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Significance of radical oxygen production in sorus development and zoospore germination in *Saccharina japonica* (Phaeophyceae)

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Abstract

We investigated production of radical oxygen species (ROS) in sorus development of sporophyte discs and during zoospore germination of *Saccharina japonica* (Phaeophyceae) using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Quantitative analysis of ROS showed high intracellular production during the stages of zoosporangium formation when paraphyses elongated. In this stage, remarkable ROS release was observed. ROS production was also observed histologically (under a fluorescence microscope) in the elongating paraphyses and the sorus zoosporangia. The sorus had significantly higher phenol content, antioxidant capacity and ROS scavenging enzyme activities (ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase) than adjacent non-sorus blade sectors. Thus, intracellular ROS scavenging mechanisms are active during ROS production in sorus formation. In contrast, iodine content was lower in the sorus than in the adjacent non-sorus tissues, suggesting the existence of an antioxidant defense mechanism based on iodine efflux. We demonstrated that ROS production is an important phenomenon in the reproduction of the *S. japonica* sporophyte. Furthermore, ROS were also observed in the zoospore germination processes. We discuss the physiological and ecological roles of ROS production in sorus formation and zoospore germination.

Keywords: antioxidant; reactive oxygen; *Saccharina*; sorus; zoospore germination.

Introduction

The life cycle of kelps involves the alternation of macroscopic sporophyte and microscopic gametophyte generations. A better understanding of the reproduction mechanism is critical for overall knowledge of kelp ecology and for the maintenance of kelp forests, which are key components of coastal ecosystems in cold and cold-temperate seas other than those off Antarctica.

Many reports on the development of sporangia in *Laminaria* spp. have examined environmental influences on sporogenesis and reproductive effort (reviewed by Bartsch et al. 2008). Several physiological changes, including a decrease in photosynthesis, an increase in respiration (Matsuyama 1983, Aruga et al. 1990, Sakanishi et al. 1991, Nimura and Mizuta 2001), phlorotannin accumulation (Van Alstyne et al. 1999) and an increase in RNA/DNA ratio (Nimura and Mizuta 2001) occur in the process of sorus development. Sporulation inhibitors are thought to be involved in the mechanism of sorus formation (Buchholz and Lüning 1999, Lüning et al. 2000). Recently, it was reported that sorus formation in *Saccharina japonica* (J.E. Areschoug) C.E. Lane, C. Mayes, Druehl *et* G.W. Saunders (*syn. Laminaria japonica* Areshoug) was regulated by internal auxin level (Kai et al. 2006). In contrast, sorus formation is promoted by abscisic acid (Nimura and Mizuta 2002).

Sorus development in *Laminaria* involves the formation of zoosporangia and paraphyses originating from the epidermal cells (Abe 1939). These structures are characterized by an elongation of epidermal cells, which is promoted by cell wall loosening. Furthermore, it has been suggested that radical oxygen species (ROS) are related to cell wall strengthening (Fry 1998), and it has been shown that they play an important role in higher plant growth and development (Foreman et al. 2003, Gapper and Dolan 2006, Carol and Dolan 2006). Among seaweeds, ROS are physiologically produced in the germination of zygotes of the brown alga *Fucus* (Coelho et al. 2002, 2008). ROS also control more specialized processes, such as plant growth, defence, hormonal signalling, and development as signalling molecules (Mittler et al. 2004). Hence ROS play roles in several growth stages of kelps.

Thus, this study was conducted to confirm the occurrence of ROS production in the reproductive stage of the sporophyte of the brown seaweed *Saccharina japonica*. In addition, we discuss the significance of ROS in sorus formation and in the germination of zoospores in this species.

Materials and methods

Preparation of materials

Mature sporophytes of *Saccharina japonica* were collected from a coastal area near Hakodate, Hokkaido, Japan, and transported to the laboratory. The sporophyte was divided into vegetative and sorus parts. From the vegetative part,

discs (2 cm in diameter) were cut and cultured in a polyethylene terephthalate bottle (1 l) with Provasoli's enriched seawater (Provasoli 1968) without vitamins under aeration for 3–5 months. The culture conditions were set at 10°C, 60 μmol photons m^{-2} s^{-1} (12-h light: 12-h dark cycle). The medium was renewed every week. The seawater used as a medium was filtered through a glass fibre filter (GA-100, Toyo Roshi Kaisha Ltd., Tokyo, Japan) and autoclaved at 121°C for 20 min (ASV-2401, Tiyoda Manufacturing Co. Ltd., Tokyo, Japan). After disc surfaces began to rise, showing that the sori had begun to develop, discs were collected as required. Based on the report of Nimura and Mizuta (2001), they were divided into four stages, Stage I (vegetative stage), Stage II (early stage of paraphysis elongation), Stage III (zoosporangium formation stage with paraphysis elongation) and Stage IV (zoospore release stage). These discs were used for the analytical and histological detection of ROS production.

Other discs (2 cm in diameter) collected from other sorus parts and adjacent non-sorus blade portions were used directly to measure the activity of ROS-scavenging enzymes, including ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD), as well as antioxidant activity, phenol content and iodine content.

A portion of the sorus was cut from the mature sporophyte with a scalpel to obtain zoospores. These portions were wiped with a paper towel, wrapped in newspaper and stored overnight at 4°C in a refrigerator. After 24 h, the sorus was placed in 200 ml of seawater to release zoospores. The seawater containing zoospores was poured into a polystyrene vessel (square shape, 8.5 cm×17.5 cm×3.5 cm) containing a glass slide fragment (ca. 2×2 cm) on the bottom. To allow zoospores to settle onto the glass slide fragment, the vessel was placed in an incubator set at 5°C, under 60 μmol photons m^{-2} s^{-1} on a 12-h light:12-h dark cycle. After the zoospores had settled, the seawater was exchanged with Provasoli's enriched seawater without vitamins. Some of the glass slides were used for the histological detection of ROS in the germination process.

Analytical and histological detection of ROS production

Sporophyte discs (2 cm in diameter) at different stages of sorus formation were incubated in seawater (2 ml) containing 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Wako Chemicals, Osaka, Japan, final concentration 50 μM) for 1 h. The incubated discs were ground in a mill with liquid N_2 and extracted in 1 ml of Tris-HCl buffer (40 mM, pH 7.0). After agitation for 5 min and centrifugation at 10,000×*g* for 5 min, 500 μl of the supernatant were diluted to 2.5 ml with Tris-HCl buffer and used to measure fluorescence at 488 nm (excitation wavelength) and at 525 nm (emission wavelength) with a spectrofluorometer (FB-750, Jasco, Tokyo, Japan). The production of ROS was calculated with a standard curve for dichlorofluorescein (DCF, Wako Chemicals) and expressed in units of μg DCF per single-side area of disc. Other discs were also cultured in 2 ml of medium with 10 μM DCFH-DA for 1 h. The media at the start and the end of culturing were subjected to ROS analysis using a similar spectrofluorometrical procedure. ROS release was calculated as the amount of DCF produced during the culture period.

The discs at different stages of sorus formation were also cultured in seawater with 50 μM DCFH-DA for 15 min. After culturing, the discs were taken out and rinsed with sterilized seawater to remove DCFH-DA from the surfaces. The discs and their hand-cut sections were observed using a fluorescence microscope (AH2, Olympus, Tokyo, Japan) at an excitation wavelength of 380–490 nm.

Zoospores, which were attached to glass slide segments, were used for ROS histological detection. The culture for germination of zoospores was maintained at 10°C, 22.5 μmol photons m^{-2} s^{-1} (12-h light:12-h dark cycle) for 1–2 days after release. Embryospores (germinating zoospores) were placed into 1 ml of seawater containing 50 μM DCFH-DA solution and incubated in the dark for 15 min. Glass slide segments were washed with seawater lacking DCFH-DA to remove the incubation medium. The glass slide segments with embryospores attached were placed on another slide glass for observation using a fluorescence microscope, as described above.

Measurement of antioxidant activity and ROS scavenging enzymes

Discs were powdered in liquid N_2, and the powdered samples were extracted with 50 mM phosphate buffer (pH 7.0) containing 5% (w/v) polyclar AT for CAT, GR and SOD. For APX, the extraction was carried out in 50 mM phosphate buffer (pH 7.0) containing 0.5 mM ascorbate, and 5% (w/v) Polyclar AT.

Antioxidant capacity was assayed with 1,1-diphenyl-2-picrylhydrazyl (DPPH, Wako Chemicals) according to the method of Heo et al. (2005). Powdered samples were prepared in the manner described above and placed in 2 ml of ethanol. The supernatants of the ethanol extracts (0.1 ml) were mixed with 2.9 ml of DPPH solution (400 μM) and incubated at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 516 nm with a UV-VIS spectrometer (V-530, JASCO, Tokyo, Japan). ROS-scavenging activity was expressed in units of mM Trolox equivalent (TE) per gram fresh weight.

The activities of CAT, GR and SOD were assayed following Aguilera et al. (2002). Decreases of 1 μmol H_2O_2 and NADPH for 1 min were used as units of CAT and GR activities, respectively. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%.

APX activity was assayed following Chen and Asada (1989). Decrease in absorbance at 290 nm was followed for 1 min after the addition of 50 μl of extract to 950 μl of 50 mM phosphate buffer (pH 7.0) containing 0.1 mM H_2O_2 and 0.5 mM ascorbate. A decrease of 1 μmol ascorbate in 1 min was used as the unit of APX activity.

Total soluble protein (TSP) content was assayed by the Coomassie Blue G method. Coomassie Blue G staining solu-

tion (0.01%, 950 μl) was added to 50 μl of enzyme extract. The absorbance was recorded at 595 nm. The total soluble content was corrected using serum albumin solution.

Measurement of phenolic and iodine contents

The content of phenolics was determined following the method of Senevirathne et al. (2006). Discs were powdered in liquid N_2, and the fine powder was placed in vials (10 ml) with 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min, after which 1 ml of 5% Na_2CO_3 was added, followed by thorough mixing and storage in darkness for 1 h. Subsequently, the mixture was centrifuged at ca. 7000×*g* and the absorbance of the supernatant was recorded at 725 nm by UV-VIS spectrometry. To calculate phenolic contents, a gallic acid standard curve was used as a standard, and the content was expressed as gallic acid equivalents (GAE) per gram fresh weight.

Iodine content was determined following the method of Yasui et al. (1980). The disc was dried in a nickel crucible for 2 h at 110°C. Dry matter was cooled at room temperature. After the dry weight had been determined, the dry matter was fused completely for 5 h at 450°C in a muffle furnace. The ash sample was extracted in boiling water and the extract was collected through a glass fibre filter (Whatman GF/C). The extract was agitated with 0.5 ml of 36 N sulphate and 5 ml of 3% hydrogen oxide for 10 min in a separating funnel. After 10 ml of toluene had been added, the separating funnel was agitated again for 2 min. The lower layer was discarded and the upper toluene layer was added to a test tube with 1 g of anhydrous potassium sulphate. After dehydration, the absorbance of the toluene layer was measured at 535 nm. The iodine concentration was measured using a standard curve of potassium iodide.

Statistical analysis

Data were expressed as mean±standard deviation. Bartlett's test was used to test the homogeneity of variance. If homogeneous, the data were analysed by one-way analysis of variance (ANOVA) with Dunnett' multiple comparison test. If the variances were heterogeneous, data were analysed by the Kruskal-Wallis test followed by Steel's multiple comparison test. Significant differences in ROS scavenging activities and antioxidant contents between sorus and adjacent non-sorus sectors were analysed by one-tailed paired Student's t-tests. All calculations were performed using Exel Tokei 2006 statistical software (SSRI, Tokyo, Japan). The levels of significance were set at $p<0.05$.

Results

Analytical detection of ROS production

ROS production and release at different stages of sorus formation in *Saccharina japonica* are shown in Figure 1A. Discs without sori (stage I) had a low ROS production of

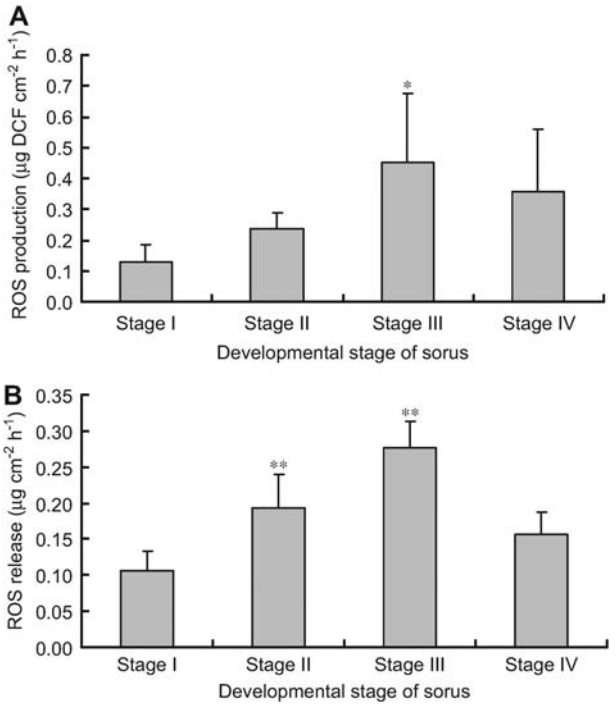

Figure 1 *Saccharina japonica*: ROS production (A) and release (B) at different developmental stages of the sorus.
Stage I: vegetative stage; Stage II: early elongation stage of paraphysis; Stage III: zoosporangium developmental stage with paraphysis elongation; Stage IV: zoospore release stage. Values are means+SD (for A: n=5; for B: n=6–8). (A) Kruskal-Wallis ANOVA: H=13.6, $p<0.01$; *$p<0.05$, compared with Stage I (Steel's test). (B) ANOVA: $F_{3,\ 22}=20.508$, $p<0.01$; **$p<0.01$, compared with Stage I (Dunnett's test).

0.13±0.06 (mean±SD) μg cm^{-2} h^{-1}. When the paraphyses started to elongate (Stage II), mean ROS production increased, but production was not significantly different from that in Stage I. During zoosporangium formation with paraphysis elongation (Stage III), highest ROS production (0.45±0.23 μg cm^{-2} h^{-1}) was observed; values were five times higher than in Stage I. Production of ROS in Stage IV was not significantly different from the level in Stage I.

Releases of ROS by discs at different stages of sorus formation are shown in Figure 1B. ROS release was significantly higher in Stage II than in Stage I. Release was the highest (0.28±0.4 μg cm^{-2} h^{-1}) in Stage III (2.6 times higher than in Stage I). In Stage IV, ROS release decreased to the non-significant level in the undeveloped sorus stage.

Histological detection of ROS production

The sorus part of the sporophyte disc had a strong green DCF fluorescence (Figure 2A,B). The non-sorus part distant from the sorus had only a red chlorophyll fluorescence; the transitional area between sorus and non-sorus sectors was scattered with weak fluorescence. Strong green DCF fluorescence was observed in the elongating paraphyses (Figure 2C); the fluorescence was located in the peripheral regions of the cytoplasm of paraphyses around the cell walls, partic-

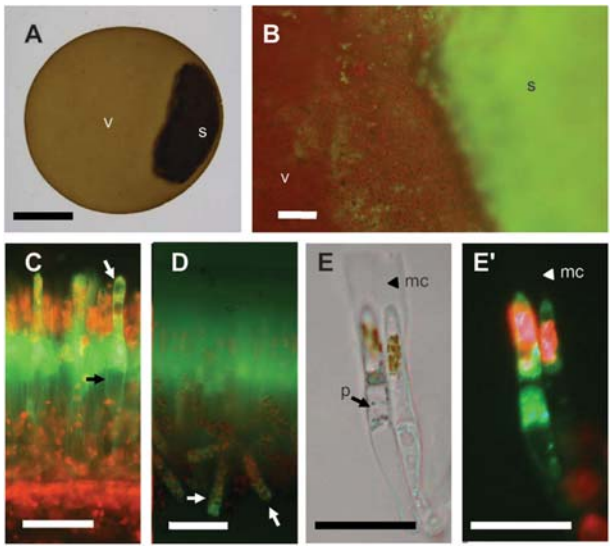

Figure 2 *Saccharina japonica*: ROS production in the sorus of a sporophyte disc loaded with DCFH-DA.
(A) Bright field visual image of a sporophyte disc with vegetative (v) and sorus (s) sectors. (B) Microscopic fluorescence image of the surface view of a transitional area between non-sorus (v) and sorus (s) sectors, showing green and red fluorescence due to DCF and chlorophyll, respectively. (C) Fluorescence image of a section of sorus, showing strong green DCF fluorescence located in the paraphyses (arrows). (D) Fluorescence image of a section of the sorus showing DCF fluorescence in zoosporangia (arrows). (E and E′) Bright field (E) and fluorescence (E′) images of separated paraphyses (p) with mucilage cup (mc). Scale bars: 1 cm in (A), 100 μm in (B), and 50 μm in (C)–(E).

ularly in the apical and central parts of the paraphyses. DCF fluorescence also occurred in zoosporangia (Figure 2D). However, the mucilage cap, which is located on the tip of the paraphyses, did not have green fluorescence (Figure 2E and 2E′).

During germination of attached zoospores, DCF fluorescence was localized in the germ tube in the early stage of its elongation (Figure 3A,B). ROS were also located at the opposite end of the germ tube in the cytoplasm (Figure 3B,C). Production of ROS was maintained in the dumbbell-shaped germlings with movement of cell contents, including chloroplasts that emitted reddish fluorescence (Figure 3C). After the distal extremity of the germ tube had become dilated by the movement of embryospore contents into it, the empty spore had weak DCF fluorescence (Figure 3D).

Antioxidant activity, ROS-scavenging enzymes, phenol content and iodine content

Comparisons of antioxidant capacity and several ROS-scavenging activities between sorus and adjacent non-sorus sectors are shown in Figure 4. The antioxidant capacity in the sorus parts was 1.5 times higher than that in the non-sorus parts (t-test; $p<0.01$) (Figure 4A). The activities of ROS-scavenging enzymes, including APX, GR, CAT and SOD, were 2.1–2.5 times higher in the sorus part than in the non-sorus part ($p<0.05$) (Figure 4B–E). The phenol content in the sorus part was 3.7 times higher than that in the non-sorus part ($p<0.01$) (Figure 4F). However, the iodine content in the sorus was 63% of the level in the non-sorus part ($p<0.05$) (Figure 4G).

Discussion

Origin of ROS

ROS production is induced by environmental stress, infection, wounding, drying, freezing and ultraviolet radiation in

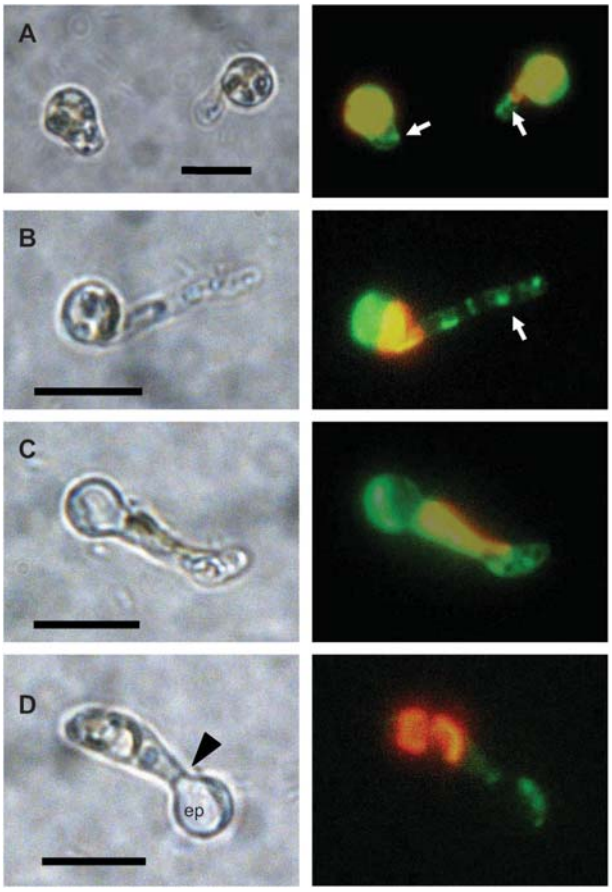

Figure 3 *Saccharina japonica*: bright field (left) and fluorescence (right) images during ROS production in different germination stages loaded with DCFH-DA.
(A) First stage in germination of zoospores with germ tubes (arrows), showing green and red fluorescence due to DCF and chlorophyll, respectively. (B) Germ tube elongation stage in the germination of zoospore showing DCF fluorescence distributed in the germ tube and in the cytoplasm at the opposite end of the germ tube. (C) Dumbbell-shaped germlings, showing movement of cell contents into the apical part and red fluorescence located in germ tube cytoplasm. (D) Later stage in germination, showing that cell contents have become separated by a septum (arrowhead), with weak DCF fluorescence in the empty spore (ep). Scale bars: 50 μm.

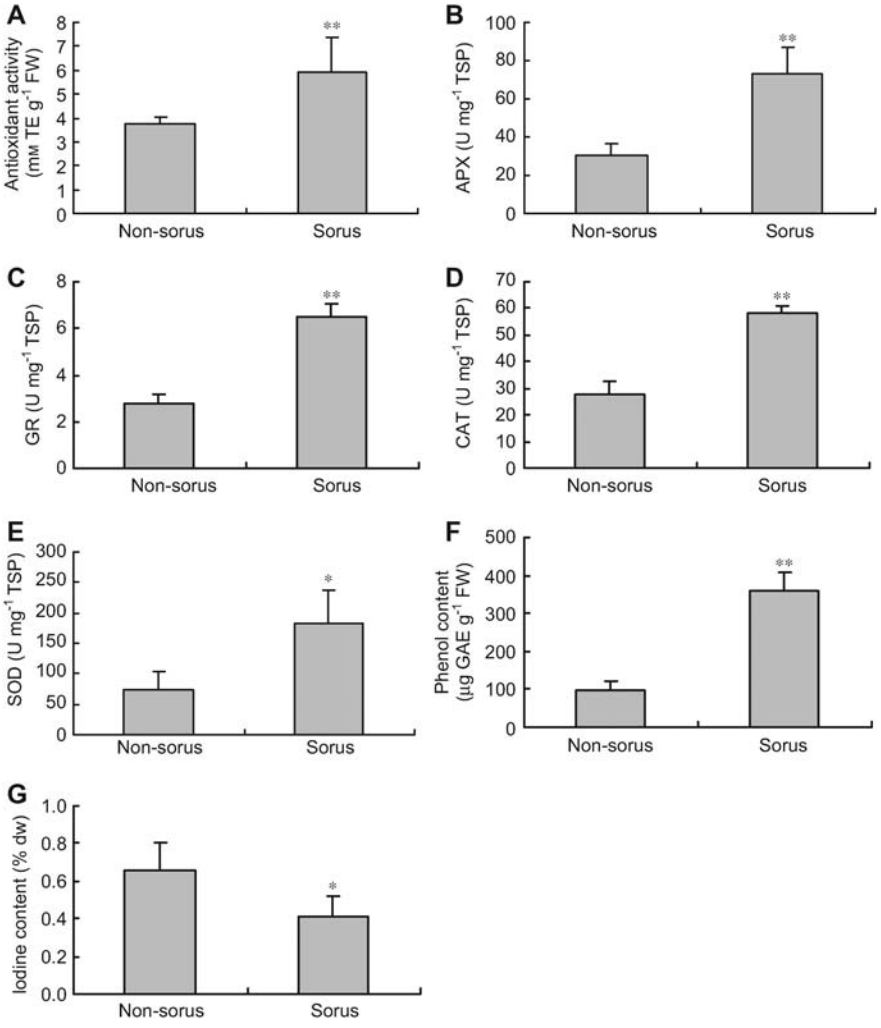

Figure 4 *Saccharina japonica*: comparison between sorus and adjacent non-sorus sectors for antioxidant activity (A), four radical oxygen scavenging enzyme activities (B–E), phenol content (F) and iodine content (G).
(B) Ascorbate peroxidase activity (APX). (C) Glutathione reductase activity (GR). (D) Catalase activity (CAT). (E) Superoxide dismutase activity (SOD). Values are means+SD (n=4). Asterisks indicate significantly different means (**$p<0.01$; *$p<0.05$, compared to non-sorus sector). TE, trolox equivalent; TSP, total soluble protein.

higher plants. Such responsiveness to stress has also been found in seaweeds (reviewed by Dring 2006). In *Saccharina japonica*, ROS production increased in parallel with sorus development, suggesting that ROS production is closely linked to sorus formation. ROS is also produced in metabolic processes of plant cells, and the major sources of ROS are organelles, such as chloroplasts and mitochondria. In the process of sorus development in *S. japonica*, ROS appeared to be distributed mostly around the plasma membrane on the insides of the cell walls of the paraphyses. In addition, we observed an extracellular release of ROS through sorus formation. In higher plants, plasma membrane NADPH oxidase plays an important role in ROS release by germinating radish seeds (Schopfer et al. 2001) and in ROS signalling for cell growth (Foreman et al. 2003, Liszkay et al. 2004). These results suggest that plasma membrane NADPH oxidase may be a major source of ROS in sorus formation of *S. japonica*.

Regulation of internal ROS level

Generally, ROS cause oxidation of lipids, denaturation of proteins and the decomposition of nucleic acids; these phenomena are life-threatening for plants. Therefore, ROS are generally maintained at a low-level in plant cells by ROS-scavenging enzymes and antioxidants. During sorus formation, ROS-scavenging enzymes were more active. High antioxidant capacity and high phenolic content was also observed. Plant phenolics are known to be important groups of natural antioxidants. Electron microscopic observations have revealed that many Golgi vesicles and physodes appear in paraphyses and zoosporangia of *Saccharina angustata* (Kjellman) C.E. Lane, C. Mayes, Druehl *et* G.W. Saunders (Motomura 1993). Physodes accumulated large amounts of phenolic substances, suggesting that the synthesis and accumulation of phenolics occur actively in the cytoplasm of

paraphyses and zoosporangia. Hence, phenolic antioxidants likely play an important role in the regulation of intracellular ROS levels.

Extracellular release of ROS was also observed in the sorus developmental process in *Saccharina japonica*. This release probably plays an important role in regulating intracellular ROS level (in conjunction with antioxidant substances and ROS-scavenging enzymes). In addition, iodine, which is stored largely as iodide ion in the apoplast, functions as an inorganic antioxidant (Küpper et al. 2008). Iodine release by macroalgae is thought to occur in response to oxidative stress (Küpper et al. 2001, 2008). Therefore, iodine probably contributes to controlling intracellular ROS levels when it is released. In *S. japonica*, lower iodine content in sorus than adjacent non-sorus sectors suggests that low levels of iodine are present because it is released in the ROS-producing process of sorus formation. However, non-sorus sectors released 80% of the ROS produced; 44–61% of these were released in Stages III and IV. The low ratio of ROS released to ROS produced in these stages suggests that a substantial part of the ROS produced is scavenged by iodine. Therefore, the contribution of iodine to scavenging ROS is likely to be greater during the sorus-formation process than in the vegetative stage.

Physiological functions of ROS

The functions of ROS as important regulators of cell development have been elucidated in higher plants. For example, ROS play an important role in the loosening of the cell wall in growing tissues (Fry 1998, Potikha et al. 1999, Liszkay et al. 2004) and in the lignification of xylem cell walls (Ros Barceló 1998). The loosening of the cell wall by ROS induces cell growth. In the sporophyte of *Saccharina japonica*, ROS are also normally produced in the development of the reproductive organs. In particular, ROS production seems to be active in the elongation of the paraphyses and zoosporangia. Therefore, we suggest that ROS contribute to loosening of cell walls and to elongation of epidermal cells in sorus formation.

ROS have also been observed in zoospore and zygote germination processes. Coelho et al. (2008) reported the existence of a high ROS gradient in rhizoid tips of germinating *Fucus serratus* L. zygotes. They also observed that suppression of the ROS gradient inhibits polarized zygotic growth. Their observation indicates that embryonic polarization in this species occurs through a ROS signalling pathway. In *Saccharina japonica*, ROS were locally produced in embryospores before germination, and ROS production was remarkable in the germ tubes. Therefore, the ROS are considered to play an important role in polarization and cell wall loosening in the germination process of zoospores of *S. japonica*. In addition, excess ROS production leads to necrotic cell death and programmed cell death in plant cells (Van Breusegem and Dat 2006). The accumulation of ROS in empty spores, the contents of which had already moved into the germ tubes, therefore seemed to be closely linked to the necrotic process of spores.

Ecological functions of ROS

ROS production in sorus formation is considered to have ecological functions in addition to the physiological functions described above. For example, correlations between the efficiency of ROS metabolism and stress tolerance have been reported in some brown and red algal species (Collén and Davison 1999a,b,c, Choo et al. 2004). The correlations are due to ROS functioning as a signalling substance for biotic and abiotic stresses (Mittler et al. 2004). The function of ROS in plants is also often discussed in terms of the resistance that occurs in plant-pathogen interactions. ROS exhibit anti-bacterial and sterilizing activities because of their strong oxidizing power. This suggests that the release of ROS plays an ecological role by inhibiting the growth of pathogenic bacteria on the surface of the sorus. Similarly, Küpper et al. (2001, 2002) showed that in *Laminaria digitata* (Hudson) J.V. Lamour., the oxidative burst controls growth of epiphytic and pathogenic bacteria.

Production of ROS in kelp results in the accumulation of chemical components that play a role in defence responses. Phenolic substances have several functions, such as cellular support materials and the protection of plants against predators (Strack 1997). In *Saccharina japonica*, high production of phenolics occurred during sorus formation. Van Alstyne et al. (1999) also reported that phlorotannin levels in the reproductive tissues of kelp were higher than in the vegetative tissues. In addition to the role of phenolics in scavenging ROS, as discussed above, the accumulation of phenolics can be considered as an additional function related to the ecological success of reproduction of this seaweed. Ar Gall et al. (2004) proposed that iodine levels in kelps may reflect the antecedents of biotic and abiotic stress responses. It has also been proposed that both the oxidative burst and the concomitant iodine release participate in the early defence response to pathogenic micro-organisms (Leblanc et al. 2006). Iodine release, which is determined by lower content in the sorus than in the adjacent vegetative parts in *S. japonica,* is likely to contribute to chemical defence against microbial attack.

Conclusion

ROS were produced in the process of sorus formation in *Saccharina japonica*. ROS production is hypothesized to play a significant role in cell elongation in the paraphyses and zoosporangia. Additionally, several metabolic processes, including ROS release, antioxidant production and active ROS-scavenging activities, are activated to control the internal ROS level. These phenomena are additionally considered to function as defences against herbivores and pathogens in the alternation from the sporophyte to the gametophyte generation. In contrast, the functional role of ROS in cell elongation is considered to contribute to the formation of the germ tube in the zoospore. During germination, ROS may be related to the necrosis of the empty spore after its contents have moved to the germ tube.

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