Control of elicitor-induced oxidative burst by abscisic acid associated with growth of *Saccharina japonica* (Phaeophyta, Laminariales) sporophytes

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Abstract

In this study, the oligoguluronate elicitor-induced oxidative burst (OB) was monitored continuously in young and mature *Saccharina japonica* sporophytes based on luminol chemiluminescence using a photon counter. The iodoperoxidase (IPO) activity, abscisic acid (ABA) and polyphenol contents were also compared in the different growth stages. The elicitor-induced OB occurred within 1 min and reached its maximum in 15−20 min after treatment in all growth stages. The active elicitor-induced OB was stronger in the young sporophytes than the older sporophytes. The IPO activity in the different growth stages also exhibited a similar pattern to the elicitor-induced OB. These results suggest that the elicitor-induced OB and the subsequent high haloperoxidase activity comprise a major defense mechanism in young sporophytes. By contrast, ABA accumulated with the growth of the sporophytes. Interestingly, ABA treatment suppressed the elicitor-induced OB during growth and enhanced the elicitor-independent IPO activity even in the young sporophytes. In addition, the polyphenol content was higher in the older sporophytes than the younger sporophytes. These observations show that dramatic changes occur in the characteristic defenses against biotic stresses as the sporophyte grows, as well as suggesting that ABA is closely linked with these changes. Moreover, the IPO activity recovered slightly in the sorus, which is the reproductive tissue, thereby suggesting that a higher ABA content increases the defense activity and the success of reproduction.

Key words: abscisic acid, elicitor, growth, iodoperoxidase, kelp, oxidative burst

Introduction

Kelps are perennial brown algae and they exhibit alternating generations between microscopic gametophyte and macroscopic sporophyte. The sporophytes often grow up to several metres in length and they can form large forests in coastal waters where they are the main primary producers with high productivity (Mann 1973). Their abundant biomass has ecological importance, including functions as a nutrient stock in coastal waters, and as a rearing ground and food for many marine organisms, including fishes and benthic animals. Thus, the sporophytes are always exposed to biotic stresses such as pathogens and feeding for a long period. For example, herbivorous benthic animals such as sea urchins (Estes et al., 1988, 2004; Launchbaugh and Howery, 1993) and pathogens (Ishikawa and Saga, 1989) may comprise biotic stressors that can lead to the disappearance of kelp forests.

Saccharina japonica (Areshoug) Lane, Mayes, Druehl and Saunders (Lane et al., 2006) has been cultivated widely as a foodstuff in eastern Asia, where the cultivation process starts with seeding and culturing to the juvenile sporophyte stage in land-based hatcheries, before transplanting into natural waters. During the cultivation process, the seedling may encounter diseases such as red spot disease (Sawabe et al., 1998), as well as swollen gametophytes and filamentous fading (Peng and Li., 2013) in the microscopic stage. After transplanting the young sporophytes into natural conditions, they are exposed to various other abiotic stresses, which differ from those in artificial indoor conditions, where hole rot disease has been reported (Wang et al 2008). Therefore, effective management during the cultivation of this kelp demands an understanding of the defense mechanisms that function in the different growth stages from the juvenile to mature sporophytes.

In general, plants have the capacity to resist biotic stresses via active (induced) and passive (constitutive) resistance and kelps are no exception. It has been reported that the use of elicitor can activate the same response to the attacks by pathogens or feeding by herbivores, and thus kelps have induced defense mechanisms to resist biotic stressors. In Laminariales plants, oligoguluronate (OG) is a decomposition product of alginic acid, a major matrix component of the cell wall, and it induces an active defense response without cell death (Küpper et al., 2001, 2002). A series of active defense responses starts with the oxidative burst (OB) characterised by the production of reactive oxygen species (ROS), which induce the subsequent defense responses, including the activation of haloperoxidase (Thomas et al., 2014) and the production of halogen compounds as chemical defense mechanisms (Palmer et al., 2005; Küpper et al., 2008). Polyphenolic substances are also synthesised as secondary metabolic products (Küpper et al., 2002). Thus, the elicitor-induced OB is an important primary defense mechanism that protects against biotic stresses.

Kelps also have passive defense capacities in addition to the active defense mechanisms described above. For example, Laminariales plants produce polyphenolic compounds as a chemorepellent to avoid feeding by herbivores (Steinberg, 1984; Pavia and Toth 2000). Harbivore-induced wounding results in the production of ROS, although the ROS level is controlled by scavenging enzymes and antioxidants. In the presence of ROS and haloperoxidase, kelps can strengthen their cell wall by cross-linking polyphenolic compounds (Berglin et al 2004; Bitton et al 2007). Moreover, excess ROS production often induces hypersensitive cell death stop to the damage caused by infection or feeding. However, cell wall strengthening or hypersensitive cell death must affect growth, particularly in the young stages. In general, it has been shown that various metabolic activities, including nutrient uptake (Harrison and Druehl, 1982; Harrison et al., 1986) and photosynthesis (Wheeler, 1980), fluctuate with growth and ageing. Therefore, the stress response activity is also expected to fluctuate with growth, ageing and the physiological status in sporophyte.

In unicellular algae, it has been reported that the defense responses to stresses are related to abscisic acid (ABA), which has a role in protecting against the oxidative stress induced by salt and osmotic stresses (Saradhi et al 2000; Yoshida et al 2003, 2004). Similarly, in macroalgae, ABA acts as a signaling hormone under salt stress (Guajardo et al., 2016). In addition to abiotic stresses, biotic stresses also induce OB in a similar manner. Thus, the participation of ABA in response to biotic stresses is expected in macroalgae. However, the participation of ABA in biotic stress responses has not been reported previously.

Thus, in the present study, we aimed to clarify the time-course of the changes in the elicitor-induced OB at the time unit of seconds using a photon counter. Next, we determined the changes in the defense characteristics among the growth stages from young to mature *S. japonica* sporophytes by investigating the elicitor-induced OB. Finally, we investigated the relationships among the elicitor-induced OB, ABA content, and haloperoxidase activity.

Materials and Methods

Preparation of materials: Sporophytes of *S. japonica* were used as materials. The immature sporophytes of different growth stages (50−80cm and 150−200cm in thallus length) and mature sporophytes (ca. 1−3m) were collected from coastal waters near the Fisheries station of the Field Science Center of Hokkaido University routinely during October 12, 2015 and December 18, 2016. After collection, the sporophytes were placed in polyethylene bags, which were transported in a cool box with refrigerants to our laboratory. To prepare the young sporophytes, the reproductive parts (sori) were cut off, washed with sterilised seawater (33−35psu) that had been filtered through a glass fibre filter (Whatmann GF/C, pore size: 0.45μm), wiped with a paper towel (Nippon Paper Crecia Co. Ltd Tokyo) and wrapped in newspaper, before storing in a refrigerator (4°C). After 2−5 h, the sorus parts were placed in separate polyethylene bags with autoclaved seawater (120°C for 20 min) filtered through glass fibre filters (Whatmann GF/C) to release the zoospores. The solution containing zoospores was poured into a polystyrene container (180 × 90 × 45mm) with segments of slide glass and sterilised filtered seawater. After about 1 day, we confirmed the settlement of zoospores on the glass slide segments under an inverted microscope (Nikon TMS, Tokyo, Japan) and the segments were placed in another case containing vitamin-free Provasoli's nutrient enriched seawater (PES) (Provasoli, 1968), before culture at 10°C under 5 μmol photons m^{-2} s^{-1} (12:12 h light:dark cycle). The juvenile sporophytes that developed from the gametophyte stage were placed into 1-L transparent plastic bottles and culture was continued under aeration until the blade length of the sporophytes exceeded 1cm. The young sporophytes were used as well as naturally collected sporophytes in the following experiments. Depending on the specific experiment, the whole juvenile sporophytes (1−3 cm in length), the discs (0.5−0.8 cm in diameter) obtained using a cork borer from the central parts of the sporophyte, or the segments (ca. 1.5 cm × 5 cm) were used after pre-culture for more than 2 weeks.

Detection of OB induced by an elicitor: Prior to the experiments, OG was prepared as an elicitor according to the procedure described by Haug et al. (1974). Using the apparatus shown in Fig. 1, the OB induced by OG was detected in the sporophyte tissues. Whole juvenile sporophytes or discs of the sporophytes were placed in a reaction vessel (10 mL) containing the reaction medium, which comprised sterilised filtered seawater with added luminol at a final concentration of 15μM. The reaction was conducted at 10°C in the dark. Using a syringe pump (DR-10, AS ONE Co. Japan) with a remote controller (CT-10, AS ONE Co. Japan), the elicitor was added to the reaction vessel (3 - 5cm in diameter) at a final concentration of 150 μg mL^{-1}. The chemiluminescence produced when the luminol reacted with ROS was monitored for ca 1 h via the photomultiplier tube of a photon counter (SUC-100 ver. 1.5, SCIENTEX Inc. Japan) connected to an external computer. Chemiluminescence was also monitored in the reaction medium without luminol. After monitoring, the morphological outline of a young sporophyte was traced on tracing paper to estimate the surface area. Chemiluminescence was expressed as photon count number per base unit (cm^{-2}).

Before the experiment, the relationship was determined between the chemiluminescence response of luminol and the hydrogen peroxide concentration. When chemiluminescence was measured after adding 10mM phosphate−buffered saline (PBS, pH 7.8) with peroxidase (0.5×10^{-3} units mL^{-1}, horseradish) and hydrogen peroxide (0, 0.1, or 1mM) to 10mL of sterilised filtered seawater containing luminol.

Response to the elicitor in different growth stages: Using discs placed in a vessel containing 3 mL of sterilised seawater with 15 μM luminol, the response to the elicitor was monitored over time in the same manner described above. Gametophytes attached to glass slide segments (2.5 cm × 3.7 cm) were used to monitor the chemiluminescence response to the OG (final concentration 150 μg mL^{-1}). Measurements were obtained in a

vessel containing 10 mL of sterilised seawater with 15 μM luminol. The chemifluorescence was detected per unit surface area of the slide segment.

Measurement of iodoperoxidase (IPO): IPO is a peroxidase that reacts specifically with iodine as a substrate, and it was detected according to the methods reported by Hosoya (1963) and Mehrtens (1994). The young sporophytes, discs obtained from the immature sporophytes and sorus parts were ground separately in liquid N_2. Each powdered sample (0.16−0.3 g) was placed in a microtube (2 mL) with cold PBS (0.1 M, pH 6.0) and repeatedly agitated, before storing overnight at 4°C to extract the proteins. The solution was centrifuged at 12,000 × *g* and 4°C for 15min. Next, 200 μL of the supernatant was placed in another microtube (2 mL) and mixed with 1 mL of 0.1 M PBS (pH 6.0) containing 5 mM potassium iodine. After adding 12 μL of 40mM hydrogen peroxide at room temperature, the reaction was started and the increase in the absorbance for 3 min was measured with a spectrophotometer (V-600DS, JASCO Co., Ltd., Japan). The same procedure was performed using PBS instead of the supernatant as the blank and absorbance determined was subtracted from the increases in the absorbance. The enzyme activity was expressed as the change in the absorbance per surface area of the sporophyte or disc. An increase of 0.001 absorbance units per 1 min was expressed as an enzyme activity of 1 mU.

ABA content of sporophytes and its effects on the elicitor response and IPO activity: Sample segments obtained from young sporophytes, and the vegetative and sorus parts of the sporophytes were used for extracting ABA, as described by Nimura and Mizuta (2002). Each sample (1.5−2.0g) was wiped with a paper towel and extracted with 10mL of methanol at 4°C for 2 h. The extract was collected and the residual sample was extracted again. The combined extracts were mixed and concentrated to dryness at 45°C using an evaporator. The residue was redissolved in 10mL of distilled water, adjusted to pH 3.0, and washed twice with 3 mL of *n*-hexane. Next, the solution was extracted with 2 mL of ethyl acetate and the water layer was evaporated to dryness again. The residue was redissolved with 10 mM PBS (pH6.9) containing 0.1% ethanol for use in the bioassay as a test solution.

The ABA content was estimated using a bioassay based on the opening and closing of stomata on the undersurface of a pigeon bean (*Vica faba*) leaf (Tucker and Mansfield 1971). First, the epidermis of the leaf was sliced, and the epidermis was then placed in 10 mM PBS (pH 6.9) in the dark to close all of the stomata. Next, the epidermis segment with closed stomata was placed in 3 mL of a test solution in a 12-well tissue culture plate (Falcon®, Becton Dickinson Labware, & Company, USA) and treated with 200 μmol photons m^{-2} s^{-1} for 3 h. The degree of opening of the stomata was observed in more than 15 randamly selected stomata for each epidermal segment under a microscope. Three epidermal segments were used for each test solution. A calibration curve was obtained using commercial ABA (Wako Chemicals, Japan). The ABA content was expressed as μg equivalent ABA per g fresh weight of the sporophyte segment.

The effects of ABA on the elicitor responses and IPO activity of young sporophytes were investigated using discs. Eleven young sporophytes were incubated in 100 mL of vitamin-free PES medium with or without 10 μM ABA. The culture conditions comprised 10°C with 5 μmol photons m^{-2} s^{-1} (12:12 h light:dark cycle). After 0, 3, 5 and 7 days of culturing, each disc was placed in a plastic dish containing 3 mL of 15 μM luminol and the elicitor-induced OB was monitored as described above. Young sporophytes were also cultured in 700 mL of the medium with or without 10 μM ABA under the same conditions. The sporophytes cultured for 5 days were used to measure the IPO activity, the relative growth rate on an area basis, ROS production and the polyphenol and antioxidant contents.

Measurement of chemical components related to defense: Sample discs were obtained from young sporophytes, and vegetative and sorus parts of the sporophytes. Intracellular ROS production was measured as dichlorofluorescein (DCF) produced via the oxidation of 2′, 7′-dichlorodihydrofluorescein ($DCFH_2$-DA), according to Mizuta and Yasui (2010). The discs were cultured in sterilised seawater with 50 μM $DCFH_2$-DA at 10°C under dark conditions for 1 h. After culturing, the sample weight and area were measured, and the sample was ground in liquid N_2. The powdered sample was then weighed and inserted in a microtube (2 mL), before adding 1mL of 40 mM Tris-HCl buffer (pH 7.0) and agitating for 5 min. After centrifugation at 10000 × *g* for 5 min, 500 μL of the supernatant was diluted with 2.5 mL of 40 mM Tris-HCl buffer (pH 4.0), which was then used for measuring the intensity of fluorescence at an excitation wavelength of 488 nm and fluorescence wavelength of 525 nm using a photofluorometer (FP-750, JASCO Co., Ltd., Japan). DCF was used as a standard, and ROS production was expressed as the DCF produced per unit area.

The polyphenol contents were measured as described by Senevirathne et al (2006). Each disc was wiped with a paper towel, weighed and the outline was traced on tracing paper to estimate the area. Each sample was weighed, ground in liquid N_2, and the powder produced was collected in a microtube (2 mL) and weighed again. Next, 200 μL of ethanol and 1 mL of distilled water were added and mixed in the microtube, before adding 500 μL of 50% Folin-Ciocalteu reagent. After agitation, the microtube was allowed to cool in the dark conditions for 5 min. Subsequently, 150 μL of 5% sodium carbonate solution was added, before standing at room temperature in the dark for 1 h, and subjecting to centrifugation at 10,000 × *g* for 5 min. The supernatant was measured at a wavelength of 725 nm using a spectrophotometer (V530, JASCO Co., Ltd., Japan). The standard solution was prepared by dissolving gallic acid in 95% ethanol and the polyphenol content per unit area was expressed as gallic acid equivalents.

The antioxidant activity was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method described by Heo et al (2005). The discs were agitated in 0.5 mL of 70% ethanol and kept at 4°C for 10 min. The ethanol solution was then centrifuged at 10000 × *g* for 5 min and 50 μL of the supernatant was divided into two 2 mL tubes, where 1.5 mL of 40 μM DPPH was added to one and 1.5 mL of 70% ethanol was added to the other. After standing for 1 h, the absorbance was measured at 517 nm with a spectrophotometer. The antioxidant contents were calibrated using Trolox in 70% ethanol as the standard and expressed as Trolox equivalents per unit area.

Statistics: Comparisons between two groups were performed using the Student's-*t* test at $P<0.05$. Multiple comparisons were conducted using Bonferroni's multiple comparisons test if equal variances were confirmed with Bartlett's test. If the variances were not equal, the Kruskal−Wallis test was used for nonparametric multiple comparisons followed by the Steel-Dwass test. $P<0.05$ was considered to indicate significant differences in all tests.

Results

Luminol-dependent chemiluminescence was detected in *S. japonica* sporophytes after OG treatment (Fig. 2). The chemiluminescence was positively correlated with the H_2O_2 concentration ($y = 1743.8x + 65.35$, $r^2=0.96$, n=9), thereby confirming that the chemiluminescence was attributable to the occurrence of an elicitor-induced OB. The OB started to increase within 1 min after treatment with the elicitor and the maximum was reached within 20 min, before decreasing to a constant value, which was slightly higher than that before treatment with the elicitor. The time-dependent changes in the OB induced by the elicitor were similar in all of the sporophyte tissues during the different growth stages. However, the elicitor-induced OB was not detected in

the gametophytes (Fig. 2b), thereby demonstrating that the gametophytes were not sensitive to the elicitor. In addition, the elicitor-induced OB was not observed in young sporophytes treated with DPI, which is a known NADPH oxidase inhibitor (Fig. 2c).

The mean maximum elicitor-induced OB differed among the tissues during the various vegetative growth stages of sporophytes (Fig. 3a). The elicitor-induced OB was observed in all tissues, but the highest mean value occurred in the young sporophyte tissues. The maximal OB decreased with growth and the lowest value was detected in the vegetative tissues obtained from the sporophytes measuring 150−200 cm in length and the reproductive tissues (sori), where the OB was less than 20% of the level observed in the young sporophytes. The IPO activity was highest in the young sporophytes (94.1 ± 26.5 mU cm^{-2}) (Fig 3b). The lowest value was observed in the vegetative tissues of the mature sporophytes (15. 2± 26 mU cm^{-2}). The value was twice as high in sori (26.5 ± 26 mU cm^{-2}) compared with the vegetative tissues of the mature sporophytes. By contrast, the mean ABA content was lowest (0.007 ± 0.001µg equivalent-ABA g^{-1} fresh weight) in the young sporophytes and it increased with growth (Fig 3c). Sori had remarkably higher ABA contents, which were about 25 times higher (0.177 ± 0.073µg equivalent-ABA g^{-1} fresh weight) than those in the young sporophytes.

The effects of ABA on the elicitor-induced OB are shown in Fig. 4. Treatment with ABA gradually decreased the elicitor-induced OB throughout the culture period to about one-half of that at the start after 3 days of culture (Fig. 4a). The elicitor-induced OB in young sporophytes exposed to ABA for 7 days was less than one-tenth of that at the start of culture period. ABA treatment also inhibited the growth rate and ROS production (Table 1). The IPO activity in the young sporophytes cultured with ABA for 5 days was about 1.4 times higher than that in the control group. However, the polyphenol and antioxidant contents did not change significantly during culture for 10 days.

The differences in ROS production as well as the polyphenol and antioxidant contents among young sporophytes, and vegetative and reproductive tissues of the mature sporophytes are shown in Table 2. ROS production was lowest in the young sporophytes. The ROS production level was highest in sori, which was about seven times higher than that in the young sporophytes. The polyphenol and antioxidant contents among the tissues exhibited similar trends to that of ROS production, where both were lowest in the young sporophytes and increased with growth. The maximal values were observed in sori.

Discussion

The OG-induced OB was highest in the young sporophytes (Figs 2 and 3a), thereby suggesting that the young sporophytes were more sensitive to the elicitor. However, the elicitor-induced OB decreased with growth and its pattern was similar to that of the IPO activity. Thomas et al.(2011) showed that in wild *Laminaria digitata*, the expression level of the gene encoding haloperoxidase increased by 5−15 times about 3 h after treatment with an elicitor. Haloperoxidase catalyses the reaction between a halogen and hydrogen peroxide to produce an antibiotic halogen compound, which controls biofilm formation on the surface of *L. digitata* sporophytes (Wever et al., 1991; Borchardt et al., 2001). In addition, the high IPO activity in the young sporophytes is considered to be supported by the higher iodine content during the young stage, as reported previously (Ar Gall et al., 2004; Teas et al., 2004). Therefore, the defense mechanism in young sporophytes is characterised by the elicitor-induced OB and the subsequent increase in IPO supported by high iodine contents. It has also been suggested that other defense mechanisms compensate for the decline in the elicitor-induced response during growth.

In general, the structural strength of sporophytes increases with growth. The strengthening of the structure is achieved by activating secondary metabolism and the subsequent accumulation of metabolites, such as polyphenol substances. It has been suggested that polyphenol plays roles in strengthening the cell wall by cross-linking with ROS in the presence of haloperoxidase (Berglin et al., 2004; Bitton et al., 2007) , which also protects against feeding by benthic animals (Pavia and Toth, 2000). Therefore, the decrease in the defense capacity associated with the elicitor-induced OB during growth appears to be compensated for by increasing structural defenses and accumulating defense-related materials. We found that the polyphenol contents and potential ROS production increased with growth in *S. japonica* (Table 2) irrespective of the decrease in the IPO activity (Fig. 3). Thus, the IPO activity may be sufficient to increase the structural strength together with the accumulation of polyphenols and the production of ROS. Therefore, the defensive capacity is considered to be maintained throughout sporophyte growth, even in older sporophytes with low levels of elicitor-induced OB.

Interestingly, ABA greatly suppressed the elicitor-induced OB in young sporophytes (Fig. 4). Suppression by ABA has also been reported in some terrestrial plants (Graham and Graham, 1996; Clay et al 2009; Kusajima et al 2010; Bassaganya-Riere et al, 2011) and it was suggested that ABA has a negative (antagonistic) role in suppressing salicylic acid-mediated defense signalling but also a positive role in producing callose as a defensive material for disease resistance (Mauch-Mani and Mauch 2005). Thus, these negative and positive roles of ABA may be distributed widely from seaweeds to terrestrial plants. The young sporophytes appeared to possess an ABA-independent defense mechanism characterised by active elicitor-induced OB because the OB was suppressed by ABA. Moreover, the suppression of the elicitor-induced OB by ABA is considered to be closely linked with the function of ABA as a growth inhibitor (Schaffelke 1995 a, b). The ABA bioassay showed that the level was lower during the young stage with active growth activity (Fig. 3), as also reported by Nimura and Mizuta (2002). Thus, the low ABA content of young sporophytes is necessary to prevent suppression of the elicitor-induced OB but also for maintaining growth, where the negative effects of ABA have an important role in growth. Therefore, these observations suggest that the elicitor-induced OB is controlled by the accumulation of ABA as the growth activity decreases, where the defensive mechanism changes from ABA-independent to ABA-dependent defense. We hypothesise that there are closerelationships among the elicitor-induced defense mechanism, the function of ABA, and the growth of sporophyte.

The contribution of ABA to abiotic stresses is characterised by increases in the ROS scavenging activity by enzymes including catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) (Guajardo et al., 2016). In the present study, we found that ABA treatment decreased ROS production (Table 1), thereby indicating that ABA acts to mitigate oxidative stress where this function is supported by the accumulation of ABA with growth. In particular, the ABA contents were higher in the sori (Fig. 3). Higher ROS scavenging activities, including CAT, GR and SOD, have also been observed in sori (Mizuta and Yasui, 2010). These observations suggest that ABA is strongly related to the oxidative stress that occurs under various stress conditions. Moreover, the IPO activity was slightly higher in sori, where the level did not reach that observed in the young sporophytes, although the elicitor-induced OB activity was low. In addition, ABA enhanced the IPO activity (Table 1), thereby indicating the participation of ABA in the responses to abiotic stresses such as desiccation (Guajardo et al., 2016) but also in biotic stress responses. Previous studies have shown that some kelps possess various isoforms of IPO (Almeida et al., 1998, 2001). The increased IPO levels in the sori were probably ABA-dependent forms, whereas the active ABA-independent forms of IPO may have been present in the young sporophyte tissues. This suggests that successful maturation requires

both defensive mechanisms with an elicitor-independent IPO activity and structural defense with the accumulation of polyphenols.

As described above, the elicitor-induced OB was observed throughout sporophyte growth from the young to reproductive stages in *S. japonica*. However, this response was not found in the gametophygtes (Fig. 2), which agrees with a previous report on *Laminaria digitaita* (Küpper et al., 2001), thereby suggesting that the sporophyte-specific elicitor response is a common characteristic of Laminariales plants. The sporophyte-specific elicitor-induced OB was inhibited by DPI (Fig. 1), and there was a positive relationship between hydrogen peroxide production and the elicitor-induced chemiluminescence. These results indicate that the chemiluminescence induced by the elicitor was due to ROS production by NADPH oxidase. Kanamori et al. (2012) reported that in *S. japonica*, DPI inhibited ROS production by NADPH oxidase in the cell membrane of the epidermal cells adjacent to a wounded area when young sporophyte tissue was injured. This suggests that ROS production by NADPH is attributable to the defensive responses of epidermal cells to abiotic and biotic stresses.

In the present study, the elicitor-induced OB increased within 1 min and reached its maximum within 20 min (Fig. 2), which demonstrates that the defensive response to biotic stress occurs rapidly. Similar rapid responses are found in *L. digitata* and *Fucus vesiculosus* (Küpper et al., 2001). Thus, the sporophyte of *S. japonica* has similar OG elicitor response characteristics to other brown algae. The time required to reach the maximum ROS accumulation level appears to be slightly faster than the 20−40 min found in terrestrial plants (Hotter, 1997). Pathogens spread more rapidly in water, which does not have a buffer action like soil, so plants that live in water might respond more rapidly to infection. Thus, the start time and the duration of the OB are important indicators of the response to biotic stresses, and their characteristics influence survival under biotic stresses. Therefore, the continuous monitoring of OB using a photon counter can be employed to identify defense activators by quantifying the level of the priming effect, which is defined as an increase in the elicitor-induced OB.

In conclusion, we found that the young sporophyte stage was highly sensitive to an OG elicitor, which was characterised by the elicitor-induced OB and subsequent haloperoxidase activity. As the sporophyte grows, the elicitor-induced OB activity decreased but the accumulation of defensive materials containing polyphenols increased, thereby demonstrating that the major defense mechanisms change with growth and maturation. Moreover, our results suggest that the sorus develops the structural and chemical defenses but also activates IPO via ABA, which is accumulated as the sporophyte grows.

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Figure captions

Figure 1 Schematic diagram of the apparatus employed for monitoring the elicitor-induced oxidative burst in seaweed.

Figure 2 Changes in the chemiluminescence intensity in the vegetative and reproductive (sorus) parts of the *Saccharina japonica* sporophytes measuring ca. 5cm, 50−80cm, and 150−200cm in length (a) and the gametophytes (b) after exposure to the elicitor (arrows: time=0) (a). (c) Changes in the chemiluminescence intensity in the young sporophyte (ca. 5cm in length) without luminol and with DPI are shown.

Figure 3 Maximum chemiluminescence (a), iodoperoxidase activity (b) and abscisic acid (ABA) content (c) in the vegetative and reproductive (sorus) parts of *Saccharina japonica* sporophytes measuring ca. 5 cm (A), 50−80 cm (B), and 150−200 cm(C) in length. The different letters indicate significant differences ($P < 0.05$) among the vegetative tissues of the sporophyte. Asterisks indicate significant differences ($P<0.05$) between the vegetative and sorus parts of sporophytes measuring 150−200cm in length.

Figure 4 (a) Maximum elicitor-induced chemiluminescence after exposure to the elicitor in young *Saccharina japonica* sporophytes cultured with abscisic acid for 0, 3, 5 and 7 days. (b) Time-course changes in the chemiluminescence in sporophytes cultured with abscisic acid for 0 days (closed circle) and 7days (open circle).

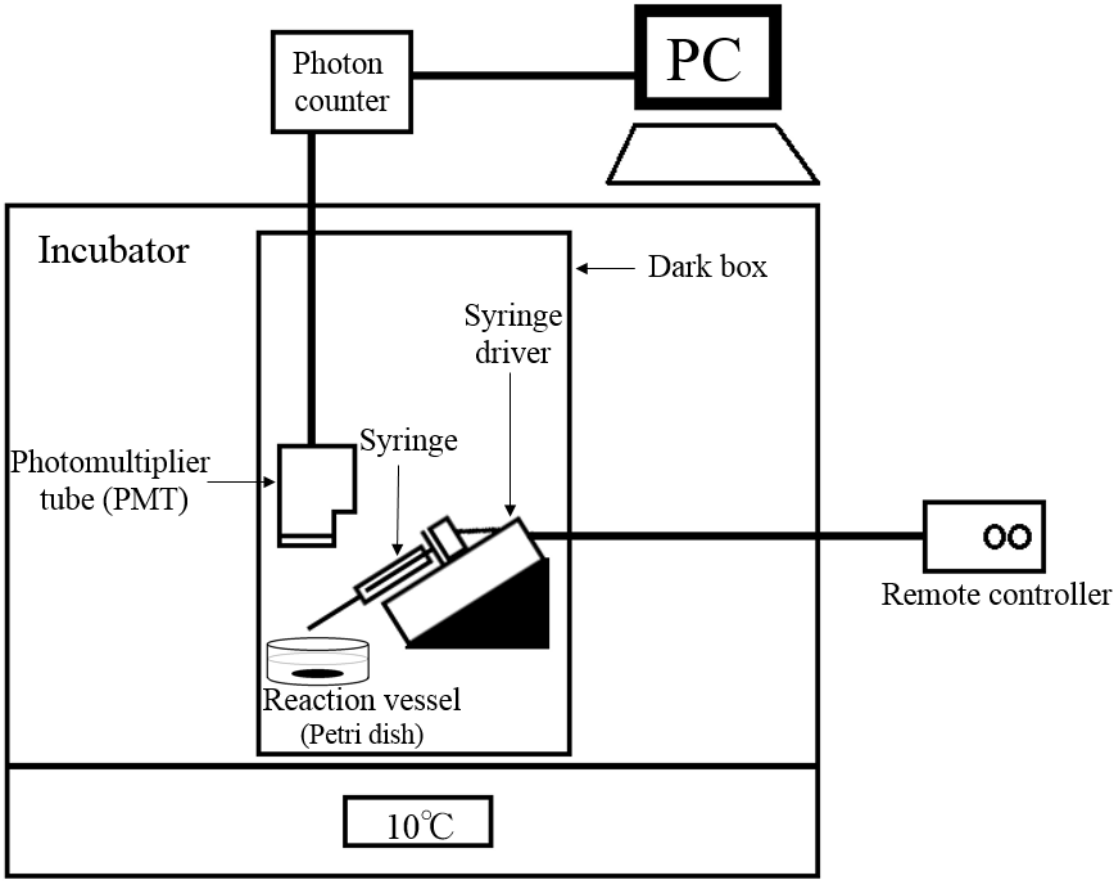
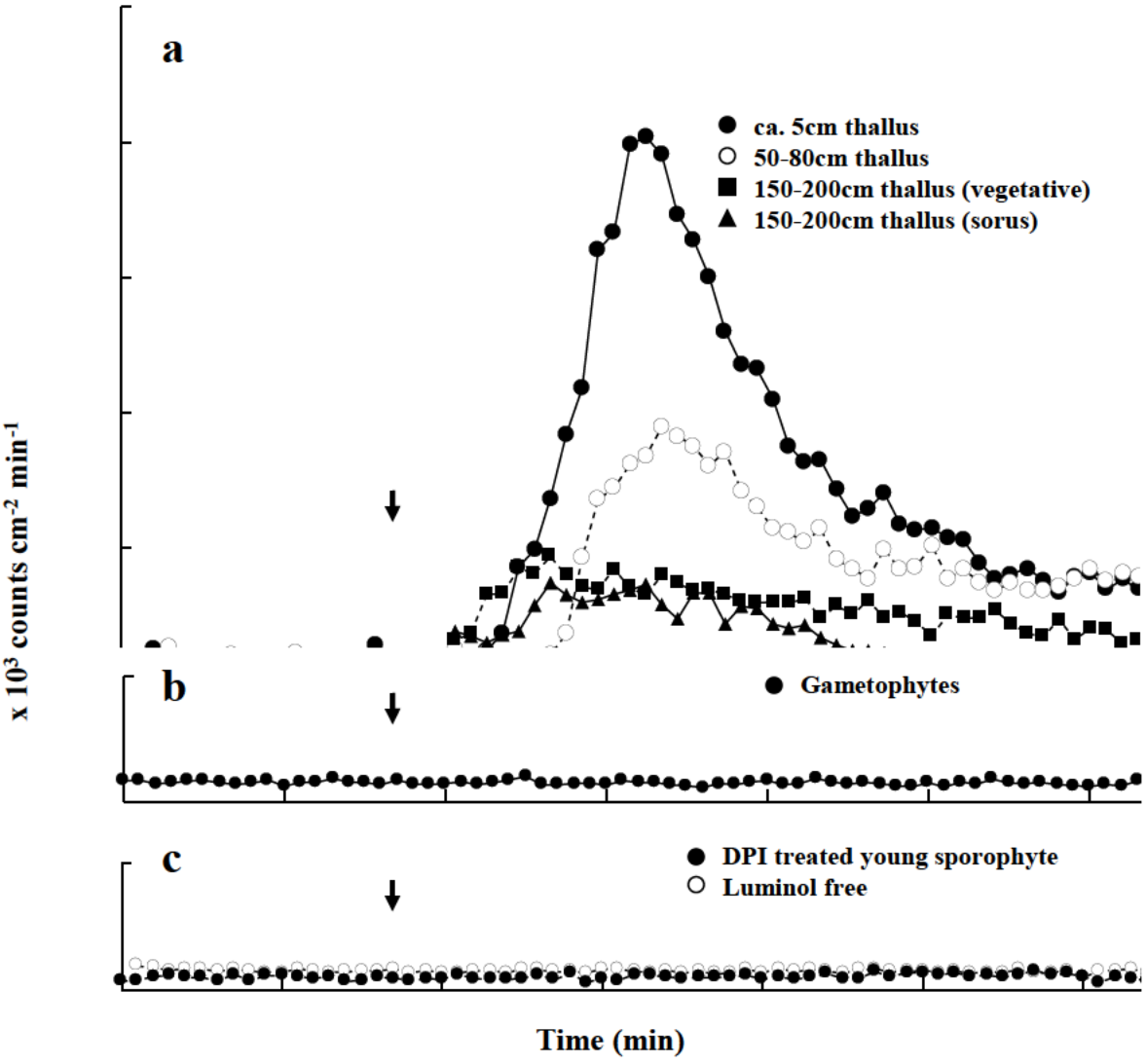


Figure 1 (Shimizu et al.)



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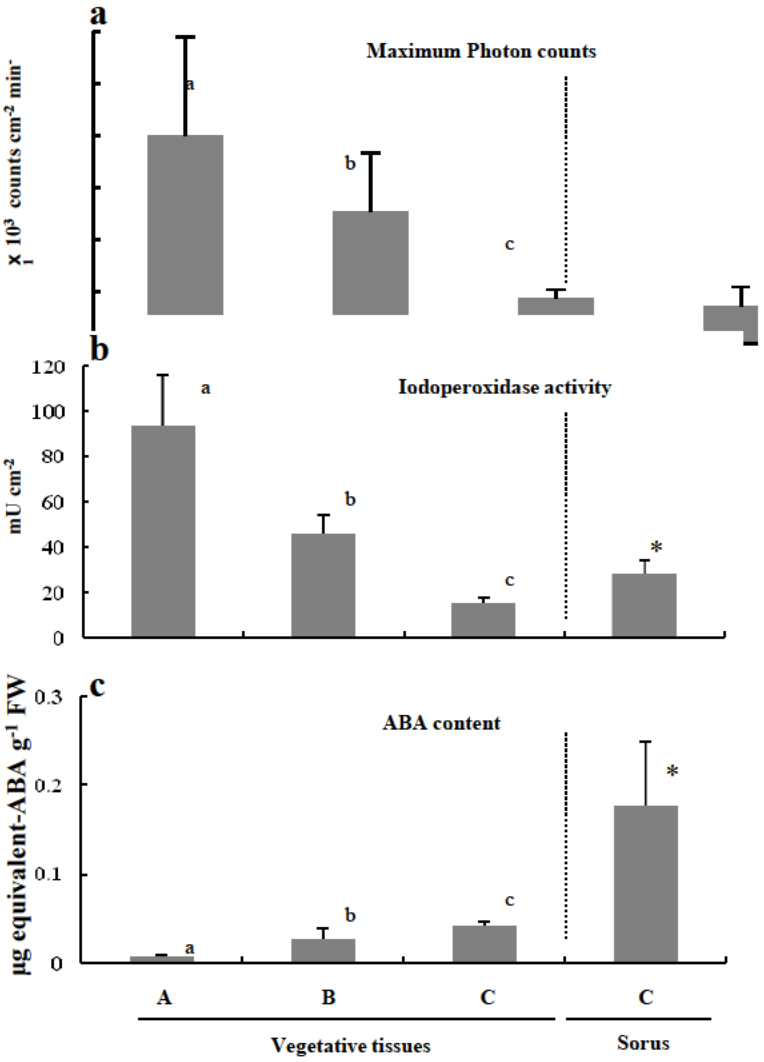

Figure 3 (Shimizu et al.)

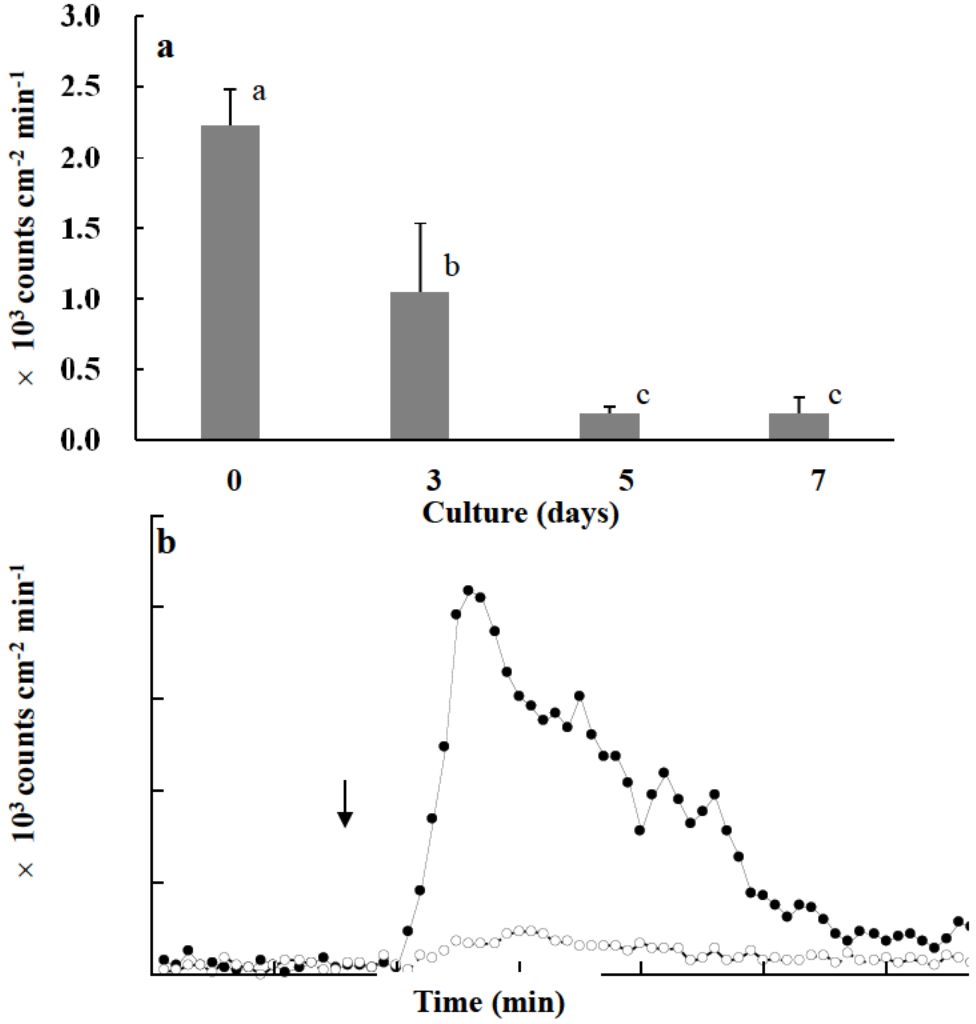

Figure 4 (Shimizu et al.)

Table 1 Effects of abscisic acid (ABA) exposure on the relative growth rate (RGR), iodoperoxidase (IPO) activity, production of reactive oxygen speceis (ROS) and the polyphenol and antioxidant contents of young *Saccharina japonica* sporophytes cultured for 10 days. Data represent the mean ± standard errors (n=4)

	RGR on an area basis ($\%^{-1}$)	IPO (mU cm^{-2})	ROS (μg DCF cm^{-2} h^{-1})	Polyphenol (μg GAE cm^{-2})	Antioxidants (μmol Trolox eq. m^{-2})
Control	13.3 ± 2.2	94.1 ± 20.5	0.095 ± 0.000	7.52 ± 0.54	0.034 ± 0.002
ABA tr.	10.7 ± 1.3*	131.9 ± 20.8*	0.048 ± 0.004**	7.78 ± 0.89	0.035 ± 0.006

Asterisks indicate significant differences in the means ($^{**}P<0.01$, $^{*}P<0.05$) compared with the control group

Table 2. Differences in production of reactive oxygen species (ROS) and the polyphenol and antioxidant contents of young and mature *Saccharina japonica* sporophytes. Data represent the mean ± standard deviation (n=4).

Growth stages	ROS production ($\mu g\ DCF\ cm^{-2}\ h^{-1}$)	Polyphenol content ($\mu mol\ Trolox\ eq.\ cm^{-2}$)	Antioxidant content ($\mu g\ GAE\ cm^{-2}$)
Young sporophytes	0.037 ± 0.005	0.030 ± 0.008	0.137 ± 0.034
Mature sporophytes			
vegitative parts	0.229 ± 0.008*	0.251 ± 0.015*	1.082 ± 0.270*
sorus parts	0.525 ± 0.131*†	0422 ± 0.106*†	1.342 ± 0.335*†

Asterisk denotes a significantly difference (*P*<0.01) from young sporophytes. Dagger denotes a significantly difference (*P*<0.01) from the vegetative parts of mature sporophytes.