1-aminocyclopropane-1-carboxylic acid and its analogs alleviate heat stress damage in the marine red alga *Neopyropia yezoensis* (Rhodophyta)

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Running title: Effects of ACC on thermotolerance in red seaweed

Abstract

Heat stress disrupts algal growth, development, and physiological processes, such as photosynthesis, and eventually decreases seaweed productivity. Previous studies of crop plants have revealed that exogenous application of phytohormones prior or parallel to stress can alleviate the negative effects of abiotic stressors, including heat stress. However, there is limited information on phytohormone-induced tolerance to abiotic stressors in seaweed. In the present study, the application the major plant hormones abscisic acid and salicylic acid failed to mitigate the negative effects of heat stress on the marine red alga *Neopyropia yezoensis*, whereas 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of the plant hormone ethylene, regulates thermotolerance. In addition, the ACC analogs 1-aminocyclobutane-1-carboxylic acid and α-aminoisobutyric acid enhanced tolerance to heat stress. ACC increased the expression of genes involved in antioxidant defense systems to protect photosynthesis and respiration. These results suggest ACC acts as a phytohormone to mitigate the impact on heat stress independent of ethylene in *N. yezoensis*.

Keywords: *Neopyropia*; red algae; heat stress; 1-aminocyclopropane-1-carboxylic acid; plant hormone

1. Introduction

Marine red algae in the order Bangiales, which includes the genera *Pyropia* and *Neopyropia* (formerly *Porphyra*), have been cultivated for several hundred years in Japan and are currently among the most successful aquaculture industries in East Asia, accounting for more than one billion USD in revenue annually (Blouin et al. 2011). Bangiales contains high level of minerals (e.g., iron and zinc), vitamins (e.g., B12 and C), and proteins (Noda 1993), in addition to the sulfated polysaccharide porphyran, which has diverse physiological activities beneficial to human health, including antitumor, immunomodulating, anti-inflammatory, and anti-hyperlipidemic effects (Isaka et al. 2015). These benefits could substantially increase the market demand for Bangiales in the near future.

Abiotic stressors affect the growth, survival, cell division, photosynthesis, and subsequent quality and yield of Bangiales. Among potential abiotic stressors, heat stress can disrupt cellular homeostasis, leading to severe retardation of growth and development, and even death. Under heat stress conditions, the survival rate of conchospore germlings of *Neoporphyra haitanensis*, was reduced to 15.9% after 15 days of culture at 28°C (Yan et al. 2010). Thus, global warming is likely to significantly impact nori cultivation. Actually, during the early seeding period, nori farms are often

55 exposed to sustained high temperatures followed by a drop to temperatures suitable for
56 conchospore release, resulting in the inhibition of germling development, the induction
57 of disease, and large-scale blade decay, resulting in a dramatic reduction in yield (Yan et
58 al. 2010; Zhang et al. 2011).

59 Phytohormones are endogenous signaling molecules that play important roles in
60 growth, development, and responses to various biotic and abiotic stressors, including
61 heat stress (Verma et al. 2016; Li et al. 2021). The major plant hormones, including
62 abscisic acid (ABA), cytokinins, salicylic acid (SA), jasmonic acid, ethylene, and
63 brassinosteroids, play crucial roles in the response of land plants to heat stress (Verma et
64 al. 2016; Li et al. 2021). Numerous studies have reported that exogenous applications of
65 plant hormones prior or parallel to heat stress regulate thermotolerance of plants through
66 the reactive oxygen species (ROS) signaling network (Khan et al. 2015; Devireddy et al.
67 2021; Kothari and Lachowiec 2021). In contrast to land plants, there have been
68 relatively few reports of the effects of phytohormones on the thermotolerance of
69 seaweed.

70 The non-proteinogenic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC),
71 the direct precursor of ethylene, is a simple two-carbon molecule with profound effects
72 in higher plants (Lin et al. 2009; Van de Poel et al. 2015). Exogenous application of

73 ACC has been used as a proxy for ethylene in numerous experiments over decades of
74 research on ethylene signaling. However, recent findings have suggested that ACC also
75 acts as a signaling molecule to regulate plant development and growth, independent of
76 ethylene in the model plant *Arabidopsis thaliana* (Polko and Kieber 2019; Van de Poel
77 2020) and the basal land plant *Marchantia polymorpha* (Li al. 2020; Katayose et al.
78 2021). Previous studies by our group showed that ACC stimulates the formation of
79 sexual cells and protects gametophytes of *Pyropia/Neopyropia* species against oxidative
80 stress (Uji et al. 2016; Yanagisawa et al. 2019). In addition, the exogenous ACC analog
81 1-aminocyclobutane-1-carboxylic acid (ACBC) induced gametogenesis and oxidative
82 stress tolerance in the same manner as ACC, but not ethephon, an ethylene-releasing
83 compound (Uji et al. 2020). Similarly, α-aminoisobutyric acid (AIB), a structural analog
84 of ACC that blocks the conversion of ACC to ethylene in higher plants, mimics the
85 effect of ACC to induce sexual reproduction without endogenous ACC accumulation
86 (Endo et al. 2021). These findings suggest a possible role of ACC as a signaling
87 molecule independent of ethylene in the regulation of sexual reproduction and stress
88 tolerance in Bangiales.
89 An effective approach to improve heat tolerance in Bangiales is important for
90 continued production in response to global warming. The objective of the present study

was to assess the effect of exogenous plant hormones, including ACC, on conferring tolerance to heat stress in *Neopyropia yezoensis*, which is widely cultivated in Japan. The results revealed that the activation of ACC signaling can promote heat tolerance of *N. yezoensis* gametophytes by activation of genes associated with antioxidant defense systems by supporting photosynthesis and respiration.

2. Materials and methods

2.1. Algal material culture and pretreatment

The leafy gametophytes of *P. yezoensis* strain TU-1 were cultured in sterile vitamin-free Provasoli's enriched seawater (PES; Provasoli, 1968) as described previously (Uji and Mizuta, 2021). Immature gametophytes (blade length, ~20 mm) were grown in a 90-mm diameter Petri dish with 40 mL of PES at 15°C under a 10:14-h light:dark photoperiod using cool white fluorescent lamps at 40 μmol photons $m^{-2}s^{-1}$. Our previous study showed that 50 μM ACC was satisfactory to induce sexual reproduction in *N. yezoensis* (Uji et al. 2020), but application of 5 μM ACC weakly promoted it. In addition, a previous experiment revealed that ACC content in the gametophytes was in fairly small amounts (Endo et al. 2021). Based on these results, prior to heat stress treatment, the

gametophytes were treated with 0, 5, 25, or 50 μM ACC (Tokyo Chemical Industry, Tokyo, Japan), 50 μM SA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), or 50 μM ABA (Tokyo Chemical Industry). To assess the effects of the ACC analogs, the gametophytes were also treated with 5 or 50 μM ACBC (Tokyo Chemical Industry) or AIB (Tokyo Chemical Industry). SA and ABA were dissolved in dimethyl sulfoxide (DMSO) to create stock solutions of 100 mM. ACC, ACBC, and AIB were dissolved in PES medium. Control experiments were performed with DMSO at concentrations corresponding to the maximum volume of the reagents.

2.2. Evaluation of tolerance to heat stress

To examine the effects of plant hormones on tolerance to heat stress, gametophytes were treated with chemical reagents for 7 days in a chamber at 15°C under a 10:14-h light:dark photoperiod at 40 μmol photons $m^{-2}s^{-1}$ and then transferred into a Petri dish containing new PES medium without chemical reagents and exposed to heat stress. The gametophytes were cultured for 2 weeks in a chamber at 28°C under a 10:14-h light:dark photoperiod at 40 μmol photons m^{-2} s^{-1}. After the stress treatments, thalli were used for measurement of the maximum photochemical efficiency of

125 photosystem II (PS II) (F_v/F_m) to evaluate heat stress-induced damage using a portable
126 chlorophyll fluorometer (Opti-Science, Inc., Hudson, NH, USA.). Data are expressed as
127 the mean ± standard deviation (SD) of five thalli for each condition.
128
129 **2.3. Transcriptional analysis**
130 Vegetative gametophytes (blade length, ~20 mm; 0.1 g fresh weight) were cultured in
131 100 mL of medium containing 50 µM ACC for 0, 3, or 7 days, then were frozen with
132 liquid nitrogen and stored at −80°C until RNA extraction. RNA extraction and
133 quantitative real-time polymerase chain reaction (qRT-PCR) analysis were performed as
134 described by Uji et al. (2019). Total RNA was extracted using the RNeasy Plant Mini
135 Kit (Qiagen, Hilden, Germany) in liquid nitrogen with a mortar and pestle, in
136 accordance with the manufacturer's instructions. The extracted RNA was purified with
137 the TURBO DNA-free kit (Invitrogen/Life Technologies, Carlsbad, CA, USA) to obtain
138 DNA-free RNA. First-strand cDNA was synthesized from 0.5 µg of total RNA using
139 the PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Inc., Shiga, Japan). For
140 qRT-PCR analysis, each 20-µL reaction volume consisted of 1.0 µL of cDNA diluted
141 by 10-fold as a template with KOD SYBR® qPCR Mix (Toyobo Co., Ltd., Osaka,

142 Japan) in accordance with the manufacturer's instructions. qRT-PCR was performed
143 with a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland) in accordance
144 with the following cycling conditions: an initial denaturation step at 95°C for 30 s,
145 followed by 40 cycles at 95°C for 5 s and at 55°C for 31 s. Relative gene expression
146 levels were calculated using the $2^{-\triangle\triangle Ct}$ method with the 18S ribosomal RNA
147 (*Ny18SrRNA*) gene as an internal reference. The relative expression level was calculated
148 as a ratio of the mRNA level to the transcription level on day 0 of ACC treatment. All
149 qRT-PCR analyses were performed in triplicate. The primers used in this study are
150 listed in Table 1. The sequences of the analyzed genes were retrieved from the genome
151 sequence data of *N. yezoensis* (Nakamura et al. 2013).

152

153 **2.4. Statistical analysis**

154 All data are expressed as the mean ± SD. The Mann–Whitney U test was used to
155 identify statistically significant differences following treatment with and without plant
156 hormones. For all analyses, $p < 0.05$ (significant) and $p < 0.01$ (highly significant) were
157 considered thresholds of statistical significance.

158

159

160 **3. Results**

161 *N. yezoensis* gametophytes cultured at 15°C were subjected to heat stress (28°C) after
162 treatment with SA, ABA, or ACC to assess the role of plant hormones in heat tolerance.
163 After heat stress treatment, spots of discoloration, which indicate a drastic decrease in
164 photosynthetic pigments, were observed in *N. yezoensis* pretreated with DMSO
165 (control), 50 μM SA, or 50 μM ABA, whereas pretreatment with 50 μM ACC mitigated
166 the discolored spots (Fig. 1). In response to heat stress, gametophytes pretreated with
167 DMSO, SA, or ABA had cells with yellow-green chloroplasts, enlarged cells or dead
168 cells. In contrast, pretreatment with ACC prevented the formation of cells with
169 yellow-green chloroplasts or enlarged cells (Fig. 1).

170 Maximum quantum efficiency (F_v/F_m) using pulse amplitude modulation techniques
171 was employed to assess the impact of heat stress on the photosynthetic capacity in *N.*
172 *yezoensis*. The F_v/F_m value of gametophytes treated with DMSO, SA, or ABA ranged
173 from 0.17 to 0.23, whereas that of gametophytes supplemented with ACC was 0.47 (Fig.
174 2).

175 Next, the effects of different concentrations of ACC against heat stress in
176 gametophytes supplemented with ACC were compared. There was a significant

177 difference in the F_v/F_m values of gametophytes pretreated with and without ACC (Fig.
178 3).The F_v/F_m value of gametophytes pretreated with a lower concentration of ACC (5
179 μM) slightly decreased as compared with those treated with higher concentrations (25
180 or 50 μM) in response to heat stress.

181 Also, the effects of the ACC analogs ACBC and AIB on heat stress tolerance in
182 gametophytes was investigated. The F_v/F_m values of thalli treated with 5 or 50 μM ACC
183 or 50 μM ACC analogs were relatively high in response to heat stress (5 μM ACC,
184 0.33; 50 μM ACC, 0.47; 50 μM ACBC, 0.46; 50 μM AIB, 0.46) (Fig. 4). However,
185 there was no significant difference in the F_v/F_m values of thalli treated with 5 μM
186 ACBC or 5 μM AIB as compared to the control (Fig. 4). These results indicate that the
187 ACC analogs were less effective than ACC, and ACC enhanced heat stress tolerance
188 independent of ethylene.

189 To understand the role of ACC treatment in acquired thermotolerance, the
190 expression profiles of genes involved in stress responses were examined (Fig. 5). Based
191 on our previous RNA-seq data in response to ACC (Uji et al., 2016), four candidate
192 genes associated with thermotolerance were selected (Table 2). The mRNA levels of
193 *NyALDH* (encoding aldehyde dehydrogenase), *NyHLIP* (encoding high light-inducible
194 protein), and *NyPOD* (encoding haem peroxidase) had gradually increased after ACC

195 treatment. The exogenous application of ACC resulted in the up-regulation of *NyBCS1L*
196 (encoding BCS1-like ATPase) to similar levels at 3 and 7 days after ACC treatment.
197

198 **4. Discussion**

199 In the present study, we revealed that pretreatment with ACC for 1 week can mitigate
200 the negative impacts of heat stress in *N. yezoensis* thalli. Thus, exogenous application of
201 ACC to nori seedlings before setting seeding nets in bays and inland seas could
202 significantly increase the yields under climate change. However, previous studies by our
203 group showed that ACC treatment inhibited the growth of *N. yezoensis* (Uji et al. 2016;
204 2019). Organisms have evolved diverse mechanisms of "tradeoff" that enable the
205 allocation of resources for growth to adapt life-threatening stress (Takatsuji 2017).
206 Elucidating the mechanisms mediating tradeoffs between the stress tolerance and the
207 growth under ACC signaling should be necessary to apply ACC as a tool in nori
208 aquaculture.

209 Phytohormones are endogenous signaling molecules that play important roles in
210 various aspects of plant development, growth, and stress responses (Verma et al. 2016;
211 Gray 2004; Yu et al. 2020). Many studies have found that exogenous application of

212 phytohormones, such as ABA and SA, significantly ameliorated heat-induced damage
213 and improved heat tolerance in land plants (Li et al. 2021; Devireddy et al. 2021).
214 Although current knowledge of plant hormone-mediated stress tolerance in algae
215 remains limited, SA is reported to alleviate the adverse effects of high-temperature
216 stress in the red alga *Gracilariopsis lemaneiformis* (Wang et al. 2017). In the current
217 study, SA and ABA had no effect on heat stress tolerance in *N. yezoensis*. In contrast,
218 the exogenous application of ACC as well as the analogs ACBC and AIB induced the
219 acquisition of heat stress tolerance. In addition to classical phytohormones, recent
220 studies have proposed that ACC, the direct precursor of the plant hormone ethylene, acts
221 as a signaling molecule to regulate development and growth independent of ethylene
222 biosynthesis in land plants and red algae (Van de Poel 2020). This report is the first to
223 describe the involvement of ACC signaling in tolerance to heat stress of land plants and
224 algae, independent of ethylene signaling.

225 Heat stress enhances production of ROS (superoxide [O^{-}_{2}], hydroxyl radicals
226 [OH^{-}], hydrogen peroxide [H_2O_2], and singlet oxygen [$^1O_2$]), which leads to oxidative
227 damage of proteins, nucleic acids, and lipids, and eventual disruption of cellular
228 homeostasis (Suzuki and Mittler 2006; Awasthi et al. 2015). For ROS detoxification,
229 plants produce antioxidants, such as ascorbic acid (AsA) and glutathione, as well as

ROS-scavenging enzymes (Choudhury et al. 2017), such as ascorbate peroxidase (APX), which is among the most important antioxidant enzymes in land plants (Shigeoka et al. 2002). Our previous study showed that ACC increased AsA synthesis in *N. yezoensis* gametophytes, while the expression levels of three APX genes of *N. yezoensis* were only slightly increased or decreased in the thalli after ACC treatment (Uji et al. 2020). In the present study, *NyPOD* transcripts had accumulated in gametophytes after ACC treatment, implying that NyPOD may play an important role as a ROS-scavenging enzyme during abiotic stress in *N. yezoensis*.

Excessive ROS accumulation also induced the production of aldehydes, which can cause genotoxic effects, such as lipid peroxidation, resulting in the loss of membrane integrity and subsequent cellular and developmental arrest (Kotchoni et al. 2006; Stiti et al. 2011). Thus, genomes of organisms encode aldehyde dehydrogenase (ALDH) enzymes that catalyze the oxidation of various aldehydes to carboxylic acids, thereby reducing lipid peroxidation (Stiti et al. 2011). Increased fluidity of the thylakoid membranes at high temperature causes light-harvesting complexes, located in the core of photosystem II (PSII), to become dislodged from the thylakoid membrane (Mathur et al. 2014). Previous studies of gain- and loss-of-function mutations in land plants suggest that ALDH enzymes reduce lipid peroxidation of thylakoid membranes under oxidative

248 stress conditions (Kotchoni et al. 2006; Zhao et al. 2017). In addition, ALDH protein
249 expression was elevated in *N. yezoensis* under high temperature and H_2O_2 stress
250 conditions, and recombinant NyALDH enhanced salinity and oxidative stress tolerance
251 in *E. coli* (Lee and Choi, 2018). In this study, the application of ACC increased
252 *NyALDH* expression, suggesting that detoxification of aldehydes is important to protect
253 peroxidation of lipid membranes to maintain photosynthetic capacity under heat stress
254 conditions.
255 Photosynthesis is among the most heat-sensitive physiological processes
256 because chloroplast is the primary sites of ROS production in response to heat stress
257 (Hu et al. 2020). ROS, which are produced by PSI and PSII as well as the Calvin–
258 Benson cycle, can cause irreversible oxidative damage to plant cells subjected to heat
259 stress (Suzuki et al. 2012; Wang et al. 2018). In the present study, *N. yezoensis* cells
260 subjected to heat stress contained yellow-green chloroplasts and the F_v/F_m value was
261 decreased. However, ACC pretreatment mitigated these changes. As described above,
262 ACC treatment greatly increased the AsA content of *N. yezoensis* thalli (Uji et al. 2020).
263 AsA plays a protective role in photoinactivation by serving as a PSII electron donor,
264 which can alleviate photodamage to PSII reaction centers caused by the accumulation of
265 ROS under abiotic stress, including heat stress, conditions (Tóth et al. 2009; 2011).

266 Thus, an increase in AsA during ACC treatment may retard the photoinactivation of
267 PSII under heat stress in *N. yezoensis*, thereby alleviating damage to the photosynthetic
268 machinery. Furthermore, exogenous application of ACC increased the *NyHLIP*
269 transcripts encoding a homolog of HLIP of cyanobacteria, which is similar to
270 light-harvesting chlorophyll a/b-binding proteins (Komenda and Sobotka 2016).
271 Transcript levels of cyanobacterial HLIPs increase in response to high light conditions
272 (Dolganov et al. 1995; He et al. 2001) and play a protective role against generation of
273 singlet oxygen to prevent photoinactivation of PSII (Sinha et al. 2012; Komenda and
274 Sobotka 2016). In a previous report, *NyHLIP* transcripts were upregulated under high
275 irradiation and low temperature conditions, suggesting that NyHLIP functions not only
276 in response to excess light, but also to protect the cell against other stressors (Kong et al.
277 2012). In this study, the activation of NyHLIP in response to ACC may have also
278 contributed to protect the photosynthetic apparatus against heat stress.
279 In addition to chloroplasts, the mitochondria are also main targets of oxidative
280 damage under abiotic stress conditions (Bartoli et al. 2004). A previous study reported
281 that *BCS1* mRNA levels of *Arabidopsis*, an ortholog of yeast *BCS1* involved in
282 biogenesis of the cytochrome bc_1 complex (Nobrega et al. 1992), increased in response
283 to SA and H_2O_2 (Ho et al. 2008). Similarly, in the present study, exogenous application

284 of ACC upregulated *NyBCS1* expression, suggesting that BCS1 is key to protect
285 mitochondria against abiotic stress in both red and green lineage. Hence, future studies
286 are warranted to elucidate the role of BCS1 in the stress response.
287 As mentioned above, ROS can cause oxidative damage to cells during
288 environmental stress. However, ROS also play a key role as signal transduction
289 molecules in plants by mediating various stress responses through interactions with
290 hormonal signaling (Mittler et al. 2004; Torres and Dangl 2005; Suzuki et al. 2013). In
291 signal transduction-associated ROS, respiratory burst oxidase homolog (*Rboh*) genes,
292 encoding nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, are the
293 main producers of ROS (Mittler et al. 2004; Torres and Dangl 2005). Loss of function of
294 NADPH oxidase is reported to impair the heat stress response in *Arabidopsis*
295 (Larkindale et al. 2005, Miller et al. 2009). In Bangiales, the formation of sporophytes
296 (also called as the conchocelis phase), which grow in the summer, produce significantly
297 higher levels of H_2O_2 accompanied with higher activities of NADPH oxidase as
298 compared to gametophytes, which usually grow in the winter (Luo et al. 2014). In
299 addition, the induction of H_2O_2 and mRNA levels of *Rboh* was observed in Bangiale
300 species under high-temperature conditions (Luo et al. 2014). The results of our previous
301 study revealed that ROS generation in *N. yezoensis* gametophytes treated with ACC was

302 accompanied by an increase in *Rboh* transcripts (Uji et al. 2020), implying that
303 fine-tuning the balance of ROS by ACC treatment contributed to prevent oxidative
304 damage caused by heat stress in *N. yezoensis* gametophytes.
305 Heat shock proteins (HSPs), which function as molecular chaperones in
306 maintaining homeostasis in protein folding, are assumed to play a central role in
307 acquired thermotolerance in plants (Wang et al. 2004; Kotak et al. 2007). To date, five
308 families of HSPs have been defined based on molecular sizes: Hsp100, Hsp90, Hsp70,
309 Hsp60, and small HSPs (sHSPs/HSP20). In addition to HSPs, multiprotein bridging
310 factor 1 (MBF1), which functions as a non-DNA-binding transcription co-factor or a
311 bona fide transcription factor, plays an important role in response to abiotic stressors,
312 particularly heat stress, in land plants (Suzuki et al. 2011; Jaimes-Miranda and Chávez
313 Montes 2020). Although there is limited evidence of thermotolerance mechanisms in
314 seaweed, the mRNA levels of *NysHSPs* and *NyMBF1* were increased in *N. yezoensis*
315 gametophytes under heat stress conditions (Uji et al. 2013; 2019). On the other hand,
316 the transcript levels of *NysHSPs* were only slightly increased in response to ACC
317 treatment as compared to under heat stress conditions (Uji et al. 2019). Moreover, our
318 RNA-seq data showed that *NyMBF1* was not identified as an ACC-responsive gene in *N.*
319 *yezoensis*. These findings raise the possibility that ACC enhances heat tolerance in *N.*

320 *yezoensis* via a somewhat different response to heat stress. Similarly,
321 brassinosteroid-mediated thermotolerance is not necessary for the expression of HSPs in
322 land plants (Kagale et al. 2007). Future comparisons of thermoprotective mechanisms
323 acquired by ACC application and moderate heat stress will further the current
324 understanding of the pathways that protect *N. yezoensis* against stress-induced damage.
325

326 **5. Conclusion**

327 The results of this study present evidence that ACC can serve as a priming hormone that
328 enables *N. yezoensis* to resistant to heat stress. On the other hand, treatment with the
329 plant hormones ABA and SA, which are regulators of the stress responses in land plants,
330 failed to improve heat stress resistance in *N. yezoensis*. ACC can activate the expression
331 of genes associated with antioxidant defense systems, such as *NyHLIP* and *NyBSC1*, to
332 protect against impaired function of chloroplasts and mitochondria cause by
333 heat-induced oxidative damage. A future challenge will be to elucidate the
334 ACC-associated signaling pathways leading to heat stress tolerance in *N. yezoensis*,
335 which could provide opportunities to generate thermotolerant varieties of Bangiales.
336

337 **Author statement**

TU was responsible for the design of the experiments and interpretation of the data. TU performed the experiments. TU and HM wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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519

520 **Figure legends**

521 Figure 1. Evaluation of exogenous applied plant hormones for heat stress tolerance in *N.*

522 *yezoensis*.

523 Gametophytes were subjected to heat stress at 28ºC for 2 weeks after 1 week of

524 pretreatment with 0 (control), 0.05% DMSO (mock), 50 μM ABA, 50 μM SA, or 50

525 μM ACC. Gametophytes grown at 15ºC were used as controls (Non-HS). Scale bar =

526 100 mm (upper panel), 50 μm (lower panel).

527

528 Figure 2. Effect of exogenous plant hormones on the photosynthetic capacity in *N.*

529 *yezoensis* under heat stress conditions.

530 Maximum photochemical efficiency (F_v/F_m) was assessed in gametophytes subjected to

531 heat stress at 28ºC for 2 weeks after 1 week of pretreatment with 0 (control), 0.05%

532 DMSO (mock), 50 μM ABA, 50 μM SA, or 50 μM ACC. Data are expressed as the

mean ± SD of five thalli for each condition. Double asterisks indicate significant differences at $p < 0.01$ between controls and treatments.

Figure 3. Evaluation of different concentrations of exogenous ACC on the photosynthetic capacity of *N. yezoensis* under heat stress conditions.
Maximum photochemical efficiency (F_v/F_m) was assessed in gametophytes subjected to heat stress at 28ºC for 2 weeks after 1 week of pretreatment with 0 (control), 5, 20 or 50 μM ACC. Data are expressed as the mean ± SD of five thalli for each condition. Double asterisks indicate significant differences at $p < 0.01$ between controls and treatments.

Figure 4. Comparison of exogenous ACC and analogs on the photosynthetic capacity of *N. yezoensis* under heat stress conditions.
(A) Structural formulas of ACC and the analogs used in this study
(B) Maximum photochemical efficiency (F_v/F_m) was assessed in gametophytes subjected to heat stress at 28ºC for 2 weeks after 1 week of pretreatment with 0 (control), 5, 50 μM ACC, ACBC, or AIB. Data are expressed as the mean ± SD of five thalli for each condition. Asterisks and double asterisks indicate significant differences at $p <$ 0.05 or 0.01, respectively, between controls and treatments.

551

552 Figure 5. Relative expression levels of genes associated with stress tolerance of *N.*

553 *yezoensis* in response to ACC.

554 RNA samples were prepared from gametophytes treated with 50 μM ACC for 0, 3, or 7

555 days, and expression levels were normalized to *Ny18SrRNA*. The results are presented

556 as relative expression as compared to non-treated gametophytes (day 0). The data are

557 presented as the mean ± SD of three independent experiments. Asterisks indicate

558 significant differences at $p < 0.05$ between controls and treatments.

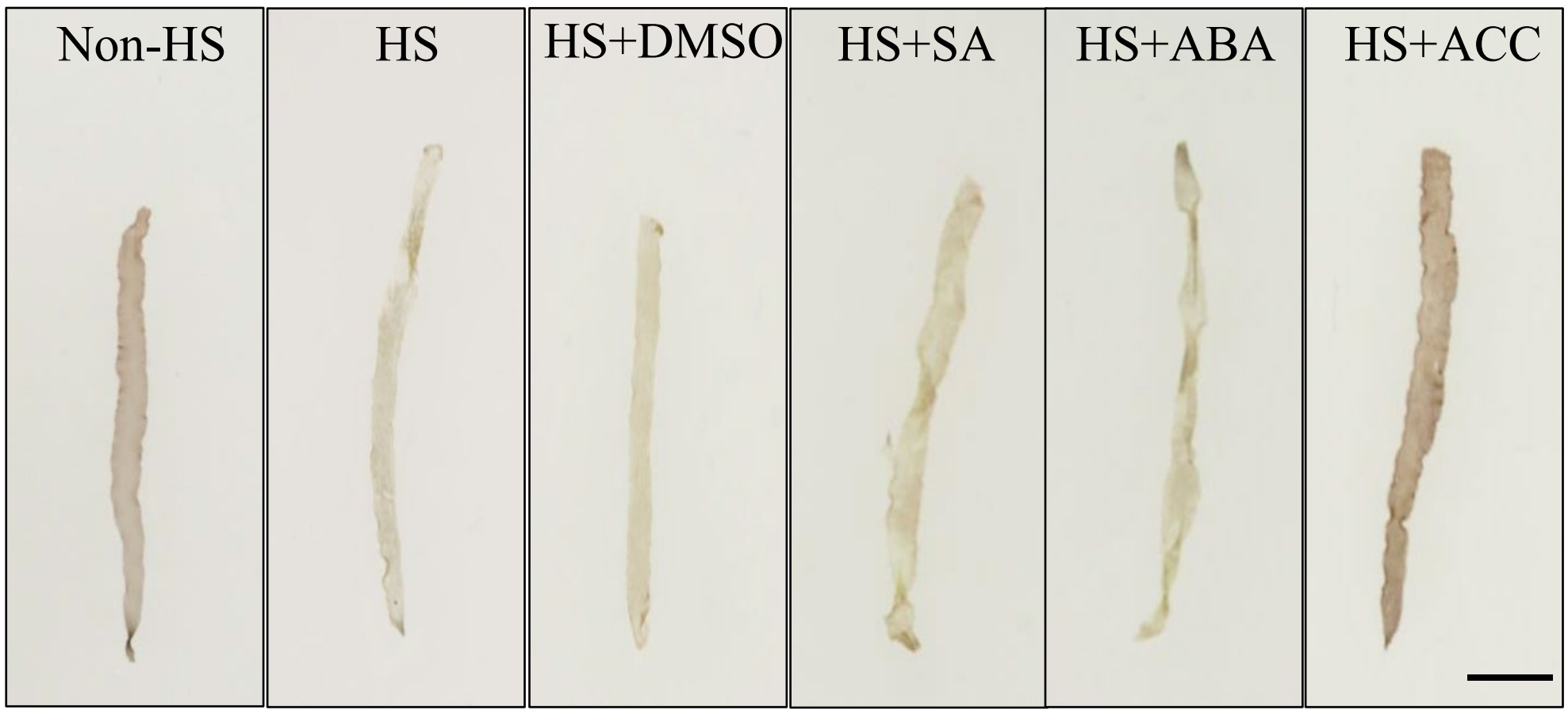

Non-HS
HS
HS+DMSO
HS+SA
HS+ABA
HS+ACC
Non-HS
HS
HS+DMSO
HS+SA
HS+ABA
HS+ACC

Fig.1

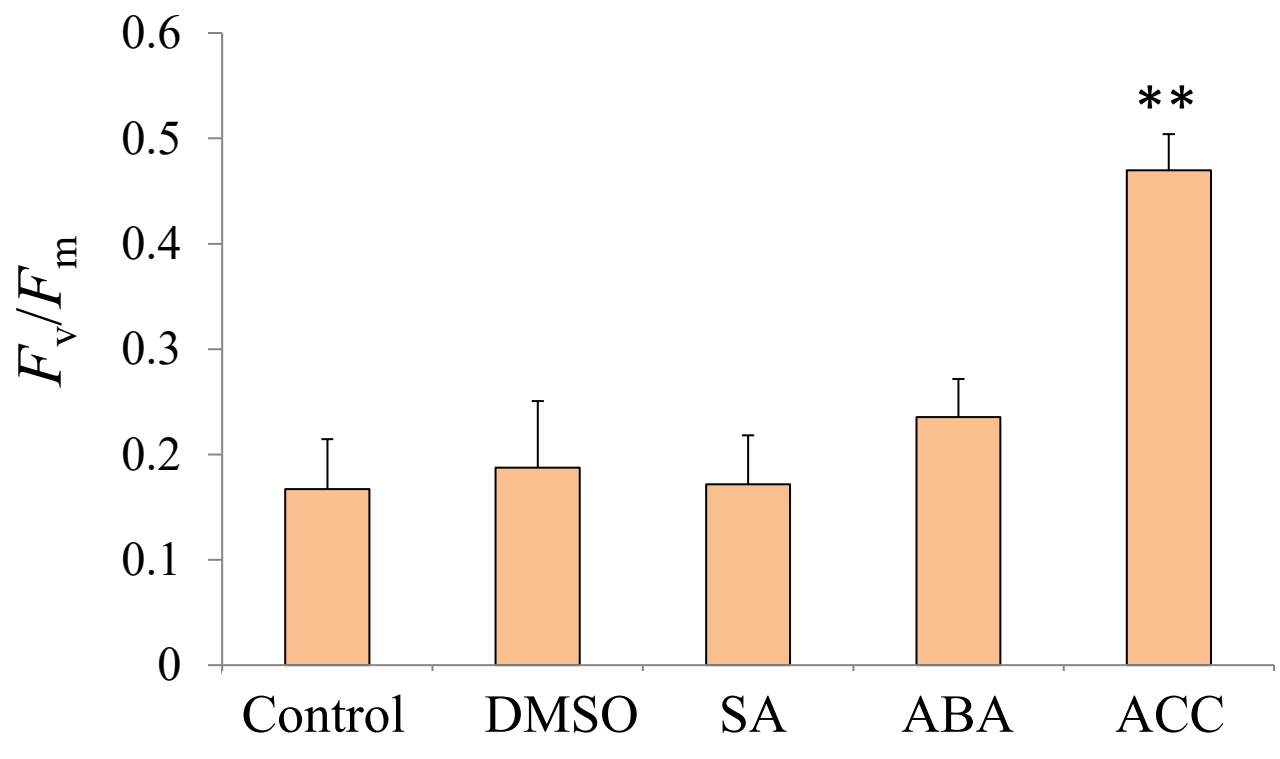


Fig.2

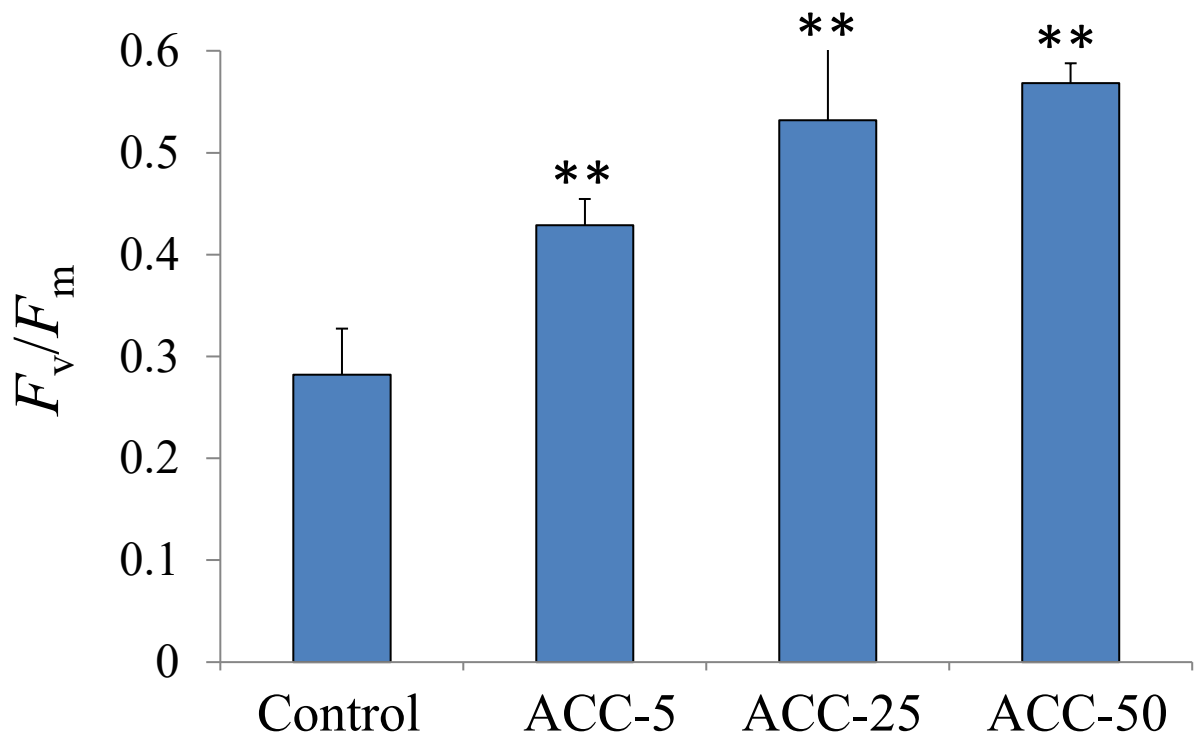
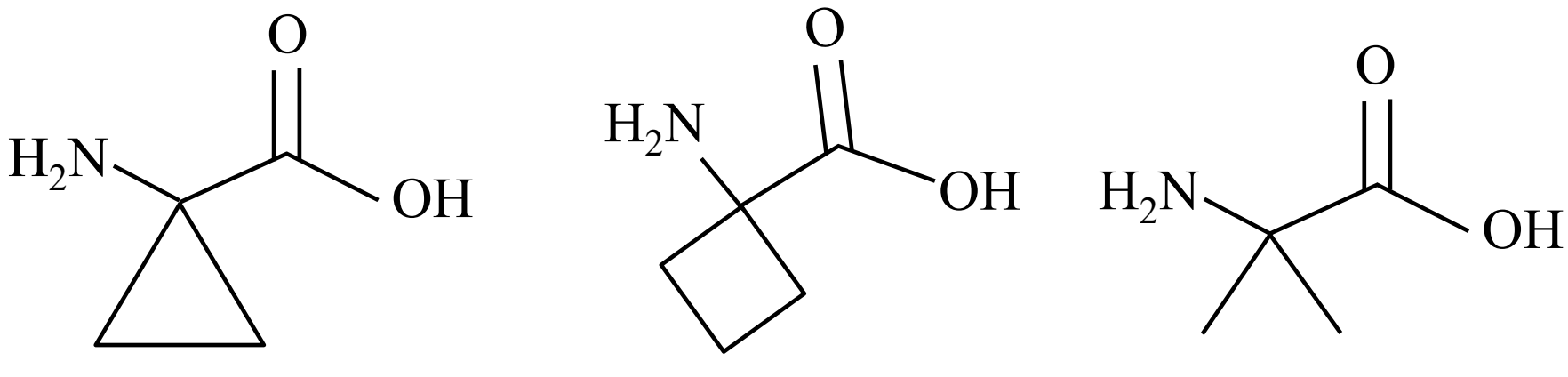


Fig.3

(A)


O
H2N
OH
ACC
ACBC
AIB

(B)

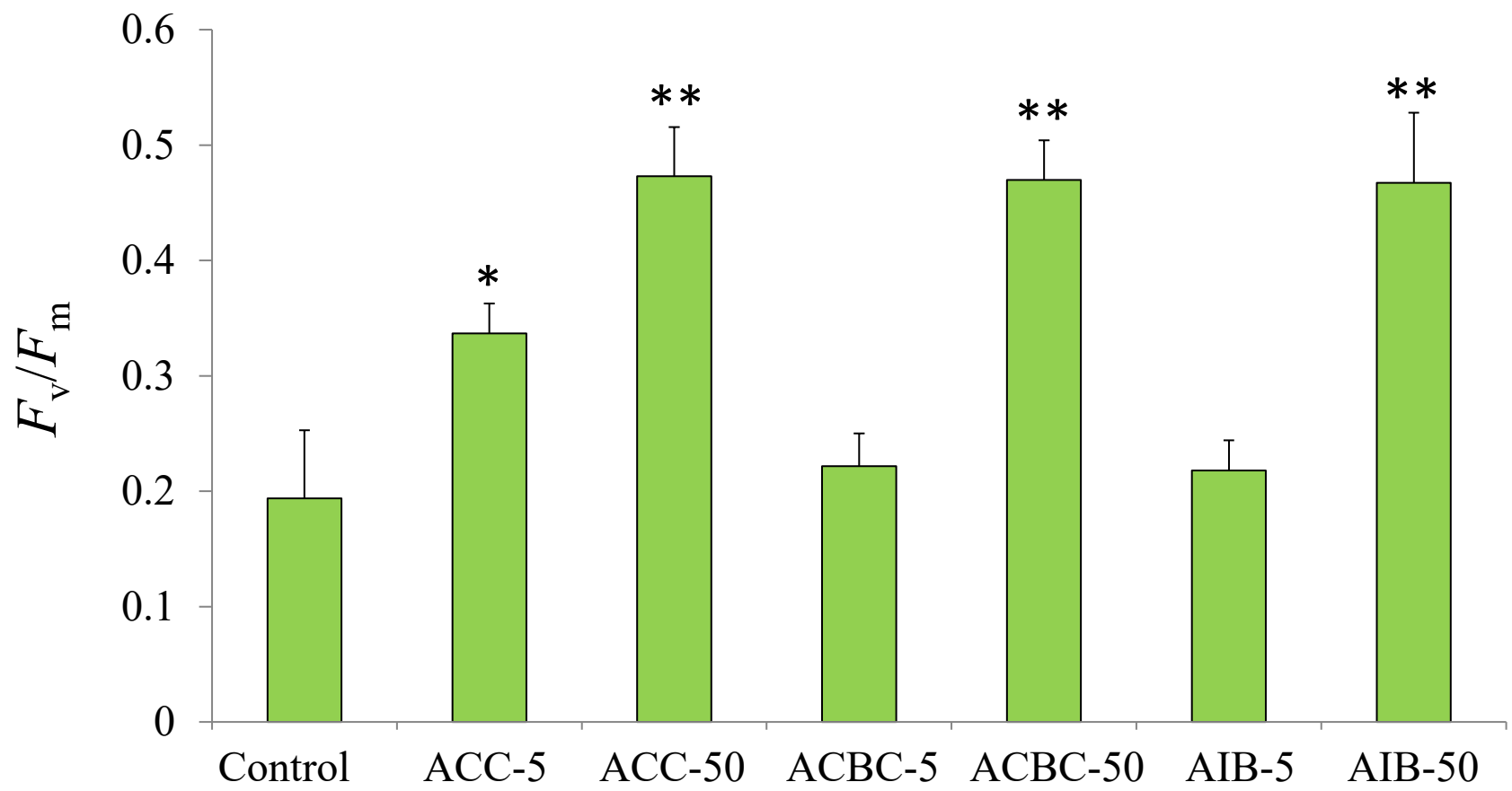
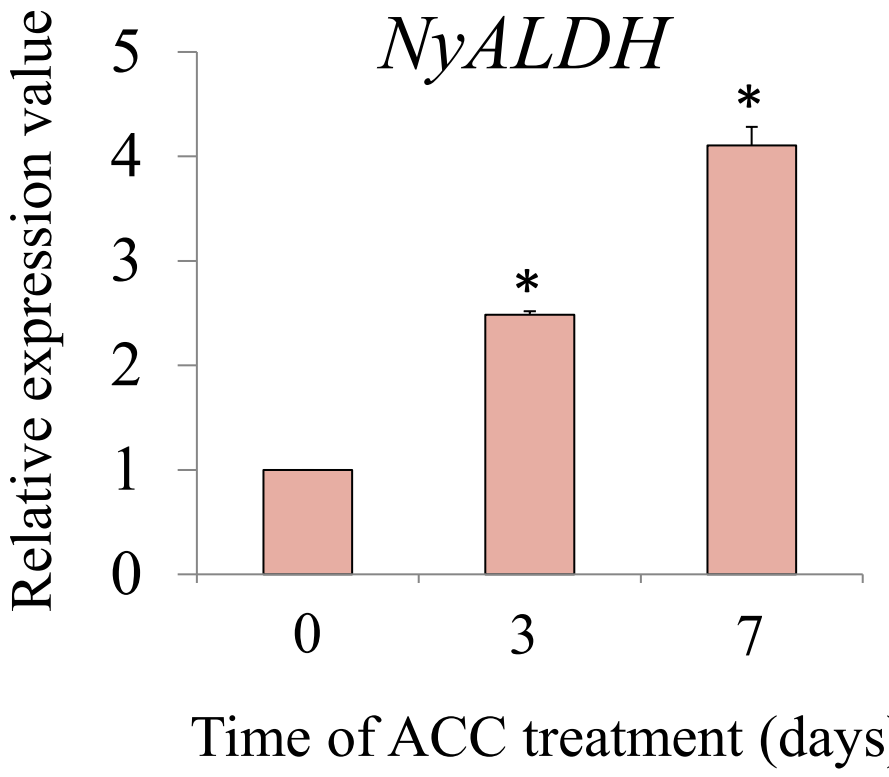
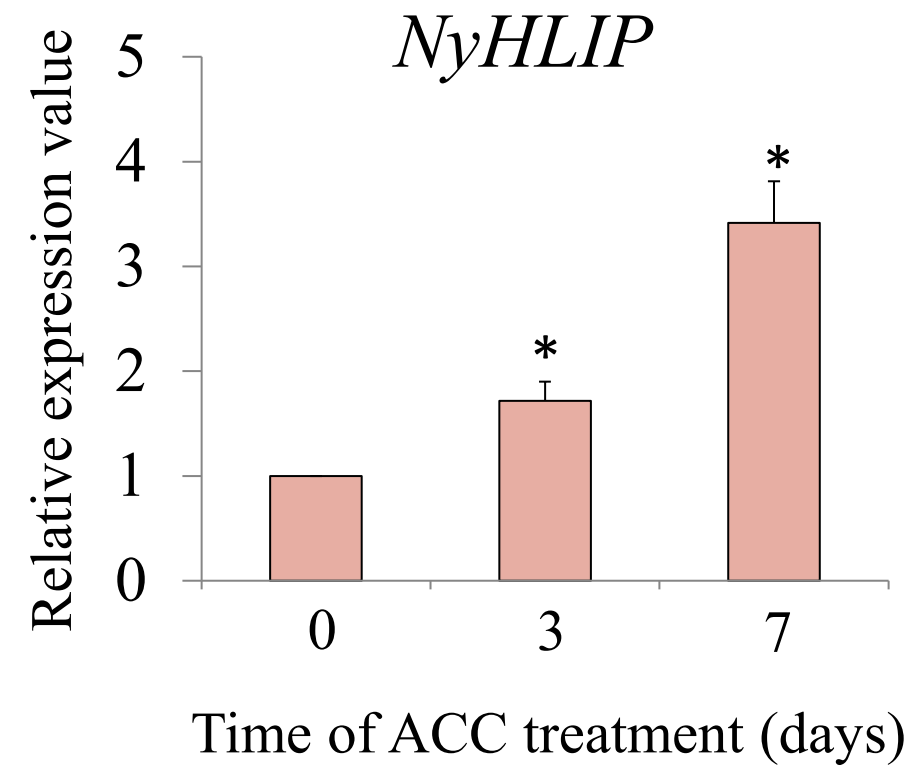
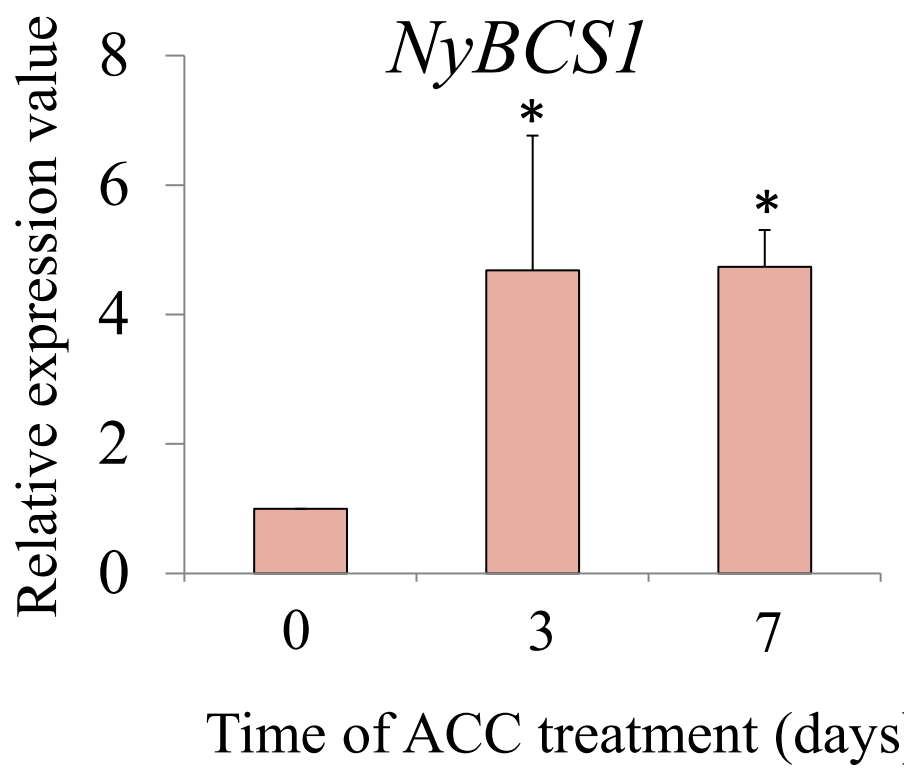

0.6
0.5
0.4
0.3
0.2
0.1
0
Fv/Fm
*
**
**
**
Control
ACC-5
ACC-50
ACBC-5
ACBC-50
AIB-5
AIB-50

Fig.4


NyALDH
Relative expression value
0
1
2
3
4
5
0
3
7
*
*
*
Time of ACC treatment (days)


NyHLIP
Relative expression value
0
1
2
3
4
5
0
3
7
*
*
*
Time of ACC treatment (days)


NyBCS1
Relative expression value
0
2
4
6
8
0
3
7
*
*
*
Time of ACC treatment (days)

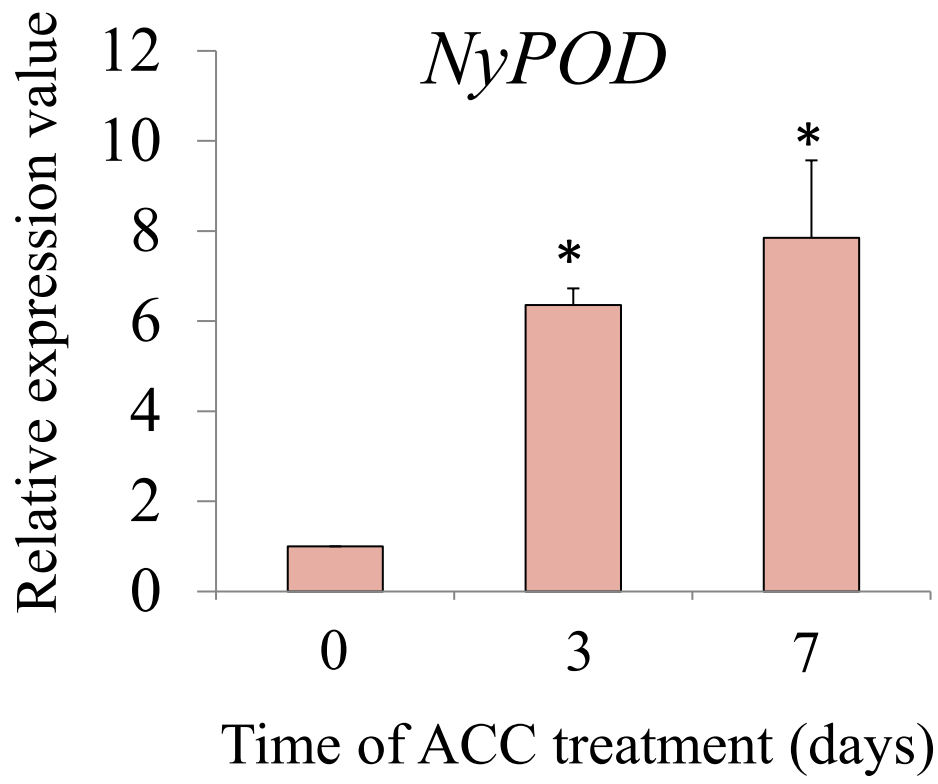

NyPOD
Relative expression value
0
2
4
6
8
10
12
0
3
7
*
*
*
Time of ACC treatment (days)

Fig.5

Table 1. The list of primers used for gene expression analysis by quantitative Real Time PCR

Primer name	contig ID	Sequence (5'-3')
NyALDH-F1	contig_6867_g1587	TACCTGGGATTGGAAAGCTG
NyALDH-R1	contig_6867_g1587	CCAATGAACAGCACATGGTC
NyPOD-F1	contig_27674_g6814	GCACGTACGGCTACCACAC
NyPOD-R1	contig_27674_g6814	CGACGACAATACCCACATCC
NyBCS1L-F1	contig_11756_g2797	AAGGAGGTGAAGCGTGATGA
NyBCS1L-R1	contig_11756_g2797	GGGGGCATACAGGAAAAATG
NyHLIP-F1	contig_21247_g5217	CTTTGTCGGCTCTGCTGTT
NyHLIP-R1	contig_21247_g5217	GGACTGCGCGTTGATCTT
Ny18S-F1	* D79976	AGGGTTGATCCGCAGGGAAG
Ny18S-R1	* D79976	GCTTGCGCCCACTCCATTAG

*Accession number in GenBank

Table 2. The list of tested genes for ACC response in *N. yezoensis*

Contig ID	Abbreviation	Functional categories	Description
contig_6867_g1587	ALDH	aldehyde scavengers	aldehyde dehydrogenase
contig_27674_g6814	POD	oxidation/reduction reaction	haem peroxidase
contig_11756_g2797	BCS1L	mitochondria chaperone	BCS1-like ATPase
contig_21247_g5217	HLIP	photoprotection of photosystem II	high light inducible protein