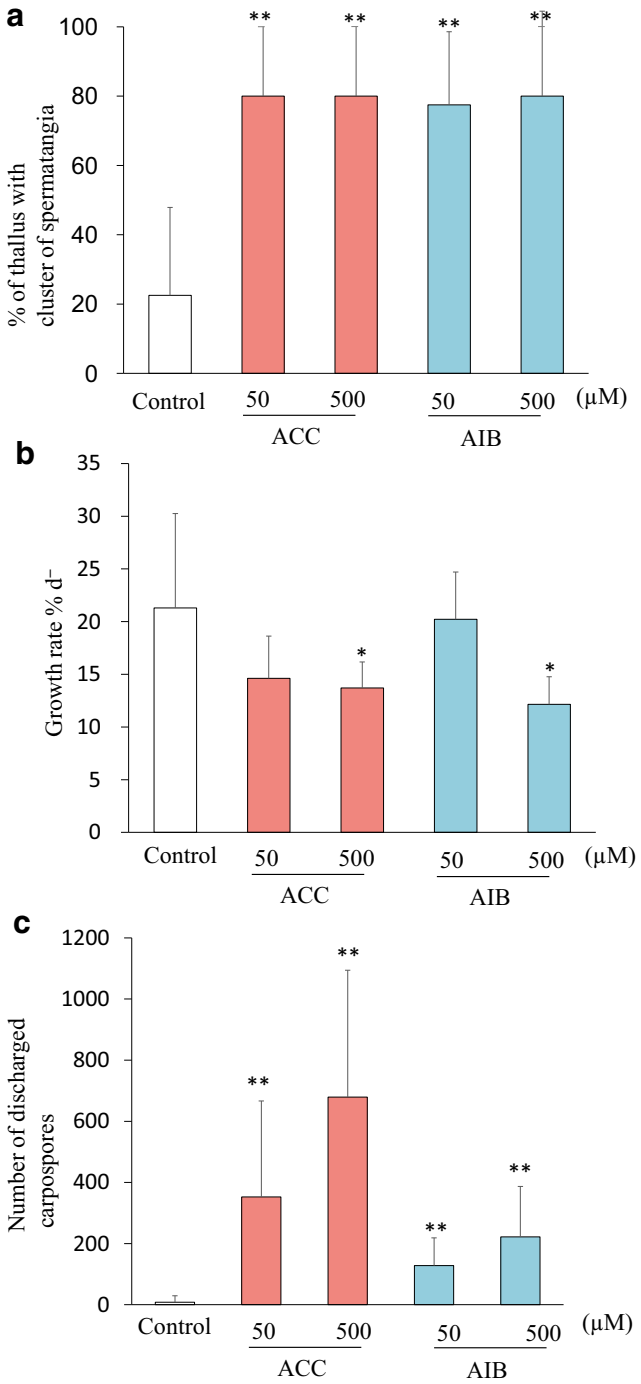

Fig. 4 α-aminoisobutyric acid (AIB) mimics the effect of ACC on the sexual reproduction in *Pyropia yezoensis* gametophytes under the short day culture condition. **a** Formation of spermatangia clusters on gametophytes after 7 day treatment with ACC or AIB. **b** Growth rate of gametophytes after 10 day treatment with ACC or AIB. **c** The number of carpospores released from gametophytes after 14 day treatment with ACC or AIB. Data are expressed as mean ± SD. of eight independent experiments with five thalli for each condition. Asterisks and double asterisks indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, between control and treatments

pathways in higher plants (Browse 2009; Zhao and Li 2012). To elucidate the role of ACC, the analysis of ACC response to

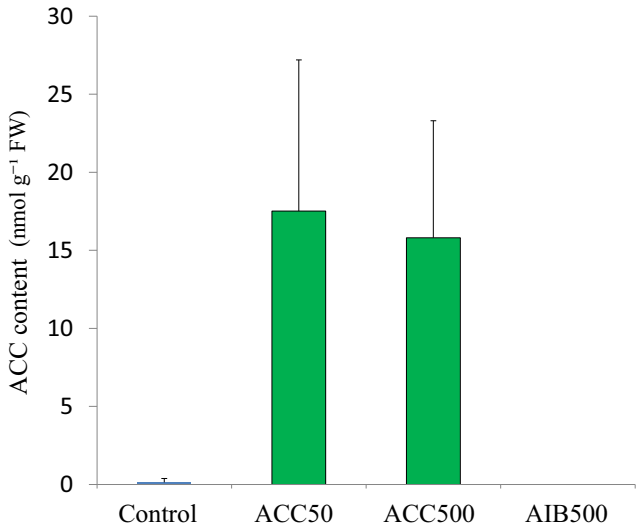

Fig. 5 Effect of α-aminoisobutyric acid (AIB) on ACC accumulation in *Pyropia yezoensis* gametophytes. Gametophytes were cultured in media containing 0 (control) or 50, 500 μM ACC, or 500 μM AIB. The ACC production is expressed as mean ± SD (nmol g^{-1} fresh wt.)

ethylene-insensitive mutant contributed to the understanding of the ACC dependent pathway (Tsuchisaka et al. 2009; Tsang et al. 2011). However, a rapid and simple method of obtaining stable mutants has not yet been established for red macroalgae. Thus, knowledge about the effects of small molecules that act as agonists, antagonists, and inhibitors of biosynthetic pathways for plant hormones is indispensable to advance macroalgal plant hormones research. In the previous study, exogenous application of ACC induced ethylene emission in *P. yezoensis*, indicating that the effect of ACC treatment is indistinguishable between the ACC- and ethylene-dependent pathways (Uji et al. 2016a, b). In contrast, AIB mimics the effect of ACC in *P. yezoensis* gametophytes and thereby discriminates between ACC- and ethylene-responses, indicating that AIB could provide a useful approach to elucidate the ACC-dependent, and ethylene-independent pathways in red algae.

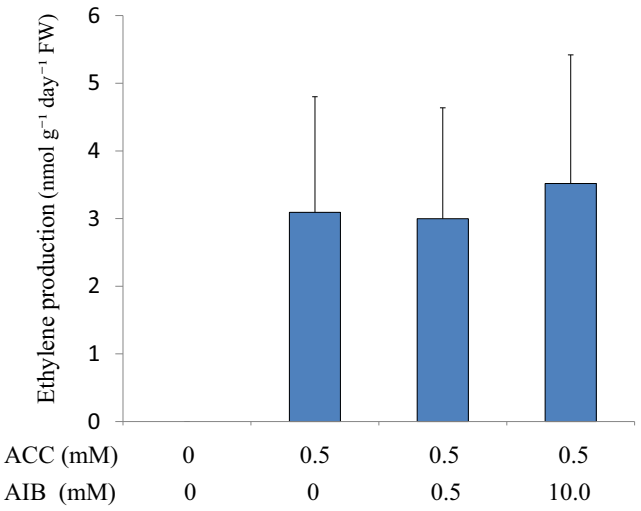

Fig. 6 Effect of α-aminoisobutyric acid (AIB) on ethylene production in *Pyropia yezoensis* gametophytes treated with ACC. The ethylene production is expressed as mean ± SD (nmol day^{-1} g^{-1} fresh wt.)

Genome sequence analyses have revealed that the homolog of the ACO gene is absent in the genomes of red algae, including *P. yezoensis*. Consistent with this, AIB application failed to inhibit ethylene production in the gametophytes (Fig. 6). In addition to the absent ACO gene, there is no homolog for the receptors and components of the ethylene signaling pathway in the red algal genome. Consistent with these findings, our recent study revealed that exogenous application of ACC promoted the formation of spermatia and carporspores in the gametophytes, whereas ethephon, an ethylene-releasing compound, did not stimulate sexual reproduction (Uji et al. 2020). In addition to the red algal genome, putative homologs for the perception and signaling of ethylene were apparently absent in chlorophyte green algae. This finding is supported by a lack of ethylene-binding activity in two chlorophytes, *Chlamydomonas reinhardtii* and *Acetabularia acetabulum* (Wang et al. 2006). In contrast, the charophyte green alga *Spirogyra pratensis* induced cell elongation in response to ethylene and possesses functionally conserved homologs of a complete set of ethylene-signaling genes (Ju et al. 2015). These findings suggest that the origin of ethylene response and signaling in plants occurred during the evolution of the charophyte lineage, prior to the colonization of land (Ju et al. 2015). However, exogenous application of ethylene promoted tetrasporogenesis in the red marine alga *Pterocladiella capillacea* (Garcia-Jimenez and Robaina 2012) and regulated gene expression during carposporogenesis in the red marine alga *Grateloupia imbricata* (Garcia-Jimenez et al. 2018). Hence, the physiological roles of ethylene in red algae require further investigation.

In the present study, treatment with ACS inhibitors generally used in higher plants, such as AVG and AOA did not have a significant effect on sexual reproduction in *P. yezoensis*. On the other hand, exogenous application of AIB induced the formation of spermatangia and carpospores without endogenous ACC accumulation, suggesting that AIB have the capacity to interact with ACC binding proteins to induce the sexual reproduction. This study supports that ACC acts as a plant hormone independent of ethylene in *Pyropia*. Further studies on the identification of ACC receptor(s) and ACC signaling pathway(s) could contribute to elucidate the role of ACC beyond the precursor of ethylene in red algae.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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