



Paper-based electroanalytical devices for in situ determination of free 3-indoleacetic acid and salicylic acid in living *Pyropia haitanensis* thallus under various environmental stresses

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Abstract

Phytohormones play a vital role in regulating plant developmental processes and signaling for defense, but little is known of the specific functions of phytohormones in *Pyropia haitanensis*. In this study, paper-based electroanalytical devices for sensitive, in situ detection of free 3-indoleacetic acid (IAA) and salicylic acid (SA) based on their electrocatalytic oxidation under differential pulse voltammetry in *P. haitanensis* thallus were built. Then, this method was used to detect free IAA and SA in *P. haitanensis* thallus under different environmental stresses, including high temperature, high irradiance, desiccation, and exposure to ultraviolet radiation. The concentrations of SA increased after the different environmental stresses were imposed, while the concentrations of IAA significantly decreased after desiccation, exposure to ultraviolet radiation and high light. During exposure to high temperature, the concentrations of IAA increased in the first hour after treatment, and then returned to the normal level for the following 5 h. All of the electrocatalytic measurements were supported by measuring expression levels of IAA and SA synthesis-related genes in *P. haitanensis*. These results help to systematically clarify that IAA acts as a negative regulatory factor in regulating plant resistance to environmental stresses except for high temperature, while SA plays the opposite role.

Keywords Paper-based electroanalytical devices · IAA · SA · Rhodophyta · Environmental stresses

Introduction

Pyropia is a seaweed with high economic value used in food and pharmaceuticals (Cao et al. 2016; Suh et al. 2017).

Qiong Wang, Xiaohui Li and Lingjuan Tang contributed equally to this work.

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Pyropia is mainly cultivated in Japan, South Korea and coastal areas of China (Li et al. 2017). *Pyropis haitanensis* is one of two main species cultivated in the southeast of China (Zhan et al. 2016). As an intertidal macroalga, *P. haitanensis* will be exposed to numerous environmental stresses during different life stages, such as desiccation, high light, high temperature, UV radiation, and so on (Kumar et al. 2011; Qian et al. 2015). To overcome these environmental stresses, *P. haitanensis* has developed many strategies. For example, *P. haitanensis* uses the rapid accumulation of floridoside as the desiccation acclimation strategy (Qian et al. 2015). Beyond that, phytohormones also play important roles in *P. haitanensis* resistance to environmental stresses.

Phytohormones participate in many aspects of algae physiological processes, including growth and sexual reproduction, resistance to abiotic and biotic stresses processes. For example, ACC (the ethylene precursor 1-aminocyclopropane-1-carboxylic acid) can promote the formation of spermatia and zygospores in the gametophytes of *Pyropia yezoensis*, whereas the growth rate was repressed after ACC treatment. Furthermore, gametophytes treated with ACC and mature gametophytes can enhance tolerance to oxidative stress (Uji et al. 2016). Hou et al. (2017) also showed that SA and

jasmonic acid (JA) could promote the growth of algae and enhance the resistance of *P. haitanensis*. Studies from the Chen lab (Wang et al. 2013; Tu et al. 2017; Chen et al. 2018, 2019) showed that agaro-oligosaccharides, flg22, 1-Octen-3-ol, and wounding can affect the contents of different phytohormones such as SA and JA. Transcriptome data showed that the phytohormones GA and ABA also played important roles in responding to temperature stress in *P. yezoensis* (Sun et al. 2015; Wang et al. 2018). Gene expression profiles of small heat shock proteins in *P. yezoensis* showed that exogenous application of ACC could significantly increase the expression levels of small heat shock proteins (Uji et al. 2019). Even so, studies of the roles of phytohormones in macroalgae are limited.

Current research of phytohormones on macroalgae is mainly based on the determination of phytohormones. The most common method to determine phytohormones is to use high-performance liquid chromatography (HPLC) hyphenated with mass spectrometry (MS). Tu et al. (2017) using LC-MS reported that the treatment of flagellin peptide flg22 could reduce the concentration of SA and induce the concentration of JA in *P. haitanensis*. Song et al. (2017) using HPLC-MS showed that high temperature induced changes in concentrations of seven different phytohormones in *P. haitanensis*. Although HPLC-MS has high sensitivity, it requires larger sample amounts and a complicated process of sample treatment, including samples being ground in liquid nitrogen, extracted with ultra-sonication, separation using chromatography columns, and drying under N₂ before being measured by HPLC-MS (Bai et al. 2010; Du et al. 2012). However, this time-consuming process is not suitable for phytohormones such as IAA and SA, because of their instability. Thus, employing the HPLC-MS method may not maintain the original amounts of phytohormones.

Recently, an alternative approach has been reported that could directly obtain the concentration of IAA and SA in plants based on electrochemical oxidation or reduction of those molecules in the liquids which requires simple sample treatment (McLamore et al. 2010; Liu et al. 2014; Seo et al. 2016). With the development of this approach, paper-based analytical devices (PADs) have been popular for chemical and biochemical analysis based on the introduction of microfabrication technology pioneered by Whitesides's research group (Lankelma et al. 2012). One of the advantages of PADs is that it could reduce the amounts of samples and the required samples decrease to be ~10 µL, which is similar to conventional microfluidic systems (Sun and Johnson 2015). The PADs were further developed and combined with disposable electrodes, which could further reduce the required samples compared with conventional electrochemical detection. By using modified PADs, the concentration of SA in tomato leaves was determined, and the concentrations of IAA and SA in

different parts of pea seedlings were measured with the weights of several milligrams (Sun et al. 2014, 2017, 2018).

The aims of this study were to test the availability of PADs in *P. haitanensis* thallus and use PADs to detect the changes of IAA and SA under different environmental stresses. To do this, PADs built-in tomato were first simulated and the concentrations of IAA and SA in *P. haitanensis* thallus were measured. The results showed that PADs built-in tomato could also be suitable to measure the concentrations of IAA and SA in *P. haitanensis* thallus.

Materials and methods

Materials and chemicals

Pyropia haitanensis thalli were collected in January 2018 from low intertidal zones on the coast of Xiangshan, Ningbo, Zhejiang Province, China, and cut into 5–10 cm long strips. The thalli samples were dehydrated at room temperature before being stored at –20 °C. Before use, they were rehydrated with filtered seawater at 20 °C and washed three times using filtered seawater that had been sterilized in 0.7% KI solution for 10 min. Healthy *P. haitanensis* thalli samples were selected and incubated in sterile seawater at 18 °C under 50 µmol photons m⁻² s⁻¹ (light-dark cycle 12:12 h) for 24 h before use in the experiments.

IAA and SA were purchased from Sigma-Aldrich (USA). The dispersion of MWCNTs (multi-walled carbon nanotubes) in water with the weight ratio of 2% was from Nanjing Xianfeng Nanomaterials Co. Ltd. (Nanjing, Jiangsu, China). The length of MWCNTs was 10–20 µm with outside diameter of > 50 nm and inside diameter of 5–15 nm. All other reagents were analytical grade. The Harris Uni-Core (Tip ID 4.0 mm) Miltex biopsy punch was from Ted Pella, Inc. (USA). The Indium tin oxide (ITO) conductive glass 355.6 mm wide, 406.4 mm long, 1.1 mm thick, and STN, 10 Ω was obtained from Nanbo Display Technology Co. LTD (Shenzhen, China). The conductive double-sided 8 mm wide, 0.16 mm thick, and 20 m long carbon adhesive tape with was from SPI Supplies (USA). The Whatman No. 1 qualitative filter paper was from GE Healthcare Bio-Sciences (USA).

Sample preparation

The stock solutions of IAA and SA (100 mM) were dissolved in ethanol and stored at 4 °C before use. The stock solutions were diluted in filtered seawater to 1 mM, and then diluted by the 0.2 M phosphate-buffered solution (pH 7.5) to 10 to 60 µM for standard curves. For high-temperature treatment, *P. haitanensis* thalli were cultivated in an incubator at 28 °C, 75% relative humidity and 50 µmol photons m⁻² s⁻¹ as

described (Qian et al., 2015). For high light treatment, thalli were transplanted in an incubator at 18 °C, 75% relative humidity, and 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For water loss treatment, algae were desiccated in an incubator at 18 °C, 75% relative humidity, and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ as described (Qian et al. 2015). For UV treatment, thalli were transplanted in an incubator at 18 °C, 75% relative humidity and irradiated with UV lamps (220 V, 30 W). *Pyropia haitanensis* thalli grown in sterile seawater at 18 °C under 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ served as control. Samples were collected and studied for the content of IAA and SA at 0, 1, and 6 h after various treatments. All treatments were repeated three times.

Hormone analysis by LC-MS

Two gram fresh weight of *P. haitanensis* thalli was collected to extract IAA and SA for HPLC–MS/MS determination according to previous protocols (Tu et al. 2017). Briefly, *P. haitanensis* thalli were extracted twice with 10 mL of acetonitrile/water/ methane acid (80:19:1, v/v/v) at –20 °C for 16 h in the dark. The supernatant obtained by centrifugation for 10 min at 12,000 rpm and 4 °C was dried by vacuum evaporation at 35 °C, and then redissolved in 500 μL methanol/water/acetic acid (90:10:0.05, v/v/v). The solution consisting of samples was passed through the activated C18 column and eluted with methanol. The elution solution was then vacuum dried at 35 °C. Finally, the residue was dissolved in 500 μL methanol/water/acetic acid (90:10:0.05, v/v/v) and analyzed by HPLC–Q q QMS (Thermo Fisher Scientific, USA). Sample solution was filtrated using a 0.22- μm membrane, and 10 μL of sample solution was injected into the C18 column (100 mm \times 2.1 mm \times 1.7 μm) at 30 °C. The mobile phase was 10 mM ammonium acetate-methanol with a gradient elution program at a flow rate of 300 $\mu\text{L min}^{-1}$. Elution was carried out with 15–100% methanol in 20 min, maintained for 5 min, then to 10% methanol in 1 min and maintained for 10 min. The ionization conditions were adjusted according to previous protocols (Tu et al. 2017). Briefly, sheath gas pressure (N_2) flow rate was 30 L min^{-1} ; aux gas pressure (N_2) flow rate was five Abs; spray voltage was 2500 V; vaporizer temperature was 300 °C; capillary temperature was 320 °C. Argon was introduced into the trap with an estimated pressure of 6×10^{-6} mbar to improve trapping efficiency and to act as the collision gas for full scan mode. The collision gas pressure was 1.5 mTorr. Results are presented as peak area. The LC-MS was operated in positive and negative modes.

Two commercial standards of IAA and SA were from Sigma-Aldrich to establish the quantitative standard curves. The hormones in the samples were identified by comparing the retention time and MS information with those of the standards.

Electrochemical detection

The modified carbon tape electrodes were prepared according to a previous report (Sun et al. 2017). Briefly, a piece of carbon tape on the conductive ITO glass was immersed in the solution of 0.01% MWCNTS (5 μL 2% MWCNTS suspension in 995 μL water) and then dried. The modified electrodes were treated under oxygen plasma for 10 min after their effective detection areas were regulated with insulated tape. A circle piece of the filter paper with a diameter of 4 mm was applied on the modified electrode for electrochemical measurements. The *P. haitanensis* thalli samples were excised by the Miltex Biopsy Punch with Plunger (Electron Microscopy Sciences, USA), weighed, and then placed on the MWCNTS modified electrode for analysis of IAA and SA contention.

For electrochemical detections, multichannel differential pulse voltammetry (DPV) was performed using a commercial CHI1040C electrochemical working station (CH Instruments, Shanghai, China). The oxygen plasma-treated MWCNTS-modified carbon tape electrode was used as the working electrode; a Ag/AgCl wire was used as the reference electrode, and a platinum wire was used as the counter electrode. DPV was carried out over the potential range from 0.2 to 1.2 V with parameters of 0.01 V increasing potential, 0.05 V amplitude, 0.02 s pulse width, 0.067 s sampling width, 0.1 s pulse period, and 20-s quiet time. Before each test, the counter electrode and reference electrode were washed thoroughly with double-distilled water. The solution of IAA and SA with a volume of 10 μL was dropped on the filter paper to optimize experimental parameters. To initiate multichannel electrochemical detection of IAA and SA in the micro samples (2–4 mg) of *P. haitanensis* thalli, 10 μL filtered seawater was dropped on the filter paper. When necessary, the recorded DPV curves were analyzed by applying the processes of baseline subtraction and peak splitting using Origin software (USA). In this way, IAA and SA peaks were separated based on fitting two Gaussian peaks having known mean and second moment features.

Gene expression

Semi-quantitative RT-PCR analysis of gene expression was carried out to help validate in situ measurements of IAA and SA. Total RNA was extracted from *P. haitanensis* thalli (0.10 g fresh weight) with TRIzol (Invitrogen, China) according to previous protocols (Li et al. 2018). Single-stranded cDNA was synthesized from total RNA using Takara PrimeScript RT reagent kit (Takara, Japan). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with the SYBR Premix Ex Taq II (Takara) on a Mastercycler ep realplex real-time PCR system (Eppendorf, Germany). The primers for *PhNIT* and *PhCHS* fragments are listed in Table 1. *Ph18S* was used as an internal

Table 1 Primers used in this work

Primer name	Sequence (5'-3')
Primers for qRT-PCR	
Ph18S-realtime-F	5'-AGTTAGGGGATCGAAGACGA-3'
Ph18S-realtime-R	5'-CAGCCTTGCACCATACTC-3'
PhNIT-realtime-F	5'-GAGATACCGTTCAGAGCTCGCT-3'
PhNIT-realtime-R	5'-CGAGAGCATGGTCCAGAGTAGG-3'
PhCHS-realtime-F	5'-GGGGTGGAGATTGTCGGGTAC-3'
PhCHS-realtime-R	5'-GACCAGCTCAATCATCCGCTCG-3'

reference gene. The PCR conditions were denaturing at 95 °C for 3 min, 40 cycles of denaturing at 95 °C for 10 s, and annealing at 58 °C for 18 s, and a final extension at 72 °C for 10 s. A dissociation curve analysis was used to determine target specificity. All reactions were performed in triplicates. Relative gene quantification was based on the comparative $2^{-\Delta\Delta C_t}$ method and normalized to *Ph18S* mRNA.

Statistical analysis

Data from IAA and SA determinations and qRT-PCR were analyzed with one-way ANOVA. All the experiments were performed at least three times.

Results

Optimization of electrochemical detection in solution

It is known that electrochemical detection of IAA and SA is normally performed in acidic or alkaline solution and the filtered seawater was alkaline (data not shown); thus, the filtered seawater is acceptable for in situ measurement of IAA and SA. Previous experiments discovered that the optimal experimental conditions for electrochemical detection of IAA and SA depended on their concentrations in PBS solution (Sun et al. 2014). Thus, whether electrochemical signals from IAA and SA in filtered seawater could scale with their concentrations under optimal experimental conditions was checked.

Figures 1 and 2 illustrate the calibration curves of IAA and SA with their concentrations under optimized experimental parameters. The linear relationship between the peak currents and the concentrations of IAA and SA from 1 to 60 μM could be observed. Based on six times of signal/noise ratio, the detection limits for IAA and SA were less than 0.1 μM . In addition, previous results showed that other components (such as citric acid, malic acid, succinic acid, abscisic acid, and methyl jasmonate etc.) in plants had no electrochemical response in the potential window for detection of IAA and SA (Sun et al. 2014, 2017). Such results suggested that the

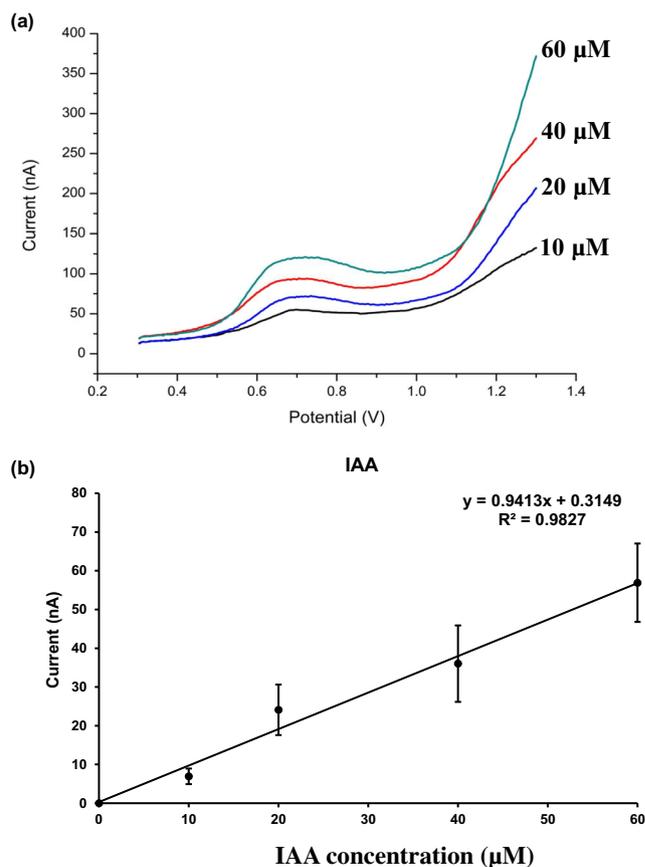


Fig. 1 a, b Calibration curves of IAA (concentrations include 10, 20, 40, and 60 μM) based on DPV detection. The average values and standard deviations were based on six separate measurements. Experimental parameters: graphene oxide concentration: 0.025 mg mL^{-1} ; plasma treatment duration, 1 min; potential range, 0.2–1.2 V; increasing potential, 0.01 V; amplitude, 0.05 V; pulse width, 0.02 s; sampling width, 0.067 s; pulse period, 0.1 s; quiet time, 20 s

presence of these components would not influence the detection of IAA and SA in this system.

To test whether PADs could be applied to *P. haitanensis* thalli, the concentrations of IAA and SA in *P. haitanensis* thalli under normal growth conditions were tested. The results showed that the concentrations of IAA and SA could simultaneously be measured using DPV (Supplemental Fig. 1A). Calculated by the established standard curve, the mean concentration of IAA in *P. haitanensis* thalli was 5.7 ng mg^{-1} , while the mean concentration of SA was 9.85 ng mg^{-1} under normal growth conditions (Supplemental Fig. 1B, C). Besides, samples were also collected at different times (0, 1, and 6 h) under normal growth conditions. The result showed that there was no significant difference of IAA and SA concentrations between samples collected at different times (Supplemental Fig. 1B, C). These results indicate that PADs could be used to measure the concentrations of IAA and SA in *P. haitanensis* thalli, and the concentrations of IAA and SA were stable in 6 h under normal growth conditions.

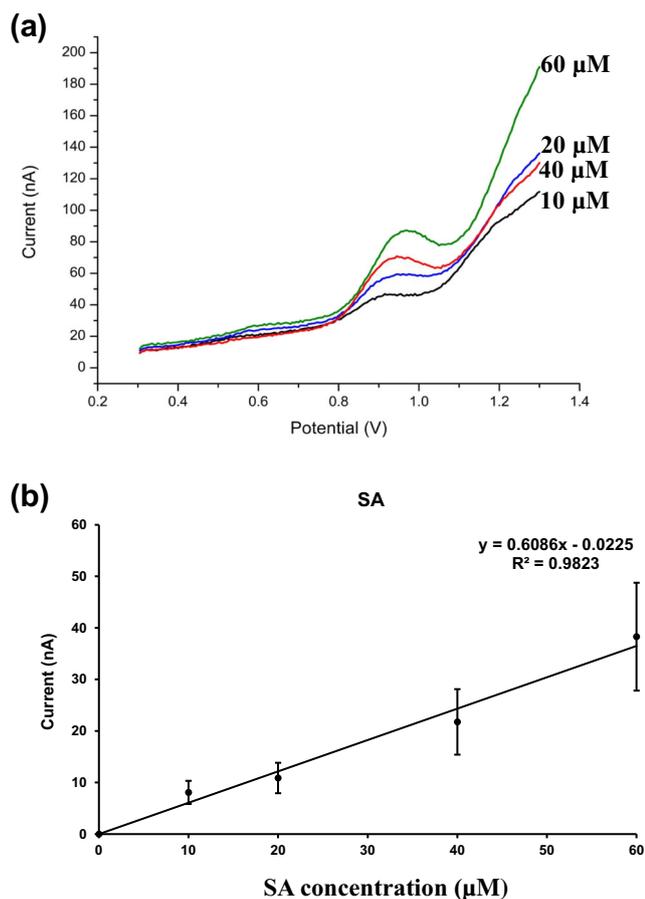


Fig. 2 a, b Calibration curves of SA (concentrations include 10, 20, 40, and 60 μM) based on DPV detection. The average values and standard deviations were based on six separate measurements. Experimental parameters: graphene oxide concentration: 0.025 mg mL^{-1} ; plasma treatment duration, 1 min; potential range, 0.2–1.2 V; increasing potential, 0.01 V; amplitude, 0.05 V; pulse width, 0.02 s; sampling width, 0.067 s; pulse period, 0.1 s; quiet time, 20 s

Concentration variations of IAA and SA under high temperature

First, the changes of IAA and SA levels under elevated temperature in *P. haitanensis* thalli using the custom paper-based electroanalytical device were tested. As shown in Fig. 3a, the DPV curves of IAA and SA changed after high temperature exposure. The concentration of IAA was calculated using a string formula, enabling us to determine that the mean concentration of IAA was 5.8 ng mg^{-1} for normal *P. haitanensis* thallus. After exposure to a temperature of 28 °C for 1 h, the mean concentration of IAA increased to 12.74 ng mg^{-1} . However, 6 h after exposure to the high temperature, the mean concentration of IAA returned to the original level (Fig. 3b). These results show that the production of IAA was influenced by high temperature.

Then, the changes in concentrations of SA under high thermal load were tracked. Using the calibration formula, the mean concentration of SA was 9.55 ng mg^{-1} for normal

P. haitanensis thallus, which was significantly higher than that of IAA. Similar to the results for IAA, 1 h after high temperature treatment, the mean concentration of SA increased to 15.95 ng mg^{-1} and 6 h after high temperature treatment, the mean concentration of SA also returned to the original level of normal *P. haitanensis* thallus (Fig. 3c). These results also suggest that the concentration of SA was affected by high temperature.

To further confirm this method, the method of sensitive HPLC-MS/MS was used to analyze IAA and SA in *P. haitanensis* thallus under high temperature stress (Supplemental Fig. 2). The concentrations of IAA and SA in *P. haitanensis* thallus after high-temperature treatment were higher than that in control leaves. These results were consistent with IAA and SA levels determined by both paper-based electroanalytical and sensitive HPLC-MS/MS. However, the paper-based electroanalytical approach introduced here can be effectively applied for determination of IAA and SA in situ.

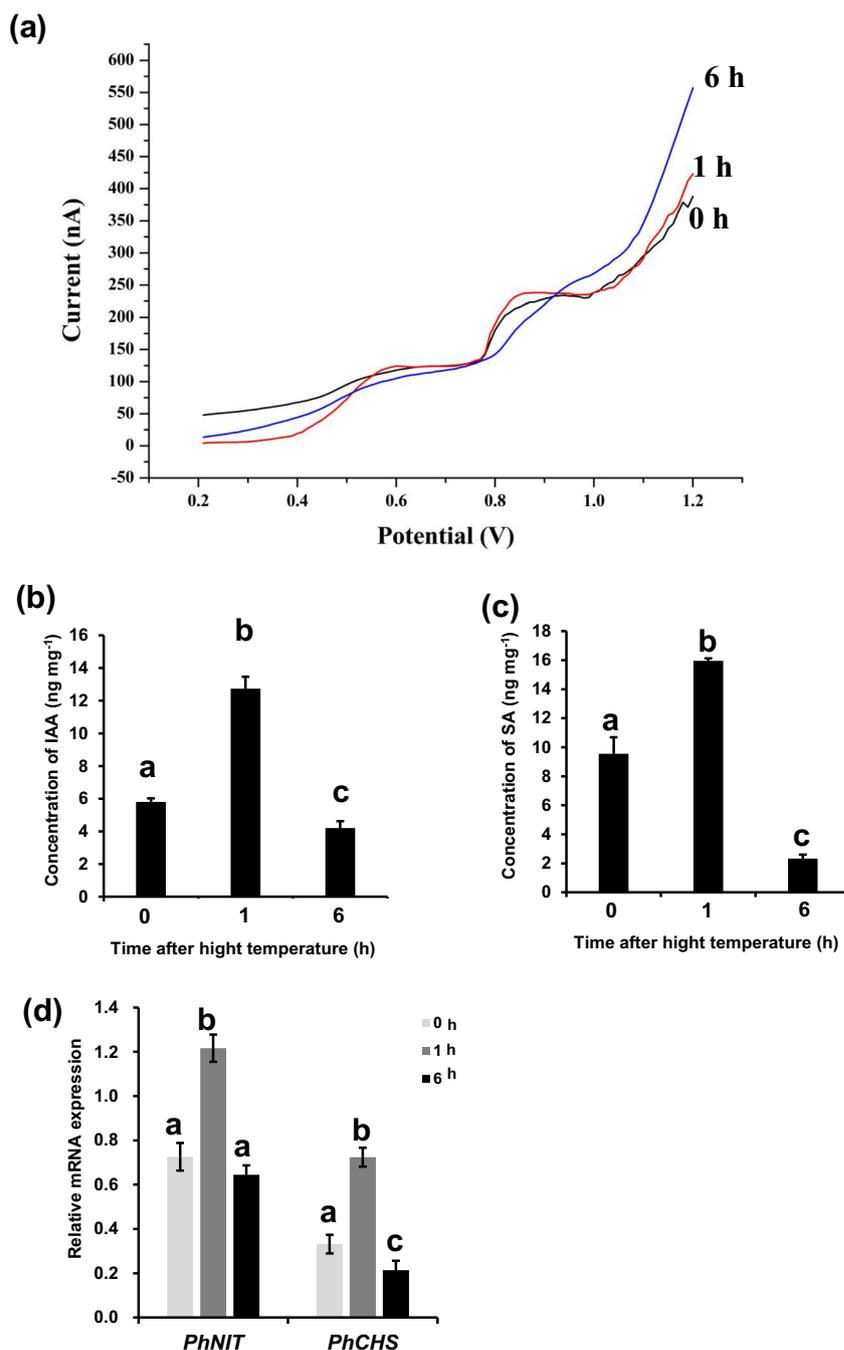
To correlate the electrochemical measurements to protein turnover, two genes *PhNIT* and *PhCHS*, which are recognized as IAA and SA synthesis-related genes, in the established transcriptome (data not shown) were chosen. The results showed that the transcription of *PhNIT* increased 1 h after high temperature exposure and then returned to normal level 6 h after treatment, which confirmed that high-temperature conditions initially induced the production of IAA and then the thallus restored its production. The transcription of *PhCHS* mRNA showed a similar trend as *PhNIT* after high temperature treatment, mirroring the measurement of SA (Fig. 3d).

Changes of concentrations of IAA and SA after high-light irradiance

Next, whether the concentrations of IAA and SA could change after high light irradiance in *P. haitanensis* thalli using PADs was tested. As shown in Fig. 4a, the results showed that the DPV response of IAA at various times after high-light exposure exhibited different peak heights, thus suggesting that the concentration of IAA changes with time after high-intensity exposure. Calculations with string formulae yielded a mean IAA concentration of 5.56 ng mg^{-1} for *P. haitanensis* thallus under normal irradiance. However, after high-light exposure, the concentrations of IAA showed a small reduction, reaching to 4.5 ng mg^{-1} 1 h post high-light exposure and 2.86 ng mg^{-1} 6 h post continuous exposure (Fig. 4b). Thus, it would appear that high light has a negative effect on IAA levels.

The concentration levels of SA after *P. haitanensis* thalli exposed to an irradiance of 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were then measured. In the custom paper-based electroanalytical cell, the DPV of SA at different times after intense light exposure showed different peaks, indicating that intense light could influence the concentration levels of SA (Fig. 4a). Thus, the

Fig. 3 IAA and SA concentration variations after high temperature exposure of *P. haitanensis* thallus. **a** In situ DPV detection of IAA and SA at different times after high temperature treatment in *P. haitanensis* thallus. **b** Histogram of IAA levels at different times after high temperature treatment. **c** Histogram of SA levels at different times after high temperature treatment. **d** Expression levels of *P. haitanensis* *PhNIT* and *PhCHS* relative to *Ph18S* in *P. haitanensis* thallus. Means \pm SEM are relative values obtained from three biological replicates; different letters represent significant differences ($P < 0.05$, Student's *t* test)



concentrations using the string formula were calculated and the results showed that the concentrations of SA increased significantly by 1 h after high light stress, and returned to the normal level of *P. haitanensis* thallus 6 h after high light treatment (Fig. 4c). Thus, opposite to IAA, SA levels are positively influenced by high light stress.

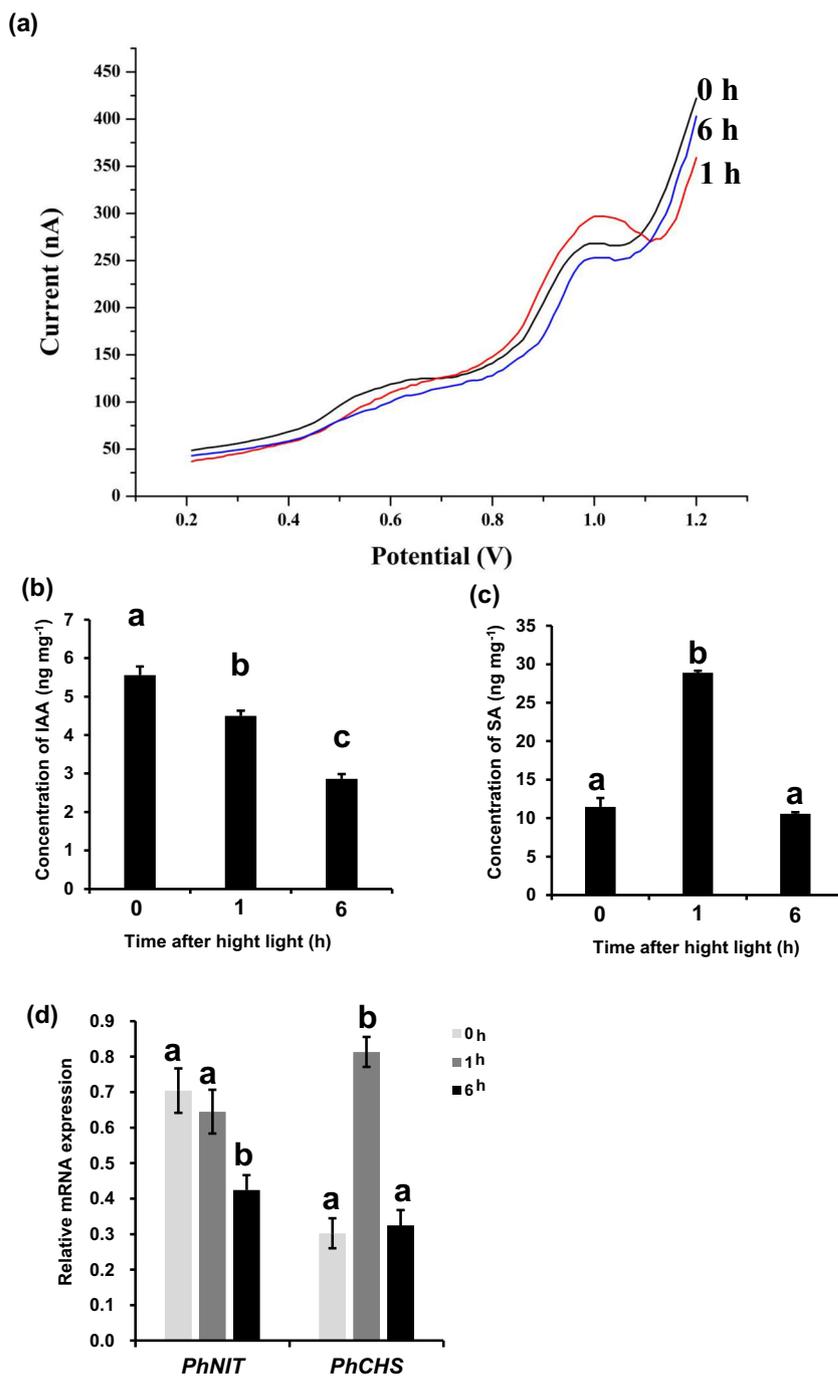
Finally, the transcriptions of *PhNIT* and *PhCHS* to explain the results of the paper electrode measurements were also compared. Although the expression of *PhNIT* was not significantly changed 1 h after high light treatment, its expression

decreased 6 h after high light treatment. In addition, the expression of *PhCHS* showed the opposite expression pattern compared with that of *PhNIT* under high light stress (Fig. 4d).

The effect of desiccation on the changes in concentrations of IAA and SA

The changes in concentrations of IAA and SA when *P. haitanensis* thalli is exposed to desiccation conditions were then measured. Similar to the results above, the DPV curves of

Fig. 4 The concentration variation of IAA and SA after high-light exposure in *P. haitanensis* thallus. **a** In situ DPV detection of IAA and SA at different times after high light exposure in *P. haitanensis* thallus. **b** Histogram of IAA levels at different times after high-light exposure. **c** Histogram of SA amounts at different times after high-light exposure. **d** Expression levels of *P. haitanensis* *PhNIT* and *PhCHS* mRNA relative to *Ph18S* mRNA in *P. haitanensis* thallus. Means \pm SEM are relative values obtained from three biological replicates; different letters represent significant differences ($P < 0.05$, Student's *t* test)

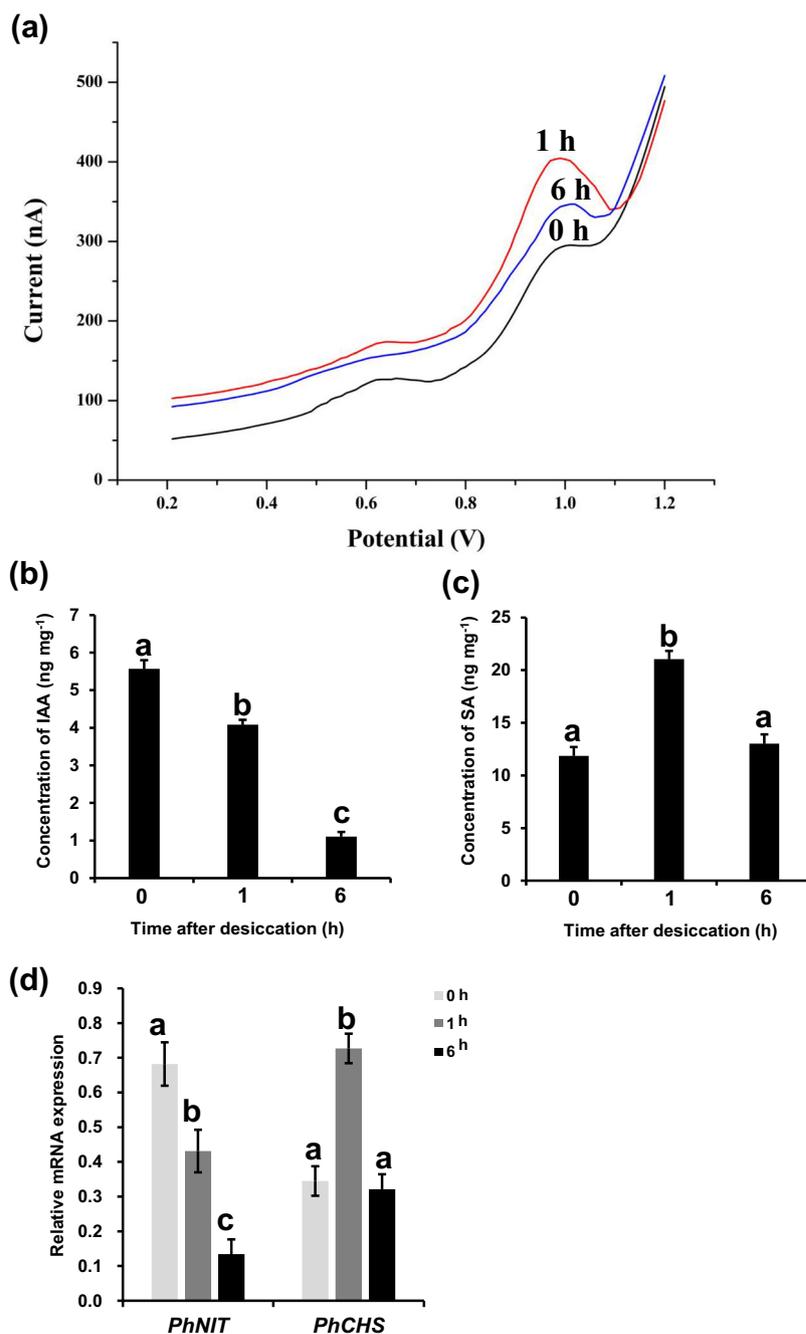


IAA and SA at varying times after desiccation exhibited different lines, indicating that the concentrations of IAA and SA also changed with the length of the desiccation interval (Fig. 5a). Calculations with the string formula allowed us to determine that under normal growth conditions, the mean concentration of IAA was 5.57 ng mg⁻¹. However, after exposure to desiccation, the concentrations of IAA decreased, reaching 4.08 ng mg⁻¹ 1 h after desiccation and 1.10 ng mg⁻¹ at 6 h after desiccation. Thus, these data suggest that the concentration of IAA might be influenced by *P. haitanensis* adaptation

to a desiccation environment (Fig. 5b). In opposition to IAA, the mean concentration of SA increased significantly after desiccation. The results showed that the mean concentration of SA was 11.86 ng mg⁻¹ for normal thallus. However, this concentration increased to 21.05 ng mg⁻¹ 1 h after desiccation and returned to 13.01 ng mg⁻¹ 6 h after desiccation (Fig. 5c).

The production of *PhNIT* and *PhCHS* mRNAs to see if the desiccation assay results were reasonable was also measured. The results showed that the transcription of *PhNIT* had led to continuously declined mRNA pools within 6 h after

Fig. 5 The concentration variation of IAA and SA after desiccation stress in *P. haitanensis* thallus. **a** In situ DPV detection of IAA and SA at different times after desiccation stress in *P. haitanensis* thallus. **b** Histogram of IAA levels at different times after desiccation stress. **c** Histogram of SA levels at different times after desiccation stress. **d** Expression levels of *P. haitanensis* *PhNIT* and *PhCHS* mRNA relative to *PhI8S* mRNA in *P. haitanensis* thallus. Means \pm SEM are relative values obtained from three biological replicates; different letters represent significant differences ($P < 0.05$, Student's *t* test)



desiccation. However, the expression level of *PhCHS* increased 1 h after desiccation and returned to normal level 5 h later (Fig. 5d).

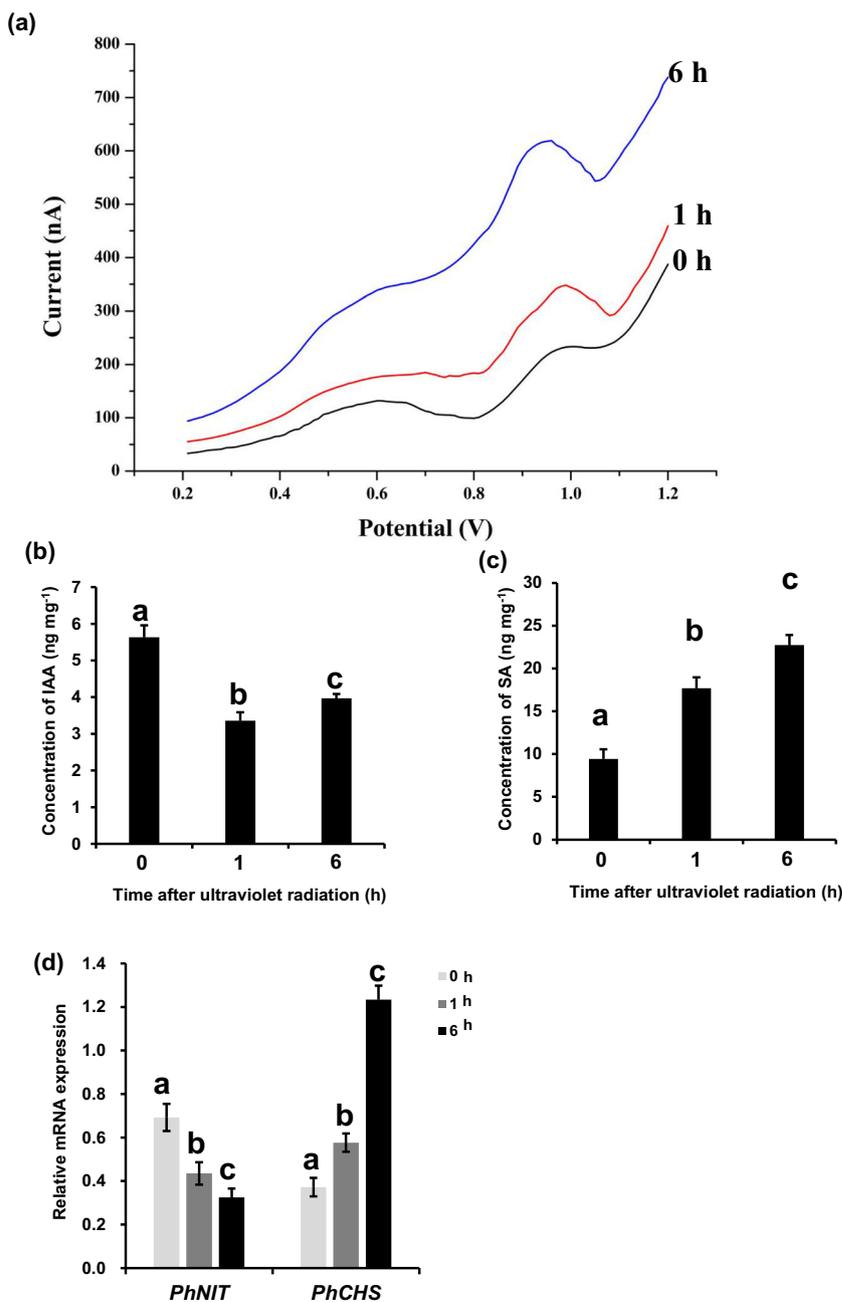
Concentration variations of IAA and SA caused by ultraviolet radiation

The variations of IAA and SA concentrations under ultraviolet radiation stress were also sought to examine. The DPV curves of IAA and SA oxidation at different times after ultraviolet radiation exposure showed that the concentrations of IAA and

SA changed continuously (Fig. 6a). Using the string formulae, the results showed that the IAA concentrations decreased monotonically at all measured intervals during the 6-h exposure (Fig. 6b). In addition, the concentrations of SA increased after UV exposure (Fig. 6c).

Pools of *PhNIT* and *PhCHS* mRNA as a function of UV exposure to check whether they showed levels that conformed to increasing or decreasing plant hormone levels were then measured. The results indicated that *PhNIT* monotonically decreased after UV exposure, whereas *PhCHS* pools continually increased after UV exposure (Fig. 6d).

Fig. 6 The variation of IAA and SA levels after UV radiation exposure in *P. haitanensis* thallus. **a** In situ DPV detection of IAA and SA levels at different times after UV radiation exposure in *P. haitanensis* thallus. **b** Histogram of IAA levels at different times after exposure to UV radiation. **c** Histogram of SA levels at different times after exposure to UV. **d** Expression levels of *P. haitanensis* *PhNIT* and *PhCHS* mRNA relative to *Ph18S* mRNA in *P. haitanensis* thallus. Means \pm SEM are relative values obtained from three biological replicates; different letters represent significant differences ($P < 0.05$, Student's *t* test)



The expression of *PhNIT* and *PhCHS* after exogenous IAA and SA administration

Finally, whether transcriptions of *PhNIT* and *PhCHS* could be induced by extracellular administration of IAA and SA was sought to test. RT-qPCR results showed that *PhNIT* transcription was enhanced by IAA administration, whereas *PhCHS* transcription decreased after IAA administration (Fig. 7a). In addition, exogenous SA did enhance the transcription of *PhCHS* but repressed the transcription of *PhNIT* (Fig. 7b). Thus, IAA and SA might play opposing roles in *P. haitanensis* adaptation to environmental stresses.

Discussion

Phytohormones engage in very important functions as effectors in triggering marine algae resistance to abiotic stresses (Bradley 1991; Tarakhovskaya et al. 2007). For example, SA promotes the growth of *Gracilaria lemaneiformis* under elevated temperature (Zhu et al. 2012). In this study, a paper-based electroanalytical device to detect the concentrations of IAA and SA was constructed and the IAA and SA concentration variations under different environmental stresses were measured. These results not only establish a new, inexpensive, and convenient method to detect concentration variations but

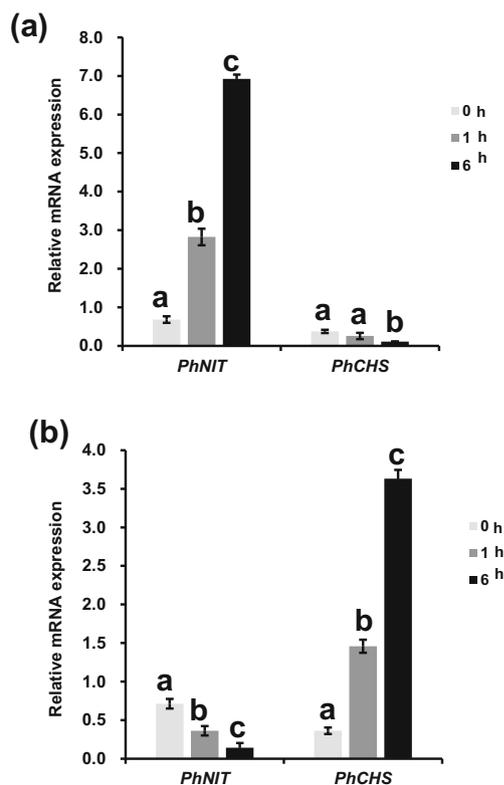


Fig. 7 The expression of *PhNIT* and *PhCHS* mRNA relative to *Ph18S* mRNA after administration of exogenous IAA and SA. **a** The relative expression of *PhNIT* and *PhCHS* vs *Ph18S* after IAA treatment. **b** The relative expression of *PhNIT* and *PhCHS* vs *Ph18S* after SA treatment. Means \pm SEM are relative values obtained from three biological replicates; different letters represent significant differences ($P < 0.05$, Student's *t* test)

also systematically and continuously monitor the concentration variations under different environmental stresses for the first time. These results will provide methods and test models for further research on phytohormones in *P. haitanensis*.

The measurement of local concentrations of phytohormones poses a major challenge in phytohormone research. HPLC-MS or HPGC-MS presently offers the main methods to measure concentrations of phytohormones in marine algae (Matsuura et al. 2018). Using LC-MS, Tu et al. (2017) found that the concentrations of SA decreased while the concentrations of JA increased after flg22 treatment. Another study from the same research group looked at the concentration levels of different phytohormones under varying growth periods in *P. haitanensis* by using HPLC-QqQ-MS (Song et al. 2017). However, in general, both HPLC and HPGC methods are complicated and time-consuming, especially considering the sample preparation that is required (Tu et al. 2017). In contrast, the electrochemical detection method that measures phytohormone concentrations by substrate oxidation using differential pulsed voltammetry in cheap, disposable cells, offers advantages far more favorable to field portability including reduced effort for sample extraction and isolation. Sun

et al. measured the concentrations of SA after inoculation of *Botrytis cinerea* in tomato plants using a paper-based electro-analytical cell and miniature processor for generating and injecting the voltage waveform across a punched sample of 1.5 mm diameter of tomato leaves (Sun et al. 2014). They also analyzed the concentration variations of IAA and SA after exposure to high salt stress in pea samples weighing from 2.9 to 7.4 mg using these dedicated devices (Sun et al. 2017). Although IAA and SA using paper-based electroanalytical devices in *P. haitanensis* thallus samples weighing 2.1 mg were also detected, the results showed that the concentrations of IAA and SA measured by the device in *P. haitanensis* thallus samples under normal growth conditions were higher than the same phytohormones measured using HPLC-MS (Supplemental Fig. 2). This indicates that paper-based electroanalytical devices are more sensitive than HPLC-MS and thus enable measurement of the concentrations of IAA and SA with greater precision. Except for high mass sensitivity, the level of effort involved in preparing samples, preparing separation media, and the expense of acquiring and maintaining the instrumentation are additional advantages of utilizing this approach whenever possible. Despite these differences, the two methods showed similar data trends for both IAA and SA hormones under high-temperature stress (Fig. 3, Supplemental Fig. 2). Thus, the paper-based electro-analytical cell, combined with a miniaturized DPV probe and detector, provides satisfactory sensitivity to monitor IAA and SA levels during and after different environmental stresses in marine algae.

Consistent with the present results, many other studies also showed that IAA and SA could be detected in algae. For example, Mikami et al. (2016) reported that IAA and SA could be detected in *Bangia fuscopurpurea* and *Pyropia yezoensis*, and their contents are the highest of all the phytohormones tested. This indicates that there are synthetic pathways for IAA and SA in algae, but the current synthetic pathways for IAA and SA in algae are not well understood. In higher plants, the tryptophan-dependent auxin synthesis process consists of four pathways: (i) the indole-3-acetamide (IAM) pathway, (ii) the indole-3-pyruvic acid (IPA) pathway, (iii) the tryptamine (TAM) pathway, and (iv) the indole-3-acetaldoxime (IAOX) pathway (Enders and Strader 2015). Through the transcriptome data (data not shown), the homologous genes *flavin-containing monooxygenase* and *nitrilase* (*PhNIT*) of the auxin synthesis pathway existed in *P. haitanensis*, which indicated that it might contain one or several IPA, TAM, or IAOX pathways. Similar results showed in brown alga *Ectocarpus siliculosus* that homologs of genes encoding for enzymes involved in the Trp-dependent TAM and IAOx biosynthetic pathways were identified with no molecular evidence for the IPA and IAM pathways (Le Bail et al. 2010). Besides, *PhNIT* gene was selected as the indicator gene, and the gene expression results were also consistent

with the IAA change trend under environmental stresses (Figs. 3, 4, 5, and 6). Meantime, IAA could also induce the gene expression of *PhNIT* (Fig. 7a). These results indicate that *PhNIT* might be an auxin synthesis gene in *P. haitanensis*. But, this requires further biochemistry and genetic experiments to verify.

In higher plants, there are two synthetic pathways for SA: the IC pathway and the PAL pathway (Janda and Ruelland 2015). Through the transcriptome data, a homologous gene *isochorismate synthase* (herein it was named *PhCHS*) of the SA synthesis pathway was detected, which suggests that SA might be synthesized through the IC pathway in the *P. haitanensis*. The gene expression results showed that the expression of *PhCHS* was consistent with the concentration changes of SA under environmental stresses (Figs. 3, 4, 5, and 6). Also, SA could induce the expression of *PhCHS* (Fig. 7b). These results suggest that *PhCHS* was a potential SA synthesis gene.

There are many studies which have shown that IAA can promote the growth of algae (Yokoya et al. 2014; Piotrowska-Niczyporuk et al. 2018). For example, low concentrations of IAA stimulated the growth of intercalary segments of *Chondracanthus chamissoi* (Gigartinales, Rhodophyta), but high concentrations of IAA stimulated callus formation in intercalary segments (Yokoya et al. 2014). IAA could also promote the growth of the green alga *Acutodesmus obliquus* (Piotrowska-Niczyporuk et al. 2018). Recent studies also have shown that IAA plays an important role in the interaction between algal and bacteria that bacteria could produce IAA and promote the growth of algae (Amin et al. 2015). Therefore, IAA is considered to be a “growth hormone.” It is generally believed that the concentrations of “growth hormones” will decrease during the stress-resistance process. For example, the concentration of IAA decreased under lead stress in green alga *A. obliquus* (Chlorophyceae) (Piotrowska-Niczyporuk et al. 2015). This is consistent with the present results, that the concentration of IAA decreased under various environment stresses except for high temperature (Fig. 3, 4, 5, and 6). Therefore, IAA might play a negative role in algae resistance to environmental stresses. The reason why IAA could be induced after high temperature treatment might be that in the early stage of high temperature, the fluidity of cell membrane and the exchange of substances were accelerated; thus, the synthesis of substances in *P. haitanensis* accelerated, including IAA. This is similar in higher plants: when rice encountered high-temperature stress, the concentration of IAA increased significantly, and the expression of IAA synthesis gene *YUCCA* also increased significantly (Du et al. 2013). However, the concentration of IAA returned to normal level at 6 h after high-temperature stress (Fig. 3b), which indicated the plant cells began to be damaged and the IAA synthesis decreased.

Thus, IAA mainly plays an important role in the process of stress resistance other than algae growth when *P. haitanensis* is exposed to high temperature stress.

It is well known that when algae are exposed to environmental stresses the concentration of reactive oxygen species (ROS) in algae will increase, which will destroy the cell structure (Fu et al. 2015; Qian et al. 2015). For example, the synthesis of ROS was triggered when *P. haitanensis* was under desiccation stress (Qian et al. 2015), and UV stimulation could also produce ROS in *Pseudokirchneriella subcapitata* (Fu et al. 2015). SA can scavenge ROS and stabilize the content of ROS in algae (Stirk and Van Staden 2014). Thus, algae could make use of SA to resist environmental stresses. This is similar to higher plants, in which studies have shown that environmental stresses can directly induce the metabolism of SA synthesis in *Arabidopsis* (Bandurska and Cieślak 2013). This study also showed that different environmental stresses could induce the synthesis of SA and the expression of SA synthesis genes in *P. haitanensis* (Fig. 3, 4, 5, and 6). Therefore, these results indicate that the roles of SA might be evolutionarily functionally preserved in *P. haitanensis* and higher plants. However, the roles of SA in response to environmental stresses of *P. haitanensis* need to be further clarified by exogenous application of SA on the physiological, biochemical, and molecular changes of *P. haitanensis*.

Another interesting phenomenon was that in addition to ultraviolet radiation, the concentration of SA was lower at 6 h after environmental stresses than that at 1 h (Fig. 3, 4, 5, and 6). This might be the reason that SA is involved in the early process of environmental stress tolerance of *P. haitanensis*, while other substances might be involved in the later stress-tolerant process of *P. haitanensis*. This process is similar to drought tolerance in higher plants. The concentration of JA increased significantly in the early drought (within 1 h) and returned to normal level at 2 h after drought stress. On the other hand, the content of abscisic acid, another plant hormone, started to increase at 2 h after drought stress (de Ollas et al. 2013). These data indicate that the increase of abscisic acid content after drought treatment is JA-dependent. In *P. haitanensis*, Qian et al. (2015) found that the production of floridoside started to increase at 2 h after drought treatment. Thus, SA might be an important component involved in the early process of environmental stresses and floridoside plays an important role in the later process of environmental stresses in *P. haitanensis*. The interaction between SA and floridoside during the drought process will be further verified by using genetic and molecular approaches in *P. haitanensis*.

In summary, this study demonstrates that the concentrations of SA increased under all the environmental stresses. In contrast, the concentrations of IAA decreased under most environmental stresses tested except for high-temperature stress. These are interesting results, as in higher plants, IAA is normally found to be a growth-related phytohormone,

whereas SA is a defense-related phytohormone which means that environmental stresses should reduce the concentrations of IAA and increase the concentrations of SA. That the variation of IAA and SA levels in *P. haitanensis* under different environmental stresses acts similar to the patterns seen in higher plants indicates that IAA and SA may be functionally conserved during evolution in *P. haitanensis* and higher plants. Thus, these results lay the foundation for future phytohormone research in *P. haitanensis*.

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Authors' contributions XL and QW designed the experiments. QW and LT performed the experiments. QW, YP, and YF analyzed the data. XL and LS drafted the initial manuscript. All the authors agreed on the contents of the paper. All the authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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