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Responses of lipoxygenase, jasmonic acid, and salicylic acid to temperature and exogenous phytohormone treatments in *Gracilariopsis lemaneiformis* (Rhodophyta)

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Abstract

Jasmonates (jasmonic acid (JA) and methyl jasmonate (MJ)) and salicylic acid (SA) play roles in the growth and developmental processes of plants and the defense response against adverse stresses as phytohormone signals. To investigate their roles in algae, we analyzed the sequences of two genes encoding lipoxygenase (LOX) and their expression profiles in response to temperature and phytohormone treatments in the macroalga *Gracilariopsis lemaneiformis* (Rhodophyta). The two *lox* sequences from *Gp. lemaneiformis* (*Gllox*1 and *Gllox*2) shared only 23.90% identity. The following results were observed: (1) high temperature (33 °C) strongly stimulated *Gllox*2 mRNA level, LOX activity, and endogenous JA and SA contents; (2) exogenous MJ promoted *Gllox*2 expression (4.91-fold at 6 h), LOX activity (2.44-fold at 6 h), and JA level at normal temperature (23 °C); however, it only slightly increased *Gllox*2 expression and internal JA content and inhibited LOX activity at 33 °C; (3) similarly, SA induced *Gllox*2 expression (2.32-fold at 12 h), LOX activity (approximately 2.00-fold at 6 and 12 h) and SA level at 23 °C, but it mainly decreased these parameters at 33 °C; and (4) the addition of MJ had either no effect or an inhibitory effect on endogenous SA content, and analogous effects of exogenous SA on the endogenous JA content were observed at both temperatures. From these results we conclude that lipoxygenase positively participated in the responses to high temperature and exogenous MJ or SA stimuli, and meanwhile, exogenous MJ or SA exerted a promoting effect on its corresponding endogenous phytohormone accumulation.

Keywords Rhodophyta · *Gracilariopsis lemaneiformis* · Lipoxygenase · Methyl jasmonate · Jasmonic acid · Salicylic acid · Temperature stress

Introduction

The seaweed *Gracilariopsis lemaneiformis* (Gracilariales, Rhodophyta) is the main feedstock in the agar industry and an ideal feed for abalone. Additionally, *Gp. lemaneiformis* can effectively absorb nitrogen and phosphorus and can thus be used to remediate eutrophic water environments (Zhou et al. 2006). Owing to its economic and ecological value, *Gp. lemaneiformis* is cultivated extensively along the coasts of China. The high-temperature-tolerant strain *Gp. lemaneiformis* 981 has been widely cultivated along the southeast coasts of Fujian and Guangdong Provinces. However, *Gp. lemaneiformis* in the south must be transported to the north because of the high summer temperatures in the south, resulting in the loss of industrial farming.

Jasmonates (JAs), which are oxylipin-type plant hormones derived from the fatty acid oxidation pathway, are widely present in animals, plants, bacteria and algae. JAs include diverse metabolites and derivatives such as jasmonic acid (JA), methyl jasmonate (MeJA or MJ), and jasmonic acid-isoleucine (JA-Ile). The enzymatically-derived oxylipin biosynthesis is a multi-step process commonly initiated by lipoxygenase (LOX) (Feussner and Wasternack 2002; Mosblech et al. 2009). As the key enzyme of JA synthesis, LOX is responsible for catalyzing the oxygenation of polyunsaturated fatty acids and their corresponding esters during

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lipid peroxidation. JAs play central roles in plant growth and developmental processes, including root growth, seed germination, flower development, leaf senescence, and plant defense responses to biotic and abiotic stresses (Wasternack 2007). An increasing number of reports have indicated that JAs and LOX actively participate in adverse environments such as heat, low-temperature stress, and wounding (Yang et al. 2009; Babenko et al. 2014; He et al. 2014). Analogous to their role in innate immunity in land plants, oxylipins play a pivotal role in the innate immunity of macroalgae (Bouarab et al. 2004; Barbosa et al. 2016). A MeJA-induced burst leads to the induction of a fatty acid oxidation cascade, the upregulation of the 13-lipoxygenase pathway, and other secondary metabolism processes to combat the inducing oxidative (Kumari et al. 2015). Similar to JAs, salicylic acid (SA) can alleviate the adverse effects caused by stressors including salt-, heat-, and water-stress (Hayat et al. 2010). As resistance-related phytohormones, SA and JAs have been the focus of some reports addressing the interplay between their signal pathways (Mur et al. 2006). However, the crosstalk between the JA and SA signal pathways remains unclear. One-way mutually antagonistic and synergistic effects between SA and MeJA have been revealed in *Sorghum bicolor* (Salzman et al. 2005).

In contrast to the information available for higher plants, information on LOX and its encoding genes in algae remains scarce. In red algae, *lox* genes have been identified in *Pyropia haitanensis* and *Chondrus crispus* (Collén et al. 2013; Zhu et al. 2015). To date, no *lox* gene information has been reported for *Gp. lemaneiformis*. Here, we identified two *lox* genes from *Gp. lemaneiformis* based on genome sequencing. Then, we analyzed *lox* transcription level and LOX activity and assayed the endogenous JA and SA contents under two temperature treatments and two phytohormone treatments. The findings provide insight into the roles of LOX and JAs as well as the relationships between JAs and SA.

Materials and methods

Algal material and culture conditions

Tetrasporophytes of *Gracilariuopsis lemaneiformis* 981 were collected from the coast near Xiapu (26° 65′ N, 119° 66′E), Fujian Province, China. After removal of sediment and other microalgae, the cleaned algae were cultivated in a light incubator. The purified algae were cultured in Provasoli medium at 23 °C under a 12L:12D photoperiod with a light intensity of approximately 40 μmol photons m^{-2} s^{-1}. The flasks were shaken and their positions exchanged with other flasks every 2 h to ensure consistent experimental conditions.

Experimental treatments

Two temperatures, 23 °C (normal temperature) and 33 °C (high temperature), and two phytohormone treatments, 50 μmol L^{-1} MJ and 100 μmolL^{-1} SA, were used. A total of six treatments (23 °C, 23 °C + MJ, 23 °C + SA, 33 °C, 33 °C + MJ, 33 °C + SA), each with three replications, were performed. The levels of *lox* transcription and LOX activity, and endogenous JA, SA contents were assayed at 0, 3, 6, 12, and 24 h.

Phylogenetic analysis of lipoxygenase genes

Two *lox* gene sequences, designated *Gllox*1 and *Gllox*2, were obtained from the genome information of *Gp. lemaneiformis* in our previous work. The ProtParam tool (http://web.expasy.org/protparam/) was used to predict the molecular weight (Mw) and isoelectric point (pI) of each Gllox amino acid sequence. Then, their subcellular localization and transmembrane domains (TMDs) were predicted using the PSORT program (http://psort1.hgc.jp/form.html) and the website (http://www.cbs.dtu.dk/services/TMHMM), respectively. The two Gllox sequences were aligned by BLASTP to determine their identity. Finally, 12 additional *lox* genes from *C. crispus* (Rhodophyta), *Ectocarpus siliculosus* (Phaeophyta), *Oscillatoria acuminata* (Cyanophyta), higher plants, and bacteria were aligned to construct a phylogenetic tree by the maximum likelihood method in MEGA 5.10 software.

Gllox expression analysis

Total RNAs were extracted by the RNeasy Plant Mini Kit (Qiagen, Germany) and were reverse transcribed into cDNA by the PrimeScript RT Reagent Kit with gDNA eraser (TaKaRa, China). Real-time quantitative PCR (RT-qPCR) primers were designed by Primer Premier 5.0 software (Table 1). The 18S rRNA gene was used as an internal control. The RT-qPCR system and procedures followed those described by Wang et al. (2017). The relative expression levels of the two *lox* genes were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Lipoxygenase activity determination

LOX activity was determined using a Plant Lipoxygenase Activity Assay Kit (Comin, Suzhou, China) based on the increase rate of the characteristic absorbance at 234 nm of oxidized linoleic acid.

Endogenous JA and SA content assays

The extraction, purification, and chromatographic analyses of JA and SA were carried out according to Cai et al. (2011).

Table 1 The primers used in the RT-qPCR analysis

Gene abbreviation	Gene name	Accession number	Primer sequences (5′-3′)	Amplicon size (bp)
*Gllox*1	Lipoxygenase	MF377635	F-TGGGCCGATTATTGGGTTCTG	173
			R-TGGCGATTGGATTTCAAGTAG	
*Gllox*2	Lipoxygenase	MF377636	F-CTTCTACGGAACCTGTCTCA TTAA	167
			R-CTTTGTCTGGCATCCACTCAT	
18S rRNA	18S ribosomal RNA	EU937746	F-CCTGAGAGACGGCTACCACA TCCA	167
			R-AGACTTGCCCTCTGCTGGCT CCTC	

Chromatographic grade *n*-hexane was added to the purified extracts from each treatment to obtain a final volume of 1 mL, and then the solution was filtered with a 0.22-μm organic filter, transferred to a CNW brown vial and analyzed by GC-MS (Shimadzu GC-MS QP2010, Japan) with an SPB50 polydimethylsiloxane non-polar column (30 m × 0.32 mm × 0.25 μm). Each sample was analyzed in three replicates.

Data statistics and analysis

All data were processed by Excel 2013. A one-way ANOVA (Tukey's post hoc test) for differences among time points or treatments and two-way ANOVA for the interactions between temperature and phytohormone treatments in SPSS 13.0 were used to determine significance at $P < 0.05$.

Results

Sequence characteristics and phylogenetic analysis

The *Gllox*1 sequence (accession number MF377635) contained a 2313-bp open reading frame (ORF) encoding a protein of 770 amino acids with a deduced 86.74 kD Mw and a 5.63 pI. The *Gllox*2 sequence (MF377636) consisted of a 1896-bp ORF encoding a protein of 631 amino acids with an Mw of 70.48 kD and a theoretical pI of 4.92. The two Gllox proteins were both localized to the endoplasmic reticulum with maximum certainties of 0.640 and 0.85, respectively, and no TMDs were detected. Their localizations differed from those of plant LOXs, which occur in chloroplasts, vacuoles, and the cytoplasm (Vick and Zimmerman 1987).

Among the top BLASTP hits, Gllox1 showed 56% identity to *C. crispus* (XP_005718273), with 82% coverage, and 67% identity to *Gracilaria chilensis* (AEH16747, partial sequence), with only 29% coverage; Gllox2 displayed 35% identity to the cyanobacterium *Acaryochloris marina* WP_012161442 and 37% identity to *Pseudanabaena biceps* (WP_009629598), both with 97% coverage.

The two Gllox sequences were separately distributed among the 14 total lox proteins from 10 species in the phylogenetic tree (Fig. 1). In group I, Gllox2 (MF377636) was grouped together with *P. haitanensis* (Rhodophyta), *Oscillatoria acuminata* (Cyanophyta), and bacteria, then with *Ectocarpus siliculosus* (Phaeophyta). In group II, Gllox1 (MF377635) first clustered with *C. crispus* and then was successively sister to *P. haitanensis* and *Porphyra purpurea* and to higher plants.

Gllox transcription level under different treatments

The transcription level of *Gllox*1 in the control, MJ, and SA groups remained almost unchanged over the 24 h at normal temperature (Fig. 2a). After the addition of exogenous MJ or SA, *Gllox*1 expression was slightly decreased at 3 and 6 h. The lowest expression of *Gllox*1 was detected at 6 h, with approximately 20% reduction in both the MJ and SA groups ($P < 0.05$).

Analogous to the changes at 23 °C, *Gllox*1 expression remained almost unchanged over the 24 h at high temperature, only with a slight increment observed at 6 h than other time points in the MJ treatment (Fig. 2b). After MJ or SA treatment, *Gllox*1 expression weakly decreased only at 24 h. According to the results in Fig. 2a, b under the two temperature conditions, *Gllox*1 transcript levels showed a slight reduction in response to exogenous MJ or SA treatment, and no interaction was found between temperature and phytohormone treatment (Supplementary Table 1).

In contrast to *Gllox*1, *Gllox*2 was markedly promoted by exogenous MJ and SA addition at 23 °C; however, it maintained basal expression in the control group. Moreover, *Gllox*2 expression was more influenced by MJ than by SA at 23 °C. For instance, *Gllox*2 expression reached its maximum (4.91-fold at 6 h) in the MJ group, whereas maximum *Gllox*2 expression in the SA group was 2.32 times higher than expression in the control group at 12 h (Fig. 3a).

Different from the expression profiles at normal temperature, the transcription level of *Gllox*2 was strongly stimulated by high temperature at the three groups (Fig. 3b). Under the

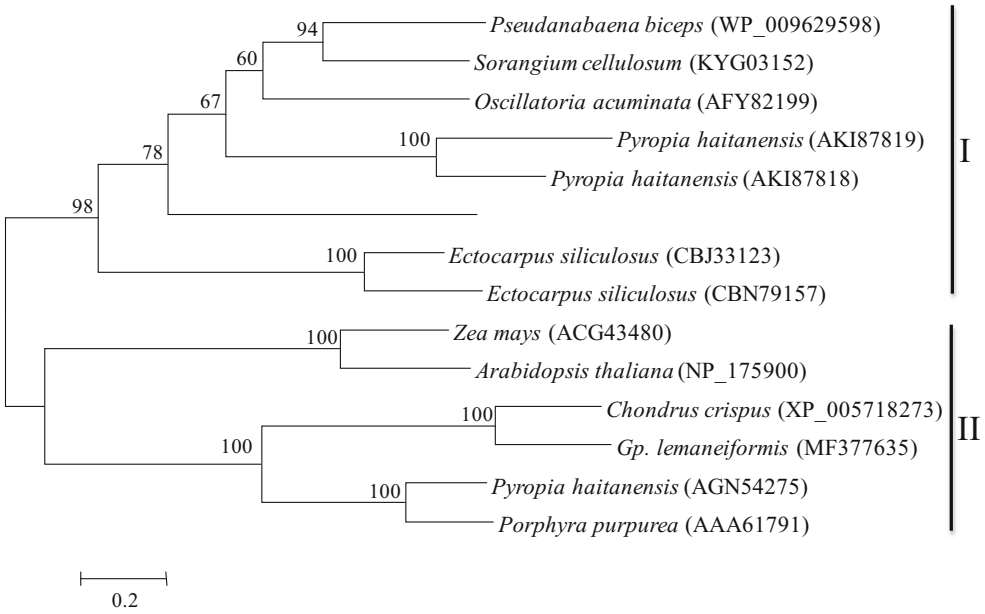

Fig. 1 Phylogenetic tree based on lipoxygenases from *Gp. lemaneiformis* and other species. The tree was constructed based on the maximum likelihood method by MEGA 5.10 software with 1000 bootstrap replications. *Gp. lemaneiformis* (MF377635) and *Gp. lemaneiformis* (MF377636) were designated as Gllox1 and Gllox2, respectively

high-temperature condition, the addition of the two kinds of phytohormone had no significant effect on *Gllox*2 expression levels relative to the respective control levels at 33 °C except for an increasing effect of MJ addition on *Gllox*2 expression to 1.35-fold that of the control at 12 h. These results indicated that MJ or SA mainly promoted *Gllox*2 expression at 23 °C and that MJ contributed slightly to the enhancement of *Gllox*2 expression at 33 °C. An interaction between temperature and phytohormone was found only in the SA treatment at 12 h ($P < 0.05$) (Supplementary Table 2).

LOX activity under different treatments

Similar to the *Gllox*2 expression profile at the same temperature, LOX activity was induced by both MJ and SA at 23 °C, with significant differences from the control activity observed from 3 to 12 h (Fig. 4a). In detail, MJ stimulated LOX activity from 3 to 12 h, which ranged from a minimum 1.80-fold to a maximum 2.61-fold the level of the respective control level. A similar trend was observed in the SA group, only with a lower fold (1.65- to 2.43-fold) than those in MJ group.

High temperature greatly enhanced LOX activity to ca. 330 U g^{-1} FW, whereas only ca. 150 U g^{-1} FW activity was detected at normal temperature (Fig. 4b). In contrast to the induction of the enzyme by two kinds of phytohormone at 23 °C, LOX activity remained almost unchanged and even decreased in the late stage at high temperature, with approximately 58 and 42% reduction relative to the control activity at 24 h in the MJ and SA treatments ($P < 0.05$), respectively. An interaction between temperature and phytohormone was detected in both the MJ and SA treatments at 6, 12, and 24 h ($P < 0.05$) (Supplementary Table 3).

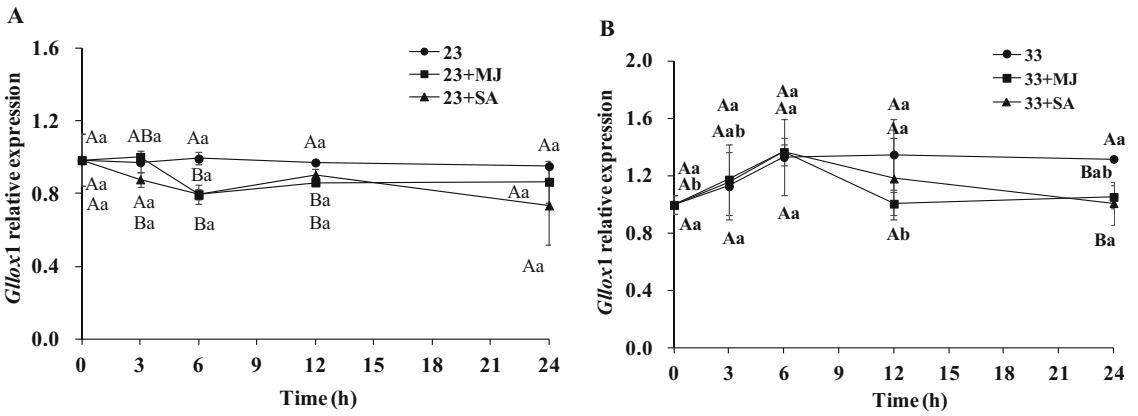

Fig. 2 *Gllox*1 expression profiles under different treatments (**a** 23 °C, **b** 33 °C). The data are displayed as the mean ± standard deviation ($n = 3$). Uppercase letters indicate differences among the three treatments at the same time point; lowercase letters indicate differences among the five time points in the same treatment group

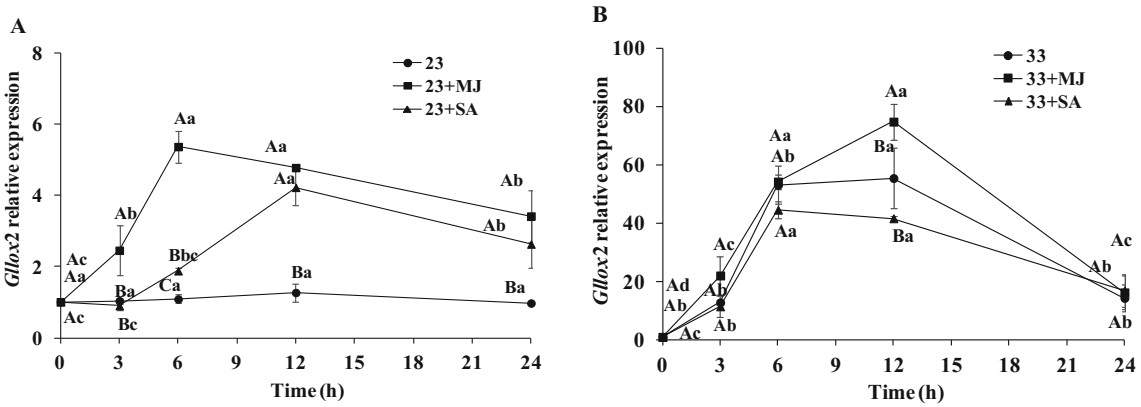

Fig. 3 *Gllox2* expression profiles under different treatments (**a** 23 °C, **b** 33 °C). The data are displayed as the mean ± standard deviation ($n = 3$). Uppercase letters indicate differences among the three treatments at the same time point; lowercase letters indicate differences among the five time points in the same treatment

Endogenous JA content under different treatments

At normal temperature, the internal JA content in *Gp. lemaneiformis* remained largely unchanged (ca. 35 ng g^{-1} FW) in the control and SA groups over the 24 h period (Fig. 5a). Exogenous MJ or SA treatment had a little effect on the endogenous JA content, e.g., the slight increments (0.17-fold at 3 h and 0.24-fold at 12 h) were observed in the MJ treatment ($P < 0.05$), and in contrast, a 0.17-fold reduction (only at 3 h) was observed in the SA treatment ($P < 0.05$).

However, high temperature stimulated endogenous JA accumulation to above 50 ng g^{-1} FW after 0 h (Fig. 5b). Similar to the effects at 23 °C, JA content showed little change other than a slight 0.24-fold increase at 12 h at 33 °C in the MJ treatment; however, JA content decreased under SA treatment, showing an approximately 30% decline at 3 and 12 h. An interaction between temperature and MJ treatment was detected at 12 and 24 h ($P < 0.05$), whereas an interaction between temperature and SA treatment was found at 3 and 12 h ($P < 0.05$) (Supplementary Table 4).

Endogenous SA content under different treatments

The endogenous SA content in *Gp. lemaneiformis* was also studied under different temperature and phytohormone treatments (Fig. 6). At normal temperature, endogenous SA accumulation was largely unaffected by exogenous MJ treatment, similar to that of the control group. However, exogenous SA significantly increased endogenous SA content from 3 to 12 h, with the maximum level of 2.32 times the control level observed at 6 h.

Higher levels of SA were observed at 33 °C than at 23 °C. Under the high-temperature condition, exogenous MJ caused ca. 33% reduction of internal SA content at 6 h ($P < 0.05$) and had no effect on SA content at the other time points. However, SA content showed an increasing trend at 33 °C, and the maximum SA content (77.12 ng g^{-1} FW) was observed at

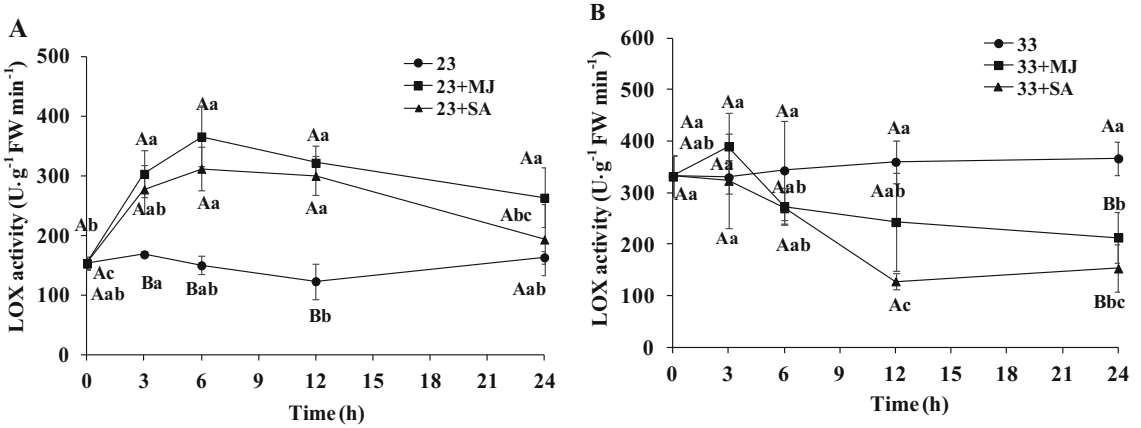

Fig. 4 LOX activity of *Gp. lemaneiformis* under different treatments (**a** 23 °C, **b** 33 °C). The data are displayed as the mean ± standard deviation ($n = 3$). Uppercase letters indicate differences among the three treatments at the same time point; lowercase letters indicate differences among the five time points in the same treatment

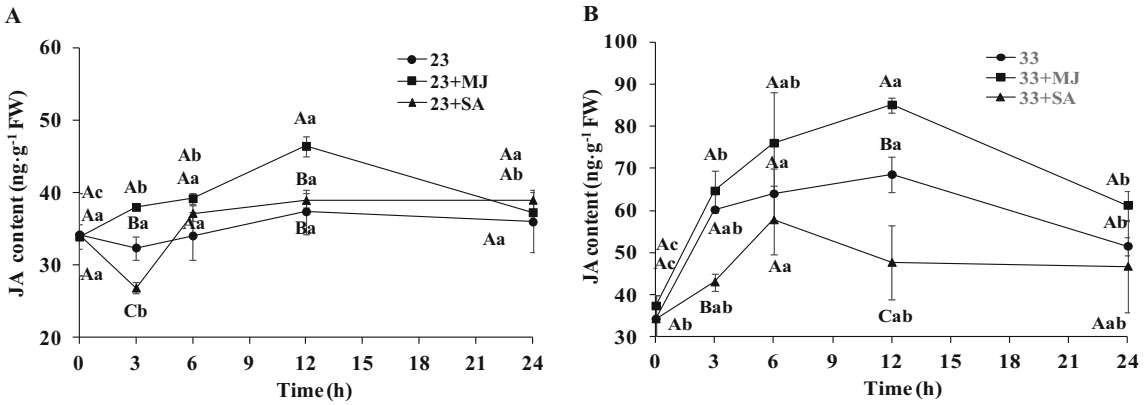

Fig. 5 Endogenous JA content of *Gp. lemaneiformis* under different treatments (**a** 23 °C, **b** 33 °C). The data are displayed as the mean ± standard deviation ($n = 3$). Uppercase letters indicate differences among the three treatments at the same time point; lowercase letters indicate differences among the five time points in the same treatment

12 h ($P > 0.05$), whereas only a 0.43-fold increment above the control level was detected at 6 h ($P < 0.05$). An interaction between temperature and MJ treatment was detected at 6 and 12 h ($P < 0.05$); however, an interaction between temperature and SA treatment was observed only at 6 h ($P < 0.05$) (Supplementary Table 5).

Discussion

LOXs in higher plants and algae

Like the oxylipins, LOXs are prevalent in mammals, microbes, higher plants and marine algae, with various catalytic substrates (Feussner and Wasternack 2002). In higher plants, various LOXs have been detected, e.g., 6 LOXs in *Arabidopsis thaliana* and 23 predicted *lox* genes in *Cucumis sativus* detected by genome sequencing (Bannenberg et al. 2009; Huang et al. 2009). In contrast to the multiple *lox* genes in higher plants, *lox* genes in algae are few, e.g., each with two in *Pyropia haitanensis* and in *Chondrus crispus*, respectively (Collén et al. 2013; Zhu et al. 2015). Here, we obtained two LOX sequences from red alga *Gp. lemaneiformis* and found that only 23.90% identity was detected between them, which is similar to the 20% identity detected between the two LOXs from *C. crispus* (Collén et al. 2013). Moreover, based on the phylogenetic tree of LOX sequences, we speculate that Gllox1 and Gllox2 might have diverged at an early stage of evolution or that the latter might have come from a symbiotic organism.

LOX levels and high-temperature stress

As a major environmental factor, temperature can affect the growth, development, and sensitivity of photosynthetic organs in plants. High temperature can cause membrane lipid peroxidation and reactive oxygen species accumulation, and it can induce the activity of antioxidant enzymes and heat shock proteins in plants to protect against these adverse effects (Noctor and Foyer 1998). An increasing number of examples show that high temperature also induces LOX activity via stress-resistance phytohormones. For instance, LOX activity and H_2O_2 content increased significantly in *Acanthopanax*

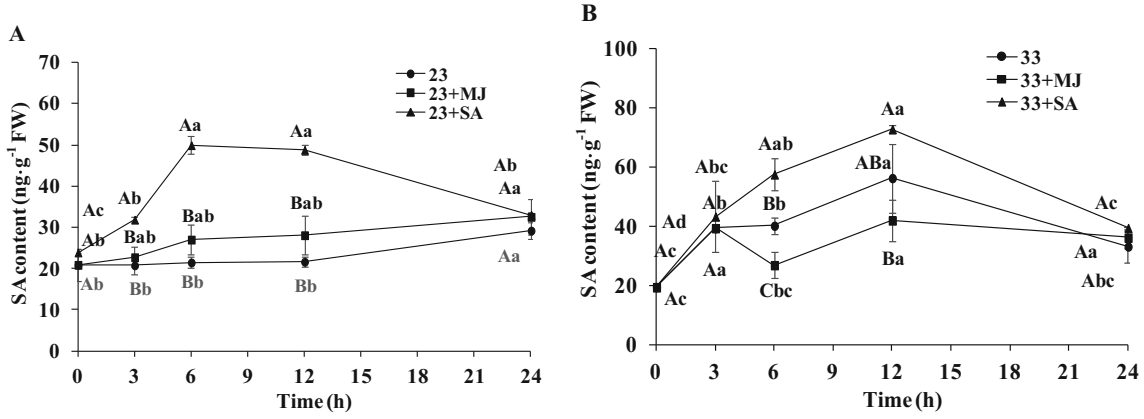

Fig. 6 Endogenous SA content of *Gp. lemaneiformis* under MJ and SA treatments (**a** 23 °C, **b** 33 °C). The data are displayed as the mean ± standard deviation ($n = 3$). Uppercase letters indicate differences among the three treatments at the same time point; lowercase letters indicate differences among the five time points in the same treatment

senticosus at 30 °C (Shohael et al. 2006), and LOX activity and MDA content were significantly higher at 30 and 40 °C than at room temperature in *Phalaenopsis aphrodite* (Ali et al. 2005). High temperature also induces *lox* expression at the transcription level. For example, a total of 3200 genes including the *lox*2 gene were induced to express in *Populus simonii* under heat stress (Song et al. 2014). The expression of *lox* was also enhanced during the ripening process in banana fruit when subjected to high temperature (Yang et al. 2010).

In *Gp. lemaneiformis*, the results are consistent with the above reports, namely, high temperature greatly enhanced LOX activity and *Gllox* mRNA accumulation. Moreover, in contrast to *Gllox*1 expression, *Gllox*2 was significantly stimulated by high temperature. Therefore, we believe that *Gllox*2 might be dominant in the synthesis of JA to resist high-temperature stress.

LOX levels and exogenous JAs/SA treatment

JAs play a key role in mediating defense responses in plants. Increasing JA levels and LOX activities contribute to tolerance to adverse stress. Reductions in LOX activity and endogenous JA content are accompanied by reduced heat tolerance and increased susceptibility to pathogenic microorganisms (Hu et al. 2015). An increasing number of studies have found that exogenous JAs can effectively influence LOX activity and its mRNA level. For instance, LOX activity increased by 28 times at 24 h after 40 μmol L^{-1} MJ addition in *Ambrosia maritima* (Zid and Orihara 2005). Exogenous 100 μmol L^{-1} MJ treatment significantly upregulated the expression levels of *Pglox*3, *Pglox*4, and *Pglox*5 in the adventitious roots of *Panax ginseng* (Bae et al. 2016). However, diverse *lox* genes exhibit distinct responses to exogenous phytohormones. In *Taxus chinensis*, *Tclox*1 and *Tclox*2 displayed different responses to exogenous MJ treatment (Li et al. 2012). In the present study, exogenous MJ largely inhibited *Gllox*1 expression and induced *Gllox*2 expression at the two temperatures and promoted LOX activity at 23 °C. Thus, these results further demonstrate that *Gllox*2 might be the major contributor to LOX activity and that *Gllox*2 might dominate in the response to exogenous MJ stimulation.

As stress-related phytohormones, SA and JAs can display similar or opposite effects. In some cases, SA and JA mainly rely on mutual antagonism (Kunkel and Brooks 2002). For instance, *lox*2 expression in wheat was inhibited by SA but induced by JA (Ding et al. 2016). In the present study, SA mostly promoted LOX activity and *Gllox*2 expression at 23 °C and mainly inhibited these traits at 33 °C, whereas it slightly decreased *Gllox*1 expression at the two temperatures. Therefore, we speculate that SA and MJ had similar effects on LOX activity and its mRNA levels. In addition, more interaction between temperature and MJ/SA occurred at the enzyme activity level.

Exogenous MJ/SA treatment and endogenous JA/SA levels

In general, exogenous JAs can stimulate intrinsic JA, e.g., pretreatment with 100–200 μmol L^{-1} MJ increased the JA content in *Pisum sativum* seedlings at both 20 and 40 °C (Shahzad et al. 2015). Similarly, exogenous SA can promote the accumulation of its endogenous content (Talieva and Kondrat'eva 2002). However, different responses can occur in different plants and even in different organs of the same plant. For instance, exogenous MJ treatments significantly increased JA concentrations in apical leaves while having no significant effect on basal leaf tissue in kale (Ku et al. 2014), and endogenous SA content was gradually reduced in shoots but not in roots after treatment with 0.05 mmol L^{-1} SA during 2-week-long seedling growth in wheat (Rakhmankulova et al. 2010). In *Gp. lemaneiformis*, analogous to the basic promoting effect of MJ on endogenous JA content, SA mainly enhanced endogenous SA content at normal or high temperature.

In plant signaling, the interactions between JAs and SA are largely reflected in their influences on each other's content. However, both synergistic and antagonistic effects have been reported. In tomato seedlings, SA content was elevated by 0.1 mmol L^{-1} MeJA treatment, and this elevation was simultaneously accompanied by a decrease of JA (Król et al. 2015). In contrast, SA content was reduced with increasing MJ concentration in *Pisum sativum* seedlings (Shahzad et al. 2015). In the present study, the addition of SA had either no effect or an inhibitory effect on endogenous JA content at 23 and 33 °C, and the addition of MJ had no impact or an inhibitory effect on endogenous SA content at some individual time points at the two temperatures. From these results, we deduce that the interaction between MJ and SA might have little effect on endogenous JA/SA levels.

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