Short communication

Treatment with heat shock protein 90 (Hsp90) inhibitors induces asexual life cycle in the marine red alga *Neopyropia yezoensis* (Rhodophyta)

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Running title: Hsp90 controls asexual reproduction in algae

ABSTRACT

The treatment with geldanamycin and radicicol, highly specific inhibitors of Heat shock protein 90 (Hsp90), induced archeosporangia formation at the marginal region of the upper parts in gametophytes of *Neopyropia yezoensis*. The archeospores discharged from archeosporangia treated with Hsp90 inhibitors prevented settlement to the substratum in the presence of the drugs. However, the archeospores transferred to media without drugs germinated after the settlement to the substratum. These results suggested that Hsp90 negatively caused a transition from vegetative to asexual reproduction. This is the first report that suggested the possibility of involvement of Hsp90 regulation in the asexual reproduction of algae.

Key index words: Asexual sporulation; Bangiales; Heat shock protein 90; *Neopyropia*; Red algae; Reproduction

Bangiales including *Neopyropia* (formerly *Pyropia*) is one of the most important marine crops worldwide, with a value of over US$1.3 billion per year (Blouin et al., 2011).The order compromises a heteromorphic life cycle between a gametophytic blade phase and a sporophytic filamentous phase. In addition to the sexual life cycle, some species in Bangiales have an asexual life cycle by producing asexual spores termed as archeospores (or monospores) or neutral spores, which reproduce gametophytic thallus (Tanaka, 1951; Drew, 1956; Kornmann, 1994). The asexual life cycles contributes to produce many gametophytic thalli on the nets during the cultivation periods, which results in the increase yield. In the future, the method on efficient induction of the asexual life cycles could lead to clonal propagation of superior strains. However, in contrast to accumulating findings on the sexual reproduction regulation (Uji et al., 2016; Yanagisawa et al., 2019; Uji et al., 2020; Endo et al., 2021), there is little available information on the molecular mechanisms by which red algae switch from vegetative to asexual reproductive stages.

Heat shock proteins (HSPs) are particularly produced when cells are exposed to environmental stress including thermal stress to serve as molecular chaperones in protein quality control (Kotak et al., 2007; Jacob et al., 2017). Among them, Hsp90 has emerged as a focus of interest because it uniquely regulates the development pathway

by serving as a molecular hub for signal transduction proteins including hormone receptors and signaling kinases (Young et al., 2001; Zhao and Houry, 2005). Hsp90 association with client proteins is regulated by the activity of the N-terminal ATPase domain, which binds and hydrolyses adenosine triphosphate (ATP) (Pearl and Prodromou, 2006). Pharmacological studies using highly selective inhibitors such as geldanamycin (GA) and radicicol (RAD) revealed that Hsp90 can involve in various pathways in the development and cell differentiation in animals and plants (Rutherford and Lindquist, 1998; Queitsch et al., 2002). Although our knowledge on HSPs including Hsp70 and small HSPs has constantly progressed in Bangiales (Park et al., 2012; Sun et al., 2015; Jin et al., 2017; Uji et al., 2019), there is no information on the Hsp90 function during the algal development and life cycle except for the specific expression in female gametophytes of *Griffithsia japonica* (Lee et al., 1998). In this study, we investigated the effect of Hsp90 inhibitors on the reproduction in *Neopyropia yezoensis* toward the goal of contributing to sustainable Bangiales farming industry.

The leafy gametophytes of *N. yezoensis* strain TU-1 were cultured in a medium of sterile vitamin-free Provasoli's enriched seawater (PES; Provasoli, 1968) at 15°C under the photoperiod regime of 10 h light:14 h dark using cool-white fluorescent lamps at 60 μmol photons m^{-2} s^{-1}. For Hsp90 inhibitor treatment, stock solutions of 100 mM

geldanamycin (GA) (Cayman Chemical Company, MI, USA) or 100 mM radicicol (RAD) (Cayman Chemical Company) were prepared by dissolving the drugs in dimethyl sulfoxide (DMSO). Five individual immature gametophytes with approximately 20 mm blade length were cultured in glass flasks (150 mL volume) to examine the effect of Hsp90 inhibitors on the asexual reproduction using 100 mL PES medium containing 5 μM GA, or 5 μM RAD. The gametophytes were cultured with the chemical reagents for 10 d and then transferred to 55 mm tissue culture dishes with PES medium in the absence of inhibitors. Four days after the transfer, the number of discharged archeospores was counted using an inverted microscope.

When the gametophytes (approximately 20 mm blade length) were grown with the Hsp90-inhibiting drugs (i.e., GA or RAD), morphological phenotypes such as blade and rhizoid shapes were not affected. However, thalli treated with 5 μM GA, or 5 μM RAD formed the archeosporangia (cluster of round shape cells) at the marginal region of the upper parts and discharged the archeospores 10 days after the inhibitor treatment (Fig. 1A, 1B). In contrast, no formation of archeosporangia was observed in gametophytes cultured in media without Hsp90 inhibitors (Fig. 1C). Theses archeospores discharged from the archeosporangia were inhibited the adhering to the substratum and the germination during the culture period (Fig. 1D), although they exhibited amoeba

movement after their release (Fig. 1E). The thalli formed archeosporangia by treatment with Hsp90 inhibitors transferred to media without drugs and subsequently discharged archeospores germinated after settlement to the substratum (Fig. 1F). Numbers of archeospores released from the gametophytes treated with 5 μM GA and 5 μM RAD were 1797 and 1971, respectively; whereas that of gametophytes treated without treatment was only 30 (Fig. 2). However, treatment with Hsp90 inhibitors did not induce the archeosporangia formation in the gametophytes with blade lengths more than 40 mm (data not shown). To date, several reports found that the suppression of Hsp90 resulted in severe defects in the transition from vegetative to reproductive phase in fungi and higher plants (Lamoth et al., 2012; Bui et al., 2016; Margaritopoulou et al., 2016). In contrast to previous reports, our study showed that Hsp90 inhibition induced asexual reproduction in *N. yezoensis*, suggesting that Hsp90 negatively regulated the transition from vegetative to asexual sporulation. The molecular mechanism of asexual reproduction in Bangiales species remains unknown, but extracellular calcium concentration can affect the production and discharge of archeospores (Takahashi et al., 2010). Besides, genetic and physical interactions between Hsp90 and calcium signaling components including calmodulin and calcineurin were observed in animals, fungi, and plants (Nguyen et al., 2009; Imai and Yahara, 2010; Virdi et al. 2011). Thus, although no

client protein for Hsp90 has been identified in red algae, Hsp90 possibly regulates the asexual reproduction of *N. yezoensis* through the calcium signaling pathway.

The archeospores discharged from thalli treated with Hsp90 inhibitors prevent settlement to the substratum in the presence of the reagents. Small and large fibrous vesicles actively are produced during asexual sporulation in Bangiales species, and released archeospores lack the cell wall but are surrounded by a layer of adhesives which presumably originate from fibrous vesicles (Hawkes, 1980). In addition, archeospores treated with brefeldin A (BFA), a vesicle trafficking inhibitor, retained the ability of amoeba movement but the cell wall was not synthesized for settlement to the substratum in *N. yezoensis* (Li et al., 2008). These findings indicate that vesicle trafficking can play a critical role in adhesive formation and release in archeospores. Previous studies also found that the secretion of adhesives in algae is regulated by calcium-based signal transduction (Roberts et al., 1994; Chin et al. 2004). Thus, Hsp90 from *N. yezoensis* may participate in the regulation of vesicular transport and exosomes responsible for the formation and release of adhesives to adhere to substratum through the calcium signaling pathway like the archeosporangia formation.

In the present study, pharmacological suppression of Hsp90 induced the archeosporangia formation and discharge of archeospores in *N. yezoensis*. Our results

suggest that Hsp90 is a negative regulator of the asexual reproduction in Bangiales species. This finding clarifies an interesting aspect of the role of Hsp90 as a central interface in the development and life cycle in red algae.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

TU was responsible for the design of the experiments, and interpretation of data. TU performed the experiments. TU and HM wrote the manuscript. All authors have read and approved the final manuscript.

ETHICAL APPROVAL

This article does not contain any studies with animals performed by the authors.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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Figure legends

Figure 1. Induction of the transition from the vegetative to asexual reproduction by Hsp90 inhibitors. Production of archeosporangia at the marginal region of the upper parts in gametophytes cultured in media with 5 μM geldanamycin (GA) (A) and 5 μM radicicol (RAD) (B). No formation of archeosporangia in gametophytes cultured in

media without drugs (C). Inhibition of settlement to the substratum in archeospores after the migration in the presence of 5 μM GA (D). Migration of archeospores discharged from archeosporangia in gametophytes treated with 5 μM GA (E). The seedlings via archeospores discharged from archeosporangia at the marginal region of the upper parts in the gametophytes treated with 5 μM GA after being transferred to media without drugs (F). Scale bar = 50 μm.

Figure 2. The number of archeospores released from gametophytes treated with Hsp90 inhibitors. Gametophytes were cultured in media containing 0, 5 μM geldanamycin (GA) or 5 μM radicicol (RAD) for 10 d. Data are expressed as mean ± standard deviation (SD) of five thalli for each condition and analyzed using the Mann–Whitney U test for treatments with and without Hsp90 inhibitors. Asterisks indicate significant differences at $p < 0.01$ between the control and treatments.

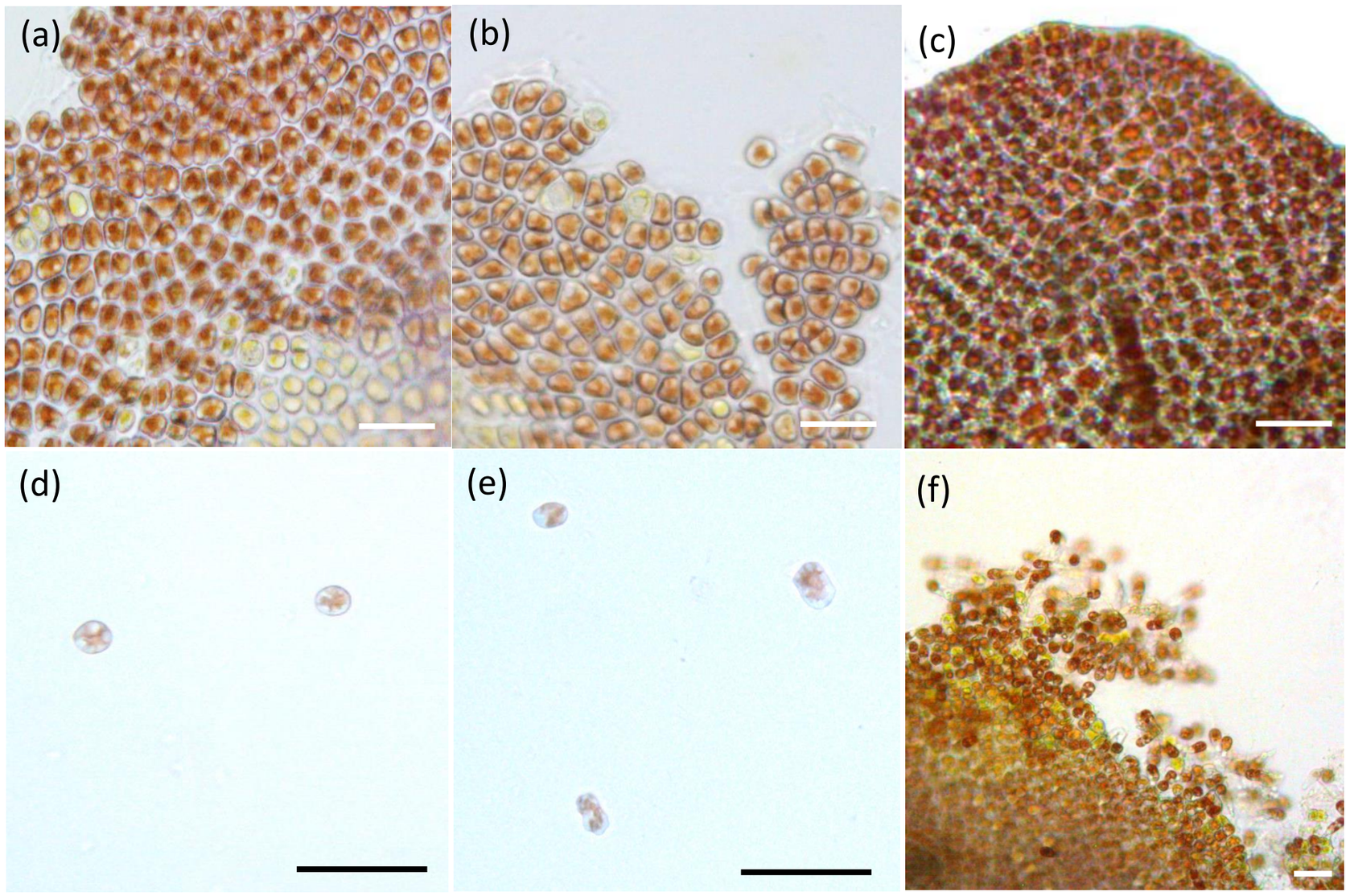

Fig. 1

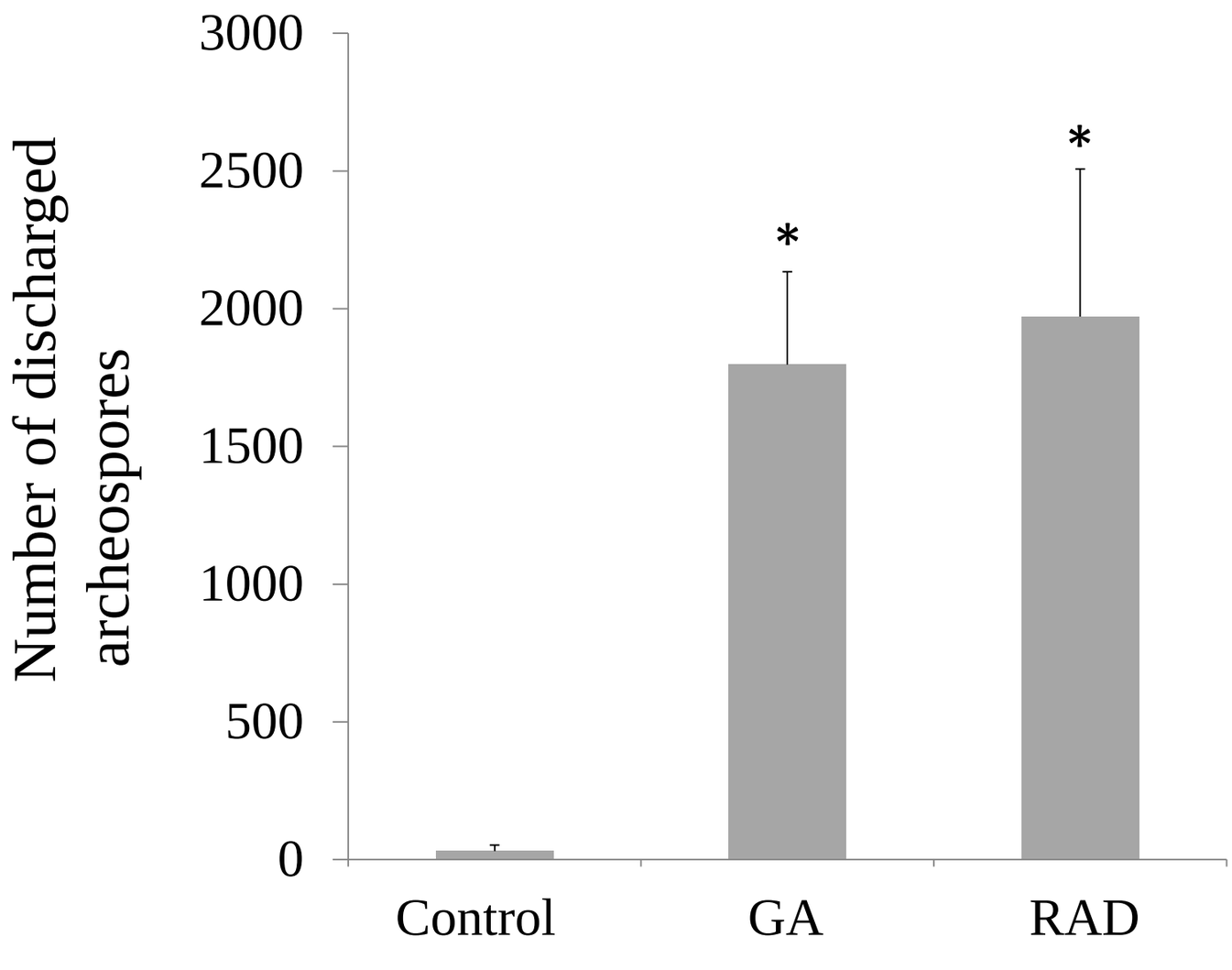
Number of discharged archeospores
3000
2500
2000
1500
1000
500
0
*
*
Control
GA
RAD

Fig. 2