



Reproductive Responses to Wounding and Heat Stress in Gametophytic Thalli of the Red Alga *Pyropia yezoensis*

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The timing of the transition from growth to reproduction is essential for the regulation of the seaweed life cycle. Variable environmental conditions can stress seaweeds and promote trade-offs between their growth and reproduction. Here, we demonstrate that reproductive responses can be induced by environmental stresses in the gametophytic thalli of the marine red alga *Pyropia yezoensis*. Excision of explants accelerated release of asexual monospores and sexual carospores. The algal sensitivity to wounding was enhanced by a 3-day dark treatment prior to the excision of the explants. By contrast, heat stress at 25°C stimulated the production of a callus, a three-dimensional aggregation of randomly divided cells with multiple cell layers. This callus produced new gametophytic thalli with a normal shape; therefore, callus formation is thought to be one of the asexual reproductive strategies used by the alga to increase the number of thalli under heat stress conditions. Our results demonstrate that wounding and heat stress can reset the timing of reproduction and that gametophytic thalli therefore use a variety of distinct reproductive strategies under different stress conditions. These findings provide insights into the induction of reproduction by environmental stresses as a life cycle trade-off in seaweeds.

Keywords: callus, heat stress, life cycle, *Pyropia yezoensis*, reproduction, wounding

INTRODUCTION

Plants and seaweeds control the timing of their growth and reproduction in response to environmental stresses, trading off these phases to optimize their life cycle progression and best adapt to the variable natural conditions they experience (Liu et al., 2017; Mohring et al., 2013; Karasov et al., 2017; Shaar-Moshe et al., 2019). The elucidation of the mechanisms regulating the trade-off between growth and reproduction in response to changes in the environmental conditions is fundamental for understanding how seaweeds integrate environmental signals and adjust their life cycle accordingly.

The Bangiales, including the marine red algal genera *Pyropia* and *Porphyra*, undergo a haploid-diploid heteromorphic life cycle, in which the haploid leafy gametophyte (thallus) and the diploid filamentous sporophyte (conchocelis) occur in a mutually exclusive manner (Blouin et al., 2011; Mikami et al., 2012; Takahashi and Mikami, 2017; Adams et al., 2018). The life cycle of *Pyropia* is in fact triphasic consisting of gametophyte, sporophyte and diploid conchospore parasitically generated on sporophyte to produce gametophytes by apospory (Mikami et al., 2019). It is well

known that the fertilization of male and female gametes, both of which develop on gametophytic thalli, results in the establishment of the sporophyte generation through the production and germination of diploid carpospores (Blouin et al., 2011; Takahashi and Mikami, 2017; Mikami et al., 2019). Gametogenesis, performed by the gametophytes, is reportedly stimulated by a temperature upshift and changes in the daylight length in the marine red alga *Pyropia yezoensis* and other Bangiophyceae species (Avila et al., 1986; Brawley and Johnson, 1992; Sidirelli-Wolf, 1992; Notoya and Nagaura, 1998; Notoya and Miyashita, 1999; Monotilla and Notoya, 2004, 2010; Kakinuma et al., 2006; Liu et al., 2017). Since we have observed that the stimulation of gametogenesis by environmental cues lowers the growth rate of the thalli by reducing the number of vegetative cells, sexual reproduction is thought to represent a life cycle trade-off in gametophytic thalli.

In addition to sexual reproduction, *P. yezoensis* also undergoes asexual reproduction, producing gametophytic clones via the differentiation of vegetative cells to asexual spores called monospores or archesporia (Li et al., 2008, 2009; Takahashi et al., 2010; Mikami et al., 2012; Takahashi and Mikami, 2017). This process also reduces the number of vegetative cells available for the growth of the existing thalli; therefore, asexual propagation also involves trade-offs between growth and reproduction. The production of monospores in *P. yezoensis* is strongly stimulated by an increase in light intensity (Ying, 1984), wounding (Hafting, 1999), hypotonic stress (Li et al., 2008, 2009), reduction in extracellular calcium ion concentrations (Takahashi et al., 2010), and oxidative stress (Takahashi and Mikami, 2017). The asexual life cycle of *P. yezoensis* is therefore activated by environmental stresses and is dependent on trade-offs between growth and reproduction.

Despite the accumulation of evidence suggesting the existence of life cycle trade-offs during reproduction, little is known about the promotion of sexual and asexual reproduction by environmental stress in the Bangiales. In this study, we examined the effects of wounding and heat stress on the life cycle trade-offs in *P. yezoensis* gametophytes. Our results demonstrate the presence of asexual and sexual life cycle trade-off strategies, which were accelerated by the wounding of the gametophytes and further enhanced by a dark treatment prior to wounding. In addition, a wounding-independent but heat-dependent activation of the asexual strategy was also observed. These findings indicate an intrinsic ability for the flexible selection of different reproductive strategies involving life cycle trade-offs in *P. yezoensis*, which maximize survival under various stress conditions.

MATERIALS AND METHODS

Algal Strain and Culture Conditions

Gametophytic blades and spores of the *P. yezoensis* strain U-51 were cultured in sterilized artificial seawater (SEALIFE; Marinetech, Tokyo, Japan) containing the nutritional mixture ESS₂ with NaNO₃ as a nitrogen source, vitamins, and trace metal elements (Takahashi et al., 2010; Li et al., 2019). The samples

were cultured in 200 ml of medium under 60–70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in a short-day photoperiod (10 h light/14 h dark) at various temperatures, aerated with air filtered through a 0.22 μm filter (Whatman; GE Healthcare, Chicago, IL, United States). The culture medium was changed weekly.

Stress Treatments

Thalli were cultured in 9 cm dishes [Asnol petri dishes: 90 mm (diameter) \times 20 mm (height); As One Corporation, Osaka, Japan] containing 30 ml seawater for a week at 15°C or 4°C in darkness or in light. A razor blade was used to excise 1 mm² gametophytic portions from the tip, middle (0.8 cm from the tip), and bottom (1.6 cm from the tip) of thalli 2.0–2.4 cm in length, inducing a wounding response. The number of spores released and adhered to the bottom of culture dishes was counted by observation using microscope. Explants were also excised from the tips of thalli 0.7, 1.7, 2.4, and 3.2 cm in length. The gametogenesis and callus formation processes were observed in all samples.

To further explore the callus formation process, nonwounded thalli were also incubated at 15°C with or without a 3-day pretreatment in the dark and at 25°C without any pretreatment. The samples were observed and imaged using an Olympus IX73 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP22 camera.

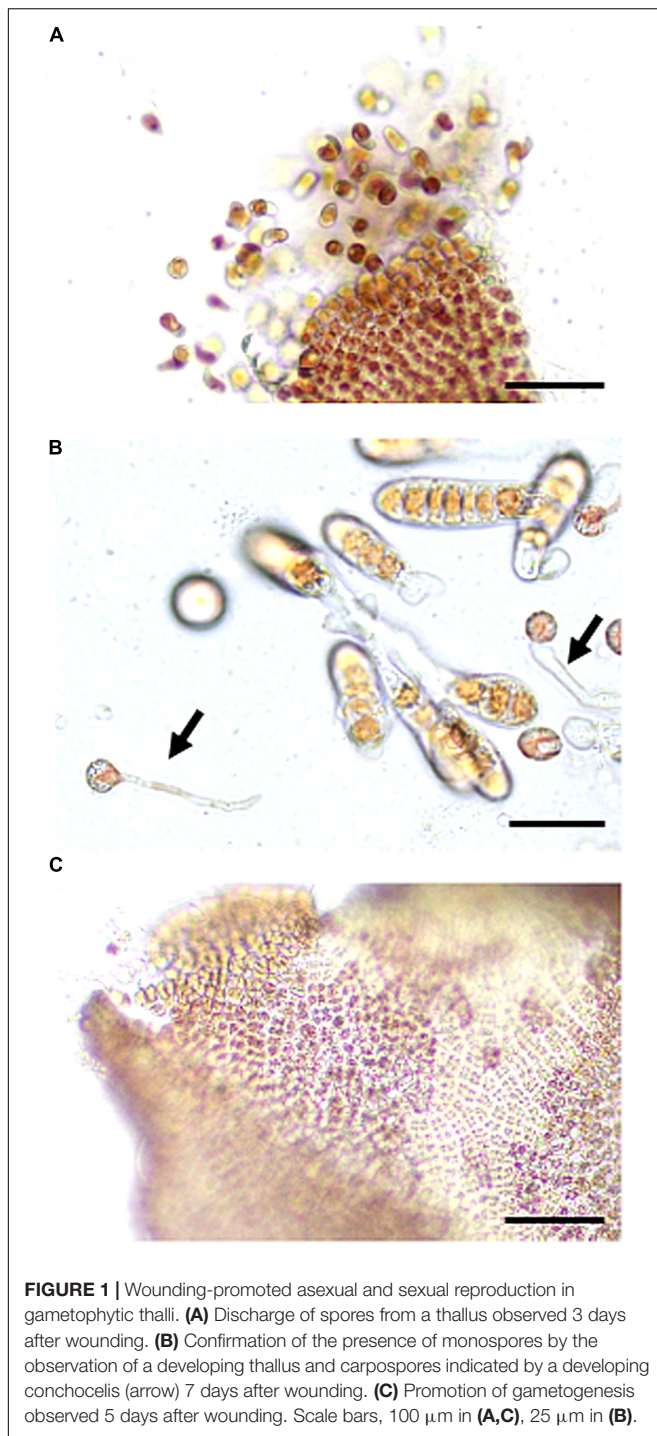
Statistics Analysis

Values are indicated with \pm SD from triplicated experiments for counting the number of released monospores. Since we proposed that effects of experimental conditions on reproductive responses is able to be evaluated by comparison of the number of released monospores among treatments every day (1–7 days) using excised portions from the same thallus as performed in our experiments, a one-way ANOVA followed by a Tukey–Kramer test was used for multiple comparisons, and significant differences were determined using a cutoff value of $p < 0.05$.

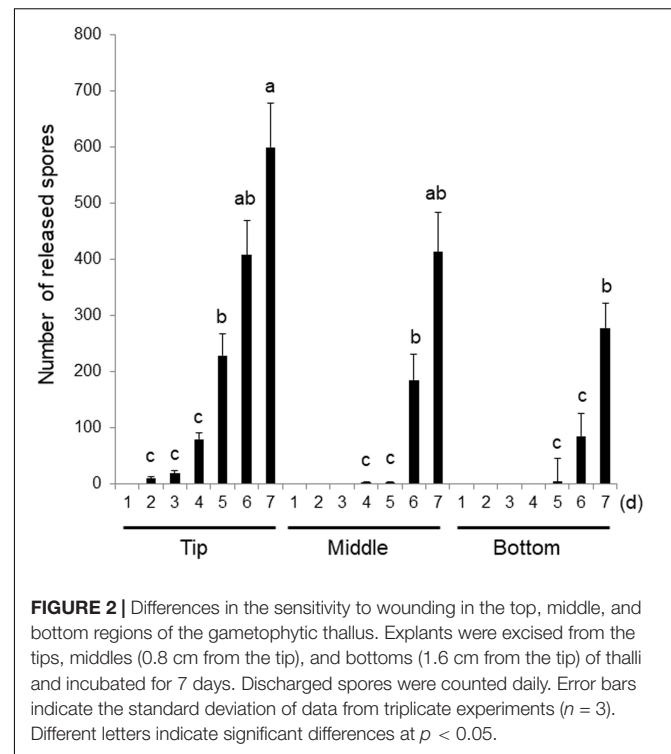
RESULTS

Wounding-Dependent Promotion of Asexual and Sexual Reproduction

First, we reexamined the wounding-dependent acceleration of asexual spore release reported by Hafting (1999). We excised 1 mm² explants from the tips of thalli grown at 15°C and incubated them at 15°C for a further seven days under short-day photoperiod conditions, resulting in the release of a large number of spores (Figures 1, 2), indicating the promotion of spore release by wounding. In addition, these spores developed into gametophytic thalli or sporophytic conchocelis (Figure 1B), the latter of which were rare and dependent on the gametogenesis and fertilization of male and female gametes in the wounded thalli (Figure 1C). Wounding therefore stimulated both asexual and sexual reproduction, resulting in the production of monospores and carpospores developing into leafy thalli and conchocelis filaments, respectively.



When explants from three different areas of the thallus were cultured for 7 days, the total number of spores released was highest from the samples excised from the tip, with the fewest spores released from the tissues taken from the bottom of the thallus (**Figure 2**). Monospores comprised the majority of the spores released by the samples extracted from all three regions (**Supplementary Figure S1**). These findings indicate that all regions of the thallus can respond to wounding through the

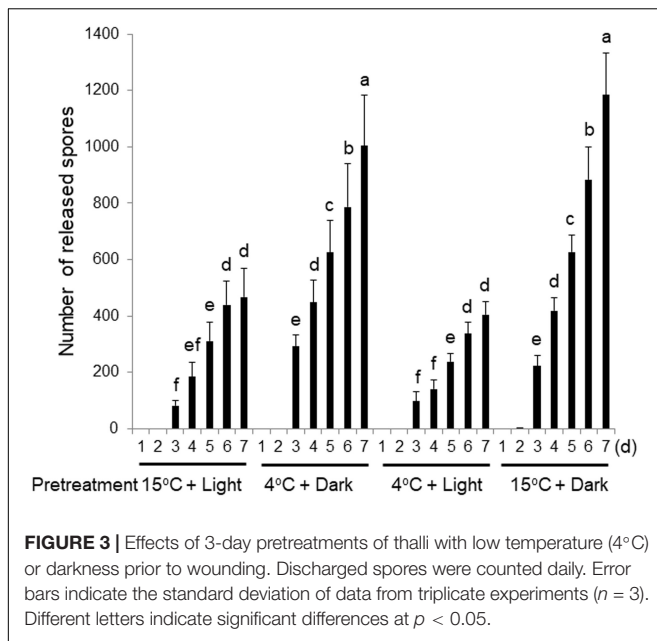


acceleration of the asexual life cycle, however, the number of reproductive spores produced in response to wounding stress varied between the portions. Moreover, when the wounding response in thalli at different growth stages was examined using the tips of 0.7, 1.7, 2.4, and 3.2 cm thalli, more spores were found to be released from the thalli 2.4 and 3.2 cm in length than by the shorter and potentially less well-developed thalli (**Supplementary Figure S2**). The sensitivity to wounding therefore increases during thallus growth development. Based on the above findings, we used explants from the tips of 2–3 cm thalli in the following experiments.

Enhancement of the Wound-Stress Response by Pretreatment in Darkness

We noticed that when we used thalli sent via a refrigerated delivery service for experiments immediately after their arrival, they released a markedly increased number of spores. Since these thalli were exposed to both cold and dark conditions for 3 days during this transportation, we hypothesized that low-temperature stress and/or dark treatment could increase the sensitivity of the thalli to the wound stress.

To address this possibility, the wounding-dependent production and release of spores was examined using thalli pretreated for 3 days with darkness or light at 4 or 15°C prior to wounding. The thalli pretreated with darkness at 4°C released more spores than those cultured under the normal conditions of 15°C and light (**Figure 3**). To distinguish whether this increased spore release was accelerated by the cold or the dark treatment, we compared the number of spores released by thalli pretreated in the light at 4°C or in darkness at 15°C. The thalli pretreated



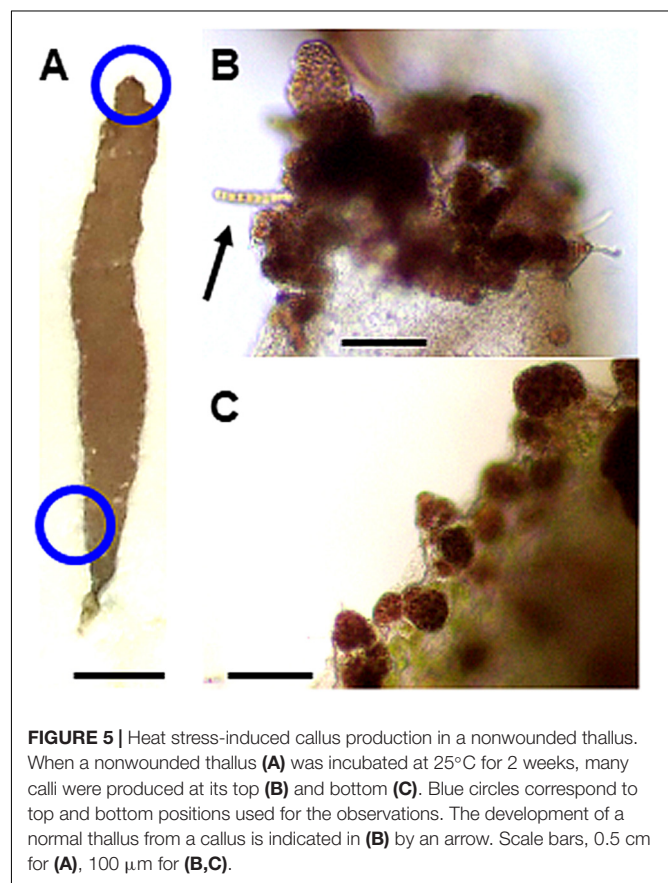
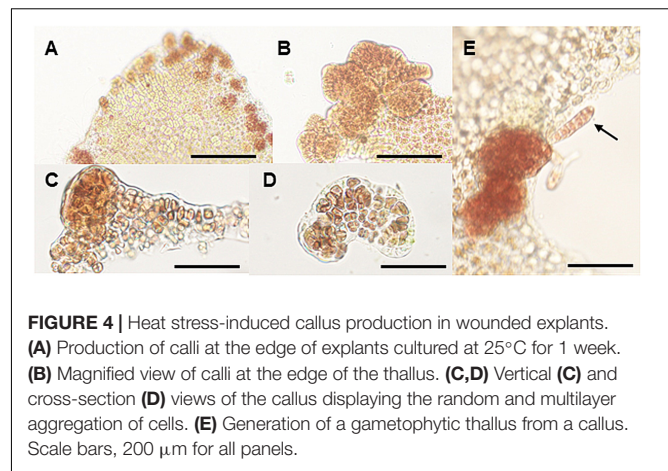
at 15°C in the dark released significantly more spores than the control thalli cultured in light, while those pretreated at 4°C in the light released a similar number of spores to the control.

We further showed that the dark treatment enhanced the release of both the monospores and carpospores (Supplementary Figure S3). Moreover, nonwounded thalli also released more spores when pretreated in darkness at 15°C (Supplementary Figure S4), although much fewer spores were released from these thalli than from the wounded explants under the same conditions (Figure 3 and Supplementary Figures S3, S4). We therefore concluded that the 3-day pretreatment in darkness is responsible for enhancing spore release and increasing the sensitivity of thalli to wounding.

Heat Stress Stimulates the Production of Calli in a Dark-Independent Manner

When the thallus explants were incubated at 25°C for 1 week, they produced calli composed of many brownish-red cells that differed from the surrounding vegetative and sexually mature cells (Figure 4A). The calli were usually produced at the edge of the explants (Figures 4B,C) and comprised a three-dimensional structure with randomly aggregated brownish-red cells (Figures 4D,E). Normal thalli developed from the calli produced at 25°C (Figure 4F), indicating that the production of calli under heat stress conditions is an asexual reproductive response to generate new gametophytic thalli.

Moreover, calli were also frequently produced by nonwounded whole thalli under heat stress conditions (Figure 5). We therefore concluded that heat stress promotes callus production to accelerate the asexual propagation of gametophytic thalli, independent of wounding. Spore release was not observed at 25°C either with or without the dark pretreatment (data not shown).



DISCUSSION

Here, we demonstrated that spore release and the gametogenesis of male and female gametes was enhanced by wounding the gametophytic thalli of *P. yezoensis* and that the formation of calli in these tissues could be induced by heat stress. These findings indicate that the gametophytic thallus has the ability to respond to environmental stresses by accelerating asexual and sexual reproduction, with different strategies of reproductive activation induced by different stresses. It is

clear that wounding directs the transition of the life history strategy from growth to reproduction. Similarly, the heat stress-induced formation of calli with accelerated and nonorganized cell division to generate three-dimensional structures was an asexual reproductive process, resulting in the production of new thalli that can survive and grow under high-temperature conditions. We therefore concluded that the gametophytic thallus *P. yezoensis* can recognize different stresses and select an asexual or sexual reproductive response as a life cycle trade-off.

As shown in **Figure 1B**, the thallus explants released both monospores and carpospores, unlike a previous report (Hafting, 1999) that did not observe wounding-promoted induction of gametogenesis and production of carpospores. Since wounding promoted gametogenesis (**Figure 1C**), we hypothesized that the carpospores were produced by the fertilization of the male and female gametes, however, we previously showed that oxidative stress-dependent diploid apogamy produced carpospores without gametogenesis in *P. yezoensis* (Takahashi and Mikami, 2017; Mikami, 2019). This means that we cannot exclude the possibility that wounding may partially promote diploid apogamy, since wounding produces reactive oxygen species (ROS) that cause oxidative stress in plants (León et al., 2001; Beneloujaephajri et al., 2013; Baxter et al., 2014) as well as in seaweeds (Mcdowell et al., 2014a,b). To explore this possibility, a new experimental system should be developed to separate spore release and gametogenesis, both of which were promoted by wounding. In this respect, mutants lacking gametogenesis could be useful for exploring the possible inducibility of apogamy by wounding, however, we are not aware of any mutants lacking gametogenesis in *Pyropia*.

The promotion of gametogenesis by wounding has also been observed in the green algal genus *Ulva*, including in *U. linza*, *U. lactuca*, *U. prolifera*, and *U. mutanilis* (Gao et al., 2010; Wichard and Oertel, 2010; Vesty et al., 2015; Katsaros et al., 2017). In *U. linza*, for example, chopping gametophytes into 3–5 mm fragments induced gametogenesis within 48 h and gamete discharge in 3 days (Vesty et al., 2015). Thus, the sexual reproductive response to wounding could be conserved in the red and green macroalgae. Species in the genus *Ulva* produce sporulation inhibitors (SI-1 and SI-2) and a swarming inhibitor (SW1) that inhibit gametogenesis and gamete discharge, respectively (Stratmann et al., 1996; Wichard and Oertel, 2010; Vesty et al., 2015). It is plausible that wounding might prevent the production or function of these inhibitors, promoting the reproductive response. It is currently unclear whether *P. yezoensis* possesses homologs of the sporulation and swarming inhibitors found in *Ulva*; therefore, it would be valuable to determine whether these factors exist in the Bangiales and to explore whether wounding or other stresses inhibit their production or activities. This would help to elucidate the mechanisms responsible for the regulation of the life cycle trade-offs in these algae.

We previously demonstrated that the asexual reproductive response in *P. yezoensis* was enhanced under hypotonic and oxidative stress conditions, as well as by a decrease in the extracellular concentration of calcium ions (Li et al., 2008, 2009; Takahashi et al., 2010; Takahashi and Mikami, 2017).

The effects of hypotonic stress on the monospore release were originally observed in *Porphyra pulchella* (Ackland et al., 2007). In addition, the promotion of the asexual life cycle by heat stress was previously observed in the red alga *Bangia fuscopurpurea* (Notoya and Iijima, 2003; Wang et al., 2008; Mikami and Kishimoto, 2018), although in this study we found that heat stress did not stimulate monospore release in *P. yezoensis* (data not shown). These findings indicate that some Bangiales species can respond to environmental stresses using life cycle trade-offs. The reproductive processes promoted by the various stresses differ between species, suggesting that different stresses target distinct regulatory components involved in the promotion of asexual or sexual reproduction in each species. To explore this possibility, it will be necessary to elucidate the systems regulating the stress-dependent promotion of asexual reproduction and the release of monospores in the Bangiales.

We demonstrated that wounding also promoted the gametogenesis in thalli to produce conchocelis filaments in *P. yezoensis* (**Figure 1**). This process involved the conversion of vegetative cells in the thalli to male and female gametangia (Takahashi and Mikami, 2017), meaning that gametogenesis usually results in the retardation of growth because of the reduction in vegetative cell numbers. Wounding therefore promotes the sexual life cycle using a growth trade-off in *P. yezoensis*. It is still unknown whether wounding promotes gametogenesis directly or if it causes growth retardation that in turn triggers gametogenesis. Little is known about regulatory mechanisms of gametogenesis in thalli; therefore, the elucidation of the wound perception and intracellular signaling pathways could help us to understand the regulation of gametogenesis and the environmental stress-promoted life cycle trade-offs in *P. yezoensis*.

Our results clearly indicated positive effects of dark pretreatment on the promotion of sexual and asexual propagation by wounding (**Figure 3** and **Supplementary Figures S3, S4**), experimentally confirming our preliminary observation of the effect of the refrigerated delivery service. Certain physiological changes may therefore be induced within 3 days in response to darkness, enhancing the sensitivity of the thalli to wounding. However, little is currently known about the factors responsible for the effect of darkness in *P. yezoensis*. As shown in **Figure 3**, the effects of wounding and darkness were almost additive but not synergistic, suggesting that these stresses make different contributions to the reproductive strategy. In terrestrial plants, darkness induces the production of ROS (Rosenvasser et al., 2006; Zhang et al., 2016), meaning that a dark-induced oxidizing status in the *P. yezoensis* cells might enhance spore release. Oxidative stress was previously shown to promote the production and release of monospores in *P. yezoensis* (Takahashi and Mikami, 2017); therefore, future studies should address whether *P. yezoensis* produces ROS in the dark and whether any dark-induced ROS production enhances gametogenesis and monospore release.

Callus production was observed in thalli under heat stress conditions (**Figures 4, 5**). Normal leafy gametophytic thalli were produced from these calli, which comprised three-dimensional tissues with aggregations of proliferating cells (**Figure 4**);

therefore, callus formation seems to be an asexual strategy to maintain the gametophyte generation. In terrestrial plants, wounding promotes callus formation, and the regeneration of tissues from calli depending on the actions of plant hormones such as auxin and cytokinin (Ikeuchi et al., 2013, 2017). We previously demonstrated the presence of plant hormones such as auxin and cytokinin in *P. yezoensis* (Mikami et al., 2016; Mori et al., 2017), although the physiological functions of these hormones are yet to be resolved. Future studies should therefore explore whether the promotion of calli formation by heat stress and the subsequent regeneration of the thalli is regulated by hormones in *P. yezoensis*.

The increase in culture temperature from 15 to 25°C stimulated the division of nonorganized cells but did not alter the identity of the life cycle generation, suggesting that heat stress can disrupt the rigidly determined direction of cell division required for the maintenance of the two-dimensional leafy thallus shape. Thus, the promotion of callus formation by heat stress could be a useful experimental system for the elucidation of unresolved important issues, such as why and how the gametophytic thalli of the Bangiales maintain a two-dimensional structure using an invariable direction of cell division. The exploration of the formation of calli and regeneration from them will therefore provide new insights into fundamental biological aspects, such as how wounding enables abnormal cell proliferation and how polarity-dependent cell division is regulated in the thalli of *P. yezoensis*.

CONCLUSION

The gametophytic thalli of *P. yezoensis* employ multiple life history strategies to cope with the negative impacts of environmental stresses by accelerating asexual and sexual reproductive responses using life cycle trade-offs. One such strategy is the promotion of changes in the life cycle strategy from vegetative growth to monospore release and gametogenesis, while the other is the production of an aberrantly growing callus as an abnormal asexual propagation mechanism. The elucidation of the regulatory mechanisms by which stresses promote the various reproductive responses in *P. yezoensis* could therefore enhance our understanding of the significance of flexible changes in life cycle strategies that enable the Bangiales to respond to and survive stressful conditions by producing asexual and sexual progenies.

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DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

KM conceptualized and designed the study, made the figures, and wrote the manuscript. MS performed the experiments, collected and analyzed the data, and made the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00394/full#supplementary-material>

FIGURE S1 | The majority of spores discharged following wounding were monospores. Explants were excised from the tips, middles (0.8 cm from the tip), and bottoms (1.6 cm from the tip) of thalli and incubated for 7 days. Discharged spores were counted daily. The pink and green bars correspond to the numbers of monospores and nongerminated spores, respectively. Error bars indicate the standard deviation of data from triplicate experiments ($n = 3$).

FIGURE S2 | Growth stage dependency of sensitivity to wounding in thalli. Explants were excised from thalli 0.7, 1.7, 2.4, or 3.2 cm in length and incubated for 7 days. Discharged spores were counted daily. Error bars indicate the standard deviation of data from triplicate experiments ($n = 3$). Different letters indicate significant differences at $p < 0.05$.

FIGURE S3 | Dark-enhanced discharge of monospores and carpospores. The thalli were pretreated at 4°C or in darkness for 3 days. Discharged spores were counted daily. Pink and green bars correspond to the numbers of monospores and nongerminated spores, respectively. Error bars indicate the standard deviation of data from triplicate experiments ($n = 3$).

FIGURE S4 | Effect of darkness on spore discharge in a wounding-independent manner. The number of spores released from nonwounded whole thalli following a 3-day pretreatment in darkness or light at 15°C. Discharged spores were counted daily. Error bars indicate the standard deviation of data from triplicate experiments ($n = 3$). Different letters indicate significant differences at $p < 0.05$.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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