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Response to wound-activated stress through a lipid oxidative metabolic pathway in *Pyropia haitanensis*

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ABSTRACT

Pyropia haitanensis inhabits the intertidal zone and is affected by rough waves and sediment, as well as herbivore-induced and mechanical damage. However, the mechanisms of *P. haitanensis* response to wounding have not been elucidated. Lipid metabolism is an important defense mechanism in algae, and the lipid defense strategy of *P. haitanensis* elicited by wound-activated stress was investigated in this study. *P. haitanensis* was damaged by cutting and allowed to recover for 30 and 60 min. Release of H_2O_2 that was stimulated by wounding could act as a primary signal to induce phospholipase A_2 activation, which catalyzes the degradation of membrane lipids to release free fatty acids. Then, several polyunsaturated fatty acids (C18:2, C20:4 and C20:5) were oxidized by PhLOXs, resulting in decreased polyunsaturated fatty acid levels and increased oxylipins, which were further transformed into short-chain volatile organic compounds. Moreover, increased jasmonic acid levels were also detected after wounding, indicating that the jasmonic acid pathway may also be involved in the defense response of *P. haitanensis* to wounding, similar to higher plants. Hence, the oxidative lipid metabolic pathway elucidated herein offers plausible physiological insights into lipid metabolism in *P. haitanensis* in response to wound-activated stress and may facilitate efficient development and improvement of *Pyropia* quality by producing cultivars resistant to wounding.

1. Introduction

Despite large differences between seaweed and terrestrial plant habitats, both of these organisms suffer from numerous environmental attacks, such as pathogenesis, desiccation, extreme temperatures or wounding by herbivores. In response to wounding, plants have evolved a variety of chemical defense strategies for protection. These strategies involve the formation of proteinaceous defense compounds and toxic compounds—such as nicotine and volatile molecules—while also initiating the jasmonic acid (JA) signal pathway. The JA family of oxylipins was discovered to occur in response to wound-activated stress. JA and methyl jasmonate (MeJA) are wound-related hormones and signal molecules that are present in most plants that, when applied exogenously, can stimulate defensive genes to increase levels of induced defense chemicals, closely matching patterns induced by wounding or insect feeding [1–3]. α-Linolenic acid (C 18:3, ALA) is as precursor of JA biosynthesis in most plants. It will be oxidized and converted into 13(*S*)-hydroperoxy-octadecatrienoic acid via 13-lipoxygenase (LOX), and then catalyzed to generate (+)-12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase. OPDA is reduced by OPDA reductase 3 and activated by OPC-8:0 CoA Ligase1. Finally undergoes β-oxidation to remove carbons from the carboxyl side chain and produce JA [4]. Hence, through the JA biosynthesis pathway in most plants, lipid peroxidation is initiated from the octadecanoid, and is essential for JA production. In addition, previous studies have demonstrated that there is cross-talk between JA and the level of reactive oxygen species (ROS), such as H_2O_2, which plays a key role as a second messenger that induces gene expression and protein synthesis [5,6].

Macroalgae have no acquired immune system, but their basic mechanisms for pathogen recognition and defense signaling were similar to that of terrestrial plants and animals. The lipid peroxidation metabolites are critical defense molecules in algae. Polyunsaturated fatty acids (PUFAs) and their oxidized derivatives—such as oxylipins,

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hormones and volatile organic compounds (VOCs)—play important roles in the signaling involved in both plant and animal stress responses [7,8]. Both plant-like and animal-like defense systems have been observed in the brown alga *Laminaria digitata* under copper-induced stress and the red alga *Chondrus crispus* recognizing cell-free extracts from *Acrochaete operculata* vegetative filaments as a defense elicitor [7,9]. Chemical defense by the invasive red alga *Gracilaria vermiculophylla* and the related noninvasive *Gracilaria chilensis* revealed a rapid lipoxygenase-mediated transformation of arachidonic acid (AA) to known and novel oxylipins [10,11]. In green alga, *Caulerpa taxifolia* reacts upon short-term wounding with a rapid transformation of its main defensive metabolite caulerpenyne to the deacetylated metabolite oxytoxin 2 [12]. In addition, a wound-activated volatile is released and contributes to chemical defense in the brown alga *Dictyota dichotoma* [13]. However, the distribution and function of JA in algae is extremely limited and largely controversial. JA and/or MeJA have been detected only in some green algae and rhodophytes [14,15], although both metabolites have been measured from *Pyropia haitanensis* [16]. Intriguingly, homologs to all of the JA biosynthesis proteins have not been observed in sequenced *Pyropia* genomes [17,18]. Therefore, the extent to which JA and its derivatives are related to wounding in marine algae is unclear and further profiling experiments are still needed.

P. haitanensis is an endemic species that is naturally distributed only along the coasts of South China, inhabiting the intertidal zone. This organism is found at the mouth of the Changjiang River and inshore coastal waters which contain a variety of grains, sands and other particles result in poor light penetration and seawater turbidity. As these areas are continuously influenced by the rough waves, as well as herbivore-induced and mechanical damage, the leaves of *P. haitanensis* are readily destroyed, thus, emphasizing the importance of understanding the wound responses of *P. haitanensis*. In this paper, after wounding in *P. haitanensis*, H_2O_2 levels, phospholipase A_2 (PLA_2) activity, and regulation of metabolites (fatty acids, oxylipins, VOCs, plant hormones) were measured in order to elucidate oxidative metabolic pathways under wound-activated stress. This data can offer plausible physiological insights into lipid metabolism in *P. haitanensis* in response to wounding stress.

2. Material and methods

2.1. Algae material and treatment

P. haitanensis "Zhedong-1" was collected from the low intertidal zones along the coast of Xiangshan, Ningbo, Zhejiang Province, China. Sampling was permitted by the local government and the local department of fisheries. *P. haitanensis* thalli that were 2–10 cm in length were dehydrated at room temperature before being stored at −20 °C. Before use, the samples were rehydrated with filtered seawater at 20 °C and sterilized by soaking in 0.7% KI solution for 15 min. The thalli were washed three times with filtered seawater to remove unwanted algae from the surface. The thalli were incubated in 300 mL of sterile seawater at 20 °C for > 48 h under cool-white fluorescent light at approximately 140 μmol photons $m^{-2} s^{-1}$ in a 12:12 h L:D cycle used as control group. In addition, in order to investigate the algal physiology of recovered *P. haitanensis*, the Fv/Fm of fresh alga and recovered alga were detected to evaluate the status of *P. haitanensis*. As a result shown in Table 1S (Supporting information), it could be found that after recovered for 24 h and 48 h of *P. haitanensis*, the chlorophyll fluorescence parameter (Fv/Fm) were nearly similar to the fresh alga which were collected from sea. Hence, it could be suggested that the status of rehydrated and recovered *P. haitanensis* was similar to the fresh alga and it is alive again [19]. The thalli of *P. haitanensis* was cut into about 4 mm * 4 mm pieces with scissors, and immediately transferred into sterile seawater at 20 °C for recovery either 30 or 60 min as the wound-activated group. At each time point the control and wounded samples were collected and freeze-dried, separately, and then stored at −80 °C until analysis.

2.2. H_2O_2 quantification

H_2O_2 quantification was performed based on the method of Miller and Kester [20]. In brief, 100 mg of fresh weight samples (control and wound-activated group) were incubated in 14 mL of artificial sterile seawater. The formulation of artificial sterile seawater was shown in Table 2S (Supporting information). After wounding, 200 μL of culture media was immediately added into the 96-well plates containing 8 μL of fluorometric reagent (6.13 μM *p*-hydroxyphenyl acetic acid, 276.9 U L^{-1} peroxidase, 8.6 μM Tris-HCl, pH 8.8) and mixed. The signal was detected with excitation and emission fluorescence at the wavelengths of 313 and 400 nm, respectively. The effect of diphenylene iodonium (DPI) on the release of H_2O_2 was tested by preincubating thalli for 10 min in the presence of 10 μM DPI, and followed by wounding in the DPI + wound-activated group. Each control and wounded-activated sample for H_2O_2 quantification and following experiments had three individual replicates.

2.3. Phospholipase activity assay

PLA_2 activity was measured according to Chandra [21]. Briefly, 4.5 mg of fresh weight samples (control and wound-activated groups) were cultivated in 96-well plates containing 200 μL of artificial sterile seawater, and then mixed with 0.1 μL of a stock of *N*-((6-(2,4-dinitrophenyl)amino) hexanoyl)-2-(4,4-difluoro-5, 7-dimethyl-4-bora-3a, 4adiaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (PED6; 1 mg mL^{-1} in dimethylsulfoxide). The PLA_2 inhibitor methyl arachidonoyl fluorophosphonate (15 μM), 0.1 μL of 1 mg mL^{-1} PED6 and 200 μL of artificial sterile seawater were added before wounding, and followed by wounding treatment as the inhibitor + wound-activated group. Each treatment was repeated in triplicate. The fluorescence signal was detected by a microplate reader with the temperature of 18 °C at excitation and emission wavelengths of 488 and 516 nm, respectively, for 1 h at 30-s steps.

2.4. Analysis of free fatty acids by GC–MS

Samples were randomly selected for free fatty acid (FFA) extraction. The fatty acid methyl esters were prepared as described previously [22] with slight modifications. Briefly, 100 mg of frozen powdered samples were extracted using 6 mL of ethyl acetate and shaken using a vortex mixer for 2 min, and then ultrasonic disruption for 5 min. Lipids were extracted by mixing on a rotary shaker for 1 h at 4 °C and then centrifugation at 3500*g* for 15 min at 4 °C. The above extraction steps were repeated 3 times and 5 mL of ice-cold water was added to the combined supernatant layer. The mixture was incubated for 5 min on ice and then centrifuged at 4 °C and 3500*g* for 15 min. After recovery of the organic phase, ethyl acetate was dried in a rotary evaporator. Subsequently, the residue was dissolved in 200 μL of acetonitrile, and prior to inter-reaction at 35 °C for 30 min, 30 μg of nonadecanoic acid (C19:0), 20 μL of 2,3,4,5,6-pentafluorobenzyl bromide and 40 μL of diisopropylethylamine were added to the solution. Finally, the reaction system was evaporated under a stream of nitrogen. The residues for GC/MS analysis was dissolved in 1 mL of hexane.

Gas chromatographic analysis (Shimadzu QP2010) was performed using a SPB-50 fused silica capillary column (Supelo, Bellefonte, PA, USA). The injection temperature was 250 °C. Highly purified helium was used as the carrier gas with flow rate of 0.81 mL min^{-1} and a pre-column pressure of 73.0 kPa. After injection, the oven temperature was held at 150 °C for 3.5 min, raised to 200 °C for 5 min at a rate of 20 °C min^{-1}, and finally raised from 250 °C to 280 °C for 30 min at a rate of 5 °C min^{-1}. The injection volume was 1 μL and the split mode