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Article

Role of NADPH-Oxidase Mediated ROS Signaling to Oomycete Infection in the Red Alga *Dasysiphonia japonica*

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Abstract: Plants are confronted with a myriad of challenges that they must mitigate without being able to move to new environments. Systemic acquired resistance (SAR) is a secondary immune response in plants to pathogen infection. Little is known about SAR in red algae. The red alga *Dasysiphonia japonica* can survive for more than six years without lethal damage when infected with the pathogen *Olpidiopsis heterosiphoniae* but dies within a month when infected with another pathogen, *Olpidiopsis dasysiphoniae*. During this process, *D. japonica* infected with *O. heterosiphoniae* accumulated reactive oxygen species (ROS, DCFH-DA staining) in the host cell membrane, whereas this was not observed with *O. dasysiphoniae* infection. Transcriptome analysis identified four NADPH-oxidase genes (*Djrboh*) that generate ROS in the cell membrane of *D. japonica*. Real-time PCR of four NADPH oxidase genes (*Djrboh*) showed that two were upregulated (*Djrboh1*, 2) at the onset of *Olpidiopsis heterosiphoniae* infection, but not during infection with *O. dasysiphoniae* in early infection that does not induce SAR. The successful SAR of *D. japonica* was mediated by low concentrations of ROS signaling and regulation of NADPH-oxidase genes.

Keywords: Rhodophyta; SAR; ROS signaling

1. Introduction

The immune response, or immunity, is the recognition of and protection against foreign invaders (Maekawa et al. 2011, Király et al. 2013); therefore, a fundamental feature of the immune response is the ability to distinguish between self and non-self (Coll et al. 2011, Alberts et al. 2015). The immune response is also classified into primary and secondary immune responses (Janeway et al. 2001, Király et al. 2013). The primary immune response is the initial response to pathogen infection, followed by a secondary immune response to infection from the same pathogen (Janeway et al. 2001, Király et al. 2013). Secondary immunity in animals is a type of cell-based immune memory, a highly effective adaptive immune response (Janeway et al. 2001, Király et al. 2013). For example, when a host is first exposed to an antigen, its B cells produce antibodies and long-lived memory B and T cells, which recognize the antigen in subsequent infections, including secondary infections, triggering an immune response to the infection that is much more effective and rapid than the primary immune response (Janeway et al. 2001, Fu and Dong 2013). Plants, however, do not have these specialized immune cells like animals, but maintain immune memory through systemic acquired resistance (SAR) for the suppression of secondary infections (Fu and Dong 2013, Király et al. 2013).

SAR is a long-term and broad-spectrum resistance occurrence throughout the whole plant after local pathogen infection (Muthamilarasan and Prasad 2013, Seyfferth and Tsuda 2014, Shine et al. 2019). During SAR, a signal is rapidly generated at the primary site of local infection and transmitted throughout the plant; a common signal molecule is salicylic acid (SA) (Mishina and Zeier 2007, Seyfferth and Tsuda 2014). In addition to SA, the hormone jasmonic acid (JA)/ethylene (ET) and its

primary and secondary metabolites; nitric oxide and reactive oxygen species (ROS) have also been reported as SAR triggers (Zhang et al. 2010, Li et al. 2019, Shine et al. 2019). SA, JA and ROS are known to transmit signaling from the local infection area to distant uninfected areas through the apoplast (Felle et al. 2005, Shine et al. 2019, Farvardin et al. 2020, Kachroo et al. 2020, Li et al. 2021).

To determine the presence or absence of SA or JA -induced immune responses in red algae, the presence of hormone synthesizing genes, the detection of endogenous hormones or a hormone-induced immune response to exogenous hormones should be observed (Seyfferth and Tsuda 2014, Radwan et al. 2007). However, in red algae, the hormones SA and JA are either not detected (Collén et al. 2006) or only observed in some species (Krupina and Dathe 1991). The genes involved SA and JA synthesis have not been identified in red algae (Collén et al. 2006, Egan et al. 2014, de Oliveira et al. 2017), and while exogenous SA is involved in growth in some red algae, it does not induce an immune response (Wang et al. 2013). However, red algae do have defense responses against diverse pathogens (Pereira and Vasconcelos 2014, Luo et al. 2015, Tang et al. 2019, Im et al. 2019). Research on innate immunity of red algae has been conducted, but it is limited to the economically important seaweed species, *Pyropia yezoensis* (Tang et al. 2019). In several multicellular red algae, some receptors and genes involved in innate immunity have been partially characterized, and defense responses such as oxidative bursts (OB) and hypersensitivity reactions (HR) have been reported (Weinberger et al. 2007, Gachon et al. 2010, Egan et al. 2014, Luo et al. 2015, de Oliveira et al. 2017, Tang et al. 2019, Im et al. 2019, Wen et al. 2023).

Both OB and HR responses are triggered by ROS, which can also be transmitted through the plant (Apel and Hirt 2004, Fujita et al. 2006). One of the main sources of ROS is the conversion of dioxygen (O_2) to superoxide anion (O_2^-), catalyzed by NADPH oxidase, with hydrogen peroxide (H_2O_2) being produced in the subsequent reaction (Lightfoot et al. 2008, Marino et al. 2012, Huang et al. 2019, Mandal et al. 2022). NADPH oxidase and ferric reductase are homologs with canonical domains but are functionally different, with iron reductase catalyzing the electron transfer from cytosolic NADPH to extracellular metal ions and NADPH oxidase catalyzing the electron transfer from cytosolic NADPH to extracellular oxygen (Zhang et al. 2013). However, NADPH oxidase is distinguished from ferric reductase by three conserved amino acids ranging from transmembrane domain 3 (TM3) to TM4 (Zhang et al. 2013). Respiratory burst oxidase homologues (RBOHs) belong to NADPH oxidase gene super-families and *rboh* genes have been also reported in red algae (Inupakutika et al. 2016, Luo et al. 2015, Im et al. 2019, Gui et al. 2022, Shim et al. 2022, Moon et al. 2022, Hong et al. 2024b). However, previously reported red algal RBOHs did not mention the presence of conserved sequences and only revealed functional domains that are difficult to distinguish from ferric reductases (Inupakutika et al. 2016, Luo et al. 2015, Im et al. 2019, Gui et al. 2022, Shim et al. 2022, Moon et al. 2022, Hong et al. 2024b).

The genus *Olpidiopsis* is one of the most common pathogens in red algae (Wen et al. 2023). The red alga *Dasysiphonia japonica* (formerly known as *Heterosiphonia japonica*), an invasive species introduced worldwide from the Asian Pacific (Choi et al. 2002, Sjøtun et al. 2008, Savoie and Saunders 2013), is also infected with *Olpidiopsis* spp. (Klochkova et al. 2017). In this study, we aimed to investigate the immune response of *D. japonica* to two species of *Olpidiopsis* (Supplementary Video S1, S2). *D. japonica* can co-exist with *O. heterosiphoniae* infection, as opposed the case of *Pyropia* in which this infection leads to lethality, and infection of another *Olpidiopsis* species (i.e., *Olpidiopsis dasysiphoniae*) which leads lethality in *Dasysiphonia japonica*.

2. Materials and Methods

2.1. Sample Collection and Culture

An *Olpidiopsis heterosiphoniae* infected *Heterosiphonia pulchra* plant was collected from Wando, Korea on 17 May 2006. Dual host-parasite cultures were established using *Dasysiphonia japonica*, collected from Wando, Korea in September 2004, Wando, Korea (Klochkova et al. 2017). *Olpidiopsis dasysiphoniae* infecting *D. japonica* was collected from Muroran, Japan on 17 August 2018 (Wen et al.

2025). To maintain a high infection rate, we transferred the *Olpidiopsis dasysiphoniae* to a *Dasysiphonia japonica* collected in September 2004 from Wando, Korea. The samples were cultured in IMR medium and renewed every three weeks. The cultures were maintained in a chamber at 15°C and 16:8 h light:dark cycle (15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

The infection experiment was performed as follows: After 4-5 days of infection, the incubated medium was filtered through a 20 μm nylon net filter (NY20, Merck, Germany) to isolate the zoospores released from infected plants. The medium containing zoospores was incubated with 1 cm indeterminate branches of *D. japonica*. The infection rate was determined by the number of infected cells in each of the six plants and average.

2.2. Microscopy Observations and Histological Staining

Samples were checked regularly using a microscope (BX50, Olympus, Japan) and recorded on a digital camera (DP72, Olympus) using the CellSens program (Olympus). The production of total reactive oxygen species (ROS) in real time was detected using 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) and was performed as in Moon et al. (2022). Accumulation of hydrogen peroxides over time (every 12 hours) was performed by 3,3'-diaminobenzidine (DAB) staining and performed as in Strittmatter et al. (2016).

2.3. NADPH-Oxidases and Calcium Influx Inhibition Experiment

Diphenylene iodonium (DPI) was used to block the activity of NADPH-oxidases (Foreman et al. 2003) and caffeine was used to block calcium influx through caffeine-sensitive Ca^{2+} channels (Länge et al. 1996). Both DPI and caffeine were pretreated to the *D. japonica* filament for 4 hours before infection.

2.4. Identification of NADPH-Oxidase from *Dasysiphonia japonica*

The known NADPH-oxidases of red algae (Thapa et al. 2020, Gui et al. 2022, Moon et al. 2022, Shim et al. 2022) were blasted in transcriptome of *D. japonica*, and a phylogenetic tree of NADPH-oxidase was constructed to identify the NADPH-oxidases of *D. japonica* (Fig. S1). Protein domains were analyzed by Interproscan in Geneious Prime. The identification of conserved sites in protein domain followed a previous study (Zhang et al. 2013).

2.5. Housekeeping Gene Testing and Quantitative PCR

Total RNA was extracted from infected *Dasysiphonia japonica* frozen samples ground in liquid nitrogen using a Qiagen RNeasy plant mini kit, following the manufacturer's protocol. Prior to cDNA synthesis, all RNA samples were treated in DNase I using the Qiagen on-column DNase kit, following the manufacturer's protocol. The integrity of the RNA was tested by agarose gel electrophoresis. The synthesis of cDNAs, from each sample, was obtained using the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Palo Alto, CA, USA) following the manufacturer's instructions.

Potential housekeeping genes (HKG) for quantitative PCR were selected from transcriptome data of *Dasysiphonia japonica*. Six genes were selected: alpha-tubulin (A-tubulin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor Tu (EF-TU), actin, isocitrate dehydrogenase (IDH), ubiquitin-conjugating enzyme E2 variant 1C (UBI) and alpha-tubulin (A-tubulin) showed the most stably expressed Ct values at different stages of infection (Fig. S2). Primers for selected genes were designed considering melting temperature and products size using the Geneious Prime and showed in the Table S1. Real-time PCR was performed using iQ SYBR Green supermix (Bio-Rad Laboratories) in a real-time PCR detection system (Bio-Rad CFX96; Bio-Rad Laboratories). cDNA samples were amplified in triplicate from the same RNA preparation. The final reaction volume was 20 μl and included 5 μl of diluted cDNA, 10 μl of iQ SYBR Green supermix and 200 nM of each primer. The reaction protocol was first set at 95°C for 2 min and then 50 cycles of 95°C

for 3 s and 60°C for 30 s. The expression of each HKG was determined from the cycle of threshold value (Ct value), which corresponds to the number of cycles required for PCR amplification to reach threshold. The selected HKGs were tested for expression stability during various infection processes, and the genes with the smallest standard deviation of Ct values were selected to determine the relative expression levels for other genes. Relative expression levels were then calculated against the housekeeping gene as a calibrator using the delta–delta C_t method.

2.6. Statistical Analysis

ANOVA was conducted to check the differences between the gene expression levels in the control and treated samples using an online website (<https://goodcalculators.com/one-way-anova-calculator/>). $P < 0.05$ was accepted as significant.

3. Results

3.1. Features of *Olpidiopsis heterosiphoniae* Infection on *Dasysiphonia japonica*

To investigate the degree of infection after exposure to *O. heterosiphoniae* zoospores, we measured infection rates at 2-day intervals, the minimum time for *O. heterosiphoniae* sporangia to mature (Fig. 1). An infected host cells was noted when intracellular sporangia were observed (Fig. 1A). After 4 days of infection, several mature sporangia were produced, and zoospores were released (Fig. 1B). On 6 (Fig. 1C) and 8-days (Fig. 1D) after infection, mostly empty sporangia were seen, and some newly produced sporangia were observed as secondary infections progressed. The number of infected cells of *Dasysiphonia japonica* increased up to day 4 and then decreased to less than 3% from day 6 onwards (Fig. 1E).

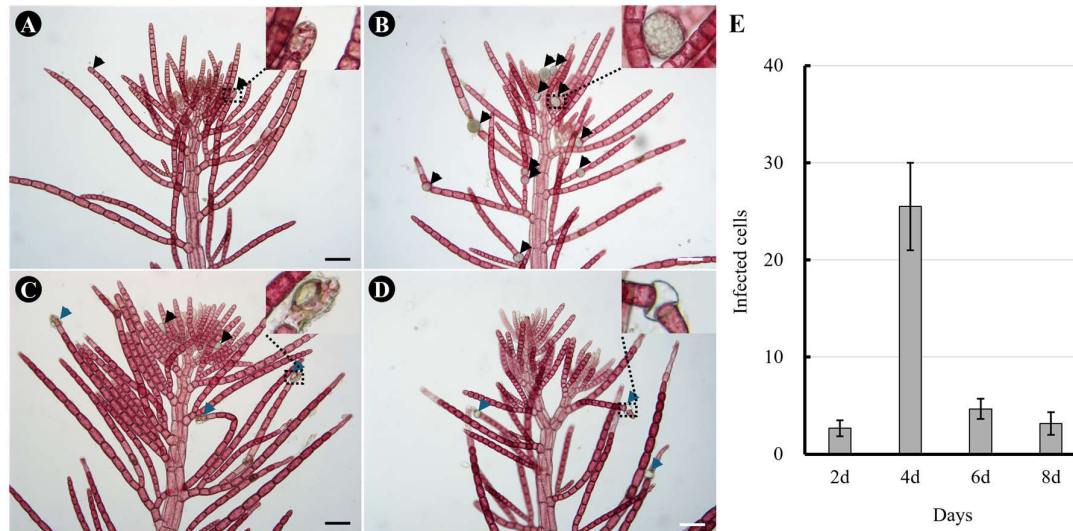


Figure 1. Infection of *Olpidiopsis heterosiphoniae* on *Dasysiphonia japonica* over time. (A) 2-days after infection. Sporangia of *O. heterosiphoniae* are seen developing inside apical cells of lateral branch (arrowheads and inset). (B) 4-days after infection, in addition to apical cells, other cells in monosiphonous branches also showed developing sporangia (arrowheads). (C) 6-days after infection with *O. heterosiphoniae*, most of the sporangia are mature, releasing zoospores and leaving empty sporangia (blue arrowheads), while some newly infected cells with developing sporangia are seen (black arrowheads). (D) 8-days after infection, empty sporangia (blue arrowhead) were observed in infected plant. (E) Changes in infection rate of *O. heterosiphoniae* on *D. japonica* over time. Data are expressed as mean $\pm$ SD ($n = 6$). Scale bars represent: A-D, 100 μ m.

3.2. Comparison of Infection Symptoms of *Olpidiopsis heterosiphoniae* and *O. dasysiphoniae* Infection in *D. japonica*

With *O. heterosiphoniae*, cell death was rarely observed around infected cells (Fig. 2A-B) even to a month after infection (Fig. 2C), plus number of infected cells remained low (see section above). However, during infection with *O. dasysiphoniae*, cell death of infected cells and adjacent cells was observed from the beginning of infection (Fig. 2D), and in many cases, cell death of multiple cells neighboring infected cells was observed (Fig. 2E). One month after the infection, the whole plant was almost dead (Fig. 2F).

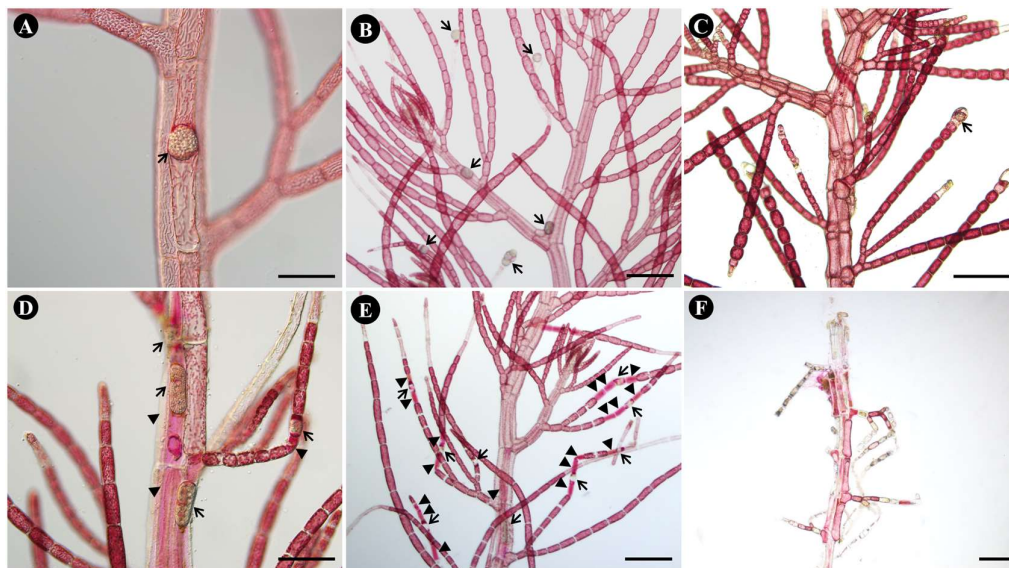


Figure 2. Symptoms of infection with *Olpidiopsis heterosiphoniae* and *O. dasysiphoniae* on *Dasysiphonia japonica*. (A-C) *O. heterosiphoniae* infection (A) Sporangium (arrow) in polysiphonous axial cell of host. (B) 4-days after infection. Arrow indicates infected cells (C) One month after infection. Arrow indicates infected cell. (D-F) *O. dasysiphoniae* infection. (D) Sporangium in polysiphonous axial cell of host. Arrow indicates infected cell. (E) 4-days after infection. Arrows indicates infected cells; arrowheads indicate adjacent dead cells. (F) One month after infection, most tissue is dead. Scale bars represent: A, D, 100 μ m; B-C, E-F, 200 μ m. .

3.3. ROS Signaling in *D. japonica* After *O. heterosiphoniae* and *O. dasysiphoniae* Infection

When ROS staining was performed for *Olpidiopsis heterosiphoniae* and *Olpidiopsis dasysiphoniae* there was a difference in ROS staining and a difference in infected cells when treated with ROS inhibitors. DCFH-DA staining of *Dasysiphonia japonica* was not detected with pre-infection (Fig. 3A) but detected around the membrane of infected host cells (Fig. 3, arrowheads), and developing pathogen, on day 2 (Fig. 3B), persisted to day 4 (Fig. 3C), and disappeared on day 8 post-infection (Fig. 3D), when most pathogen zoosporangia were empty. During infection with *Olpidiopsis dasysiphoniae*, there was no staining around the infected host cell membrane on days 2-4 but only in the developing pathogen (Fig. 3F-G). A large amount of ROS staining was observed on day 8 (Fig. 3H).

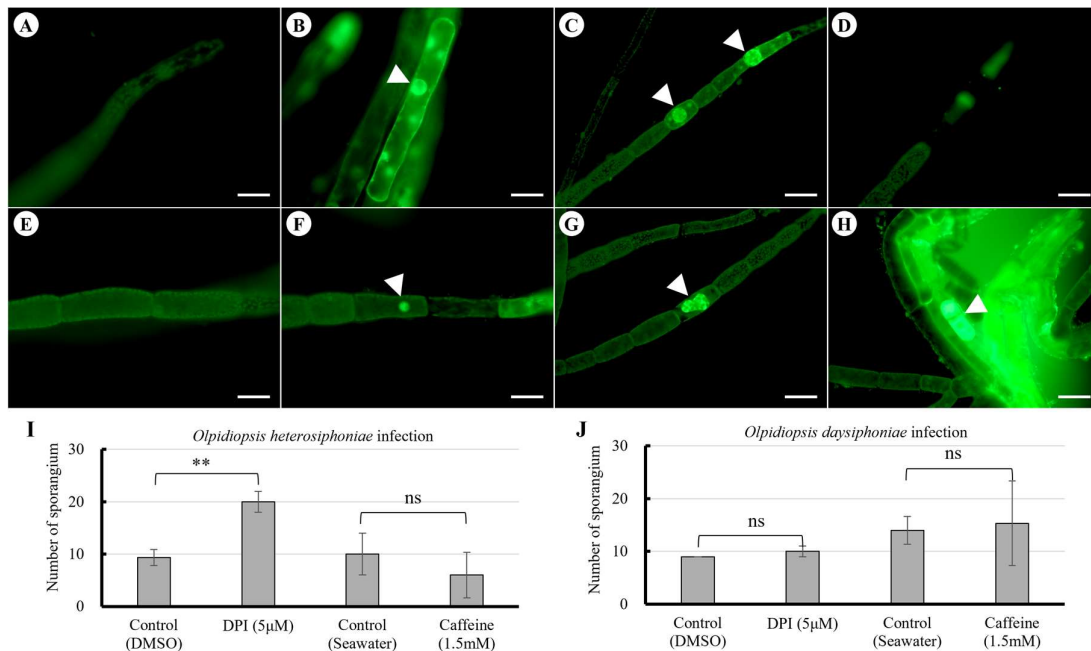


Figure 3. DCFH-DA staining of *Dasysiphonia japonica* after *Olpidiopsis heterosiphoniae* and *O. dasysiphoniae* infection (A-D) *O. heterosiphoniae* infection. (A) 0-day, pre-infection. (B) 2-day. (C) 4-day. (D) 8-day. (E-H) *O. dasysiphoniae* infection. (E) 0-day, pre-infection. (F) 2-day. (G) 4-day. (H) 8-day. Arrowhead indicates *Olpidiopsis* developing in cell. Scale bars represent: A-F, 50 μm. (I) Level of infections after ROS (DPI) and calcium channel (caffeine) inhibitors treatment on *O. heterosiphoniae* infection. Data are expressed as mean ± SD (one-way ANOVA vs. control, C, n = 6). (J) Level of infection after ROS and calcium inhibitors treatment in *O. dasysiphoniae* infection. Data are expressed as mean ± SD (one-way ANOVA vs. control, n = 3). ** p < 0.01, ns, non-significant.

Diphenylene iodonium (DPI) pretreatment of host plants increased *O. heterosiphoniae* infection rates compared to the control, but caffeine pretreatment showed no significant difference from controls (Fig. 3I). In *Olpidiopsis dasysiphoniae* infections, DPI and caffeine pretreatment of host plants did not produce any significant difference in infection rates compared to the control (Fig. 3J).

In DAB staining, in *O. heterosiphoniae* infection, staining was detected within the infected host cell on day 2 of infection (Fig. 4B, 7I), became more intense on day 4 (Fig. 4C, 4J-K), and was detected at both ends of the host cell on day 8 of infection (Fig. 4D, 7L), when most sporangia were empty. In *Olpidiopsis* sp. infections, there was no significant difference in infected cells on days 2-4 (Fig. 4E-F, 7M), and by day 8, large amounts of H₂O₂ were stained in intracellular compartments adjacent to the host cell (Fig. 4N-O).

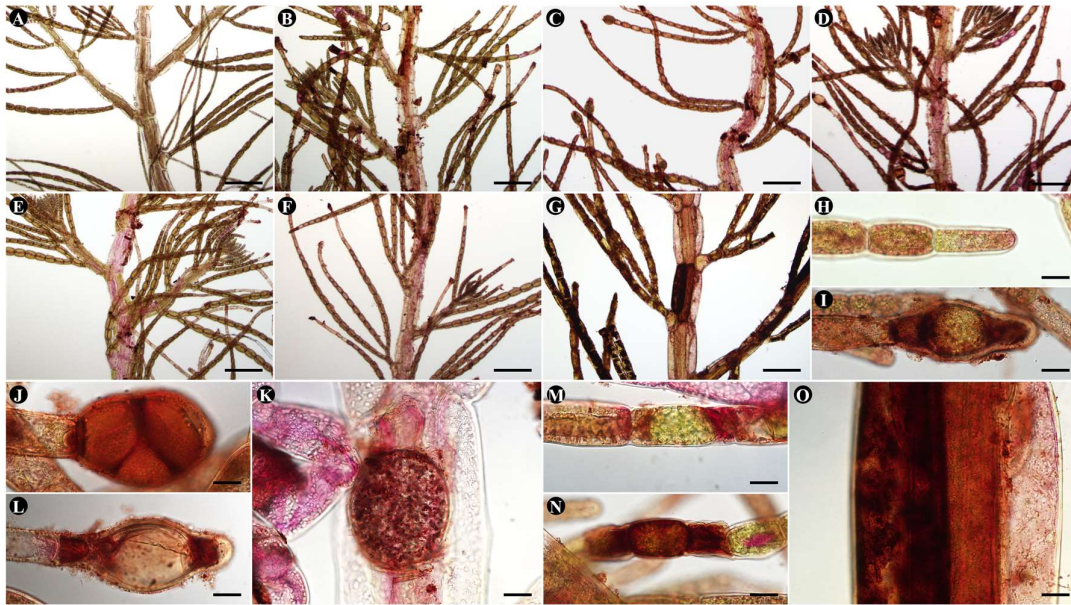


Figure 4. DAB staining of *Dasysiphonia japonica* after *Olpidiopsis* spp. infection. (A, H) Uninfected plant. (B-D, I-L) *O. heterosiphoniae* infection. (B, I) 2-day. (C, J, K) 4-day. (D, L) 8-day. (E-G, M-O) *Olpidiopsis* sp. infection. (E) 2-day. (F, M) 4-day. (G, N, O) 8-day. Scale bars represent: A-G, 200 μ m; H-O, 20 μ m.

3.4. NADPH-Oxidase Homologues and Expression Levels *O. heterosiphoniae* and *O. dasysiphoniae* Infection

Four NADPH-oxidase (*Djrboh1-4*) homologs were found in our transcriptome dataset (Fig. 5). *Djrboh*s were shown to have not only conserved sequences for the ferric reductase domain (FRD) superfamily (Fig. 5A), but also NADPH-oxidase-specific conserved sequences (i.e., conserved positions in ROS-generating homologs) that distinguish it from ferric reductases (Fig. 5B). The conserved positions in the ROS-generating homologs of *D. japonica* consist of a heme-binding histidine in transmembrane domain 3 (TM3) and a conserved threonine or serine in transmembrane domain 4 (TM4), and although not specific to NADPH-oxidase homologs, the glycine sequence is conserved in dipeptide form with the threonine or serine in transmembrane domain 4 (TM4). The above analysis provides strong evidence that *Djrboh1-4* is a protein that reduces oxygen rather than iron (Fig. 5C).

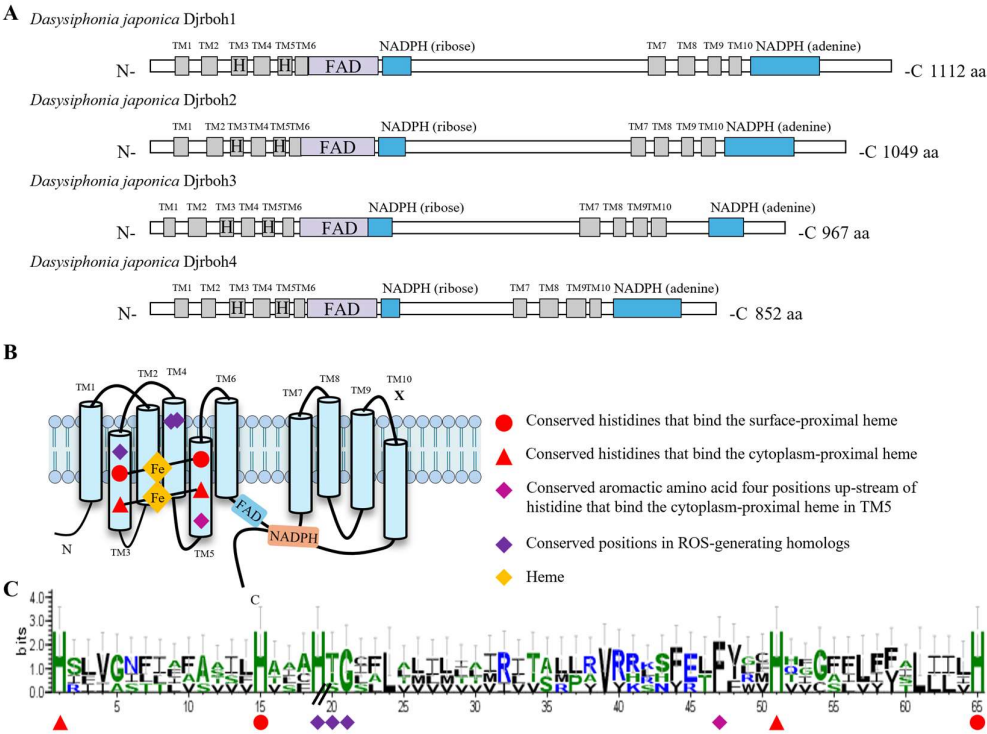


Figure 5. Structure of NADPH-oxidases in *Dasysiphonia japonica*. (A) Functional domain of NADPH-oxidase (Djrboh1-4) in *D. japonica*. (B) A schematic representation of the conserved sequences located in the transmembrane. (C) Sequence conservation logos of Djrboh1-4. The height of the stacks indicates sequence conservation, and the width of the stacks is proportional to the fraction of amino acids, thus narrowed within gapped regions.

Real-time PCR revealed that during *O. heterosiphoniae* infection, the expression of *Djrboh1* and 2 increased approximately 2-fold, over control levels, on day 4 and recovered by 8 days, while *Djrboh3*, 4 genes showed no obvious changes during the first 8 days of infection (Fig. 6A). In *O. dasysiphoniae* infection, expressions of *Djrboh1*, 2, and 3 were downregulated on day 2, expression of *Djrboh1* and of *Djrboh3* were restored by day 4 but not *Djrboh2*. Expression of *Djrboh4* did not change significantly on days 2 and 4 of infection (Fig. 6B). By day 8 post-infection, the expression of *Djrboh1*, *Djrboh3*, and *Djrboh4* all increased (Fig. 6B).

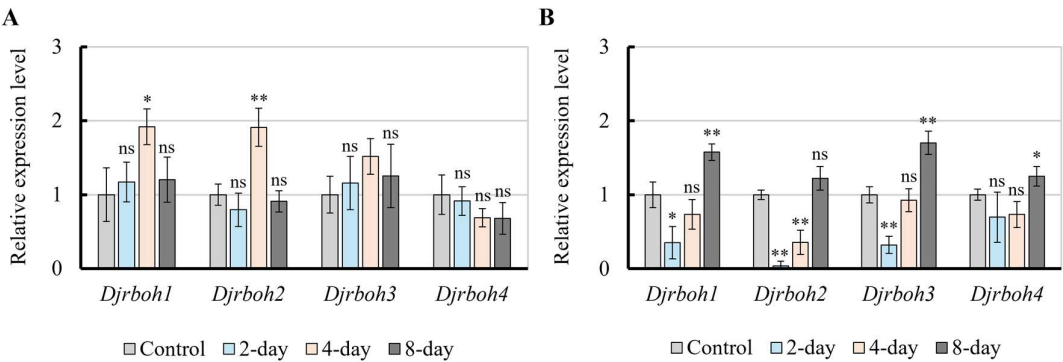


Figure 6. Relative expression level of four *Djrboh* genes (1-4) during *Olpidopsis heterosiphoniae* and *O. dasysiphoniae* infection in *Dasysiphonia japonica*. (A) Real-time PCR results of *O. heterosiphoniae* infection, showing increased expression in *Djrboh* q and 2 after days 2 and 4. (B) Real-time PCR results of *O. dasysiphoniae* infection,

showing both up-regulation or down-regulation of all copies even after 8 days. Data are expressed as mean $\pm$ SD (one-way ANOVA vs. control, $n = 3$). * $p < 0.05$, ** $p < 0.01$, ns, non-significant.

4. Discussion

In this study, red algae *Dasysiphonia japonica* infected with *O. heterosiphoniae* accumulated reactive oxygen species (ROS) in the host cell membrane, whereas this was not observed with *O. dasysiphoniae* infection. Treatment with diphenylene iodonium (DPI), an NADPH oxidase inhibitor that catalyzes ROS in the membrane, increased the infectivity of *O. heterosiphoniae* infection but did not change the infectivity of *O. dasysiphoniae*. Accumulation of hydrogen peroxide was also observed at the level of whole plant in *O. heterosiphoniae* infection, but not in *O. dasysiphoniae* infection in early infection. Four NADPH-oxidase genes (*Djrboh1-4*) were found in *D. japonica*. Real-time PCR of four NADPH oxidase genes (*Djrboh1-4*) showed that two were upregulated (*Djrboh1*, 2) at the onset of *Olpidiopsis heterosiphoniae* infection, but not during infection with *O. dasysiphoniae* in early infection that does not induce SAR.

ROS are a major cause of cellular damage, recent studies have shown that they act as key signaling molecules in most organisms (Inupakutika et al. 2016, Hong et al. 2024a). In plant immune responses, ROS signaling is always accompanied by cell death (Torres et al. 2002), while in other cellular physiological processes usually proceeds without cell death (Chapman et al. 2019, Zandalinas et al. 2020). However, infection with the pathogen *O. heterosiphoniae* in the red alga *D. japonica* resulted in the production of ROS in host cell membrane but was not accompanied by cell death, and no ROS were observed early in the infection (2, 4 days after infection) in *O. dasysiphoniae* infections that underwent cell death. Hydrogen peroxide which synthesized by NADPH-oxidase in cell membrane diffused between cells can act as a systemic signal, cause changes in other proteins to active defense strategy, and cell death as a part of such as oxidative burst (OB) and hypersensitive response (HR) (Perez and Brown 2014, Niks et al. 2015, Inupakutika et al. 2016, Wu et al. 2023). However, in *D. japonica* infected with *O. heterosiphoniae*, hydrogen peroxide accumulated throughout the plant, but no cell death was observed; conversely, in *O. dasysiphoniae* infection, hydrogen peroxide did not accumulate throughout the whole plant during the early stages of infection (2 and 4 days after infection) but cell death was observed thereafter. Pretreatment with diphenylene iodonium (DPI) inhibits NADPH oxidase activity, which mediates the generation of ROS in membranes (Moon et al. 2022), only increased infection with *O. heterosiphoniae* and did not significantly change infection with *O. dasysiphoniae* infection, suggesting that NADPH-oxidase is involved in resistance to infection with *O. heterosiphoniae* infection.

Red algae have been using NADPH oxidase to generate ROS long before land plants, and these NADPH-oxidases genes were found in diverse red algae (Inupakutika et al. 2016, Luo et al. 2015, Im et al. 2019, Gui et al. 2022, Shim et al. 2022, Moon et al. 2022, Hong et al. 2024b). The functional differentiation of RBOH family in red algae is also currently being investigated (Shim et al. 2022, Moon et al. 2022, Hong et al. 2024b). In red algae, *Bmrboh1-3* are involved in fertilization (Shim et al. 2022) and *Gmrboh1-4* are involved in cell fusion (Moon et al. 2022), but only *Gmrboh3* is specifically involved in wound healing (Hong et al. 2024b). Four other NADPH-oxidase genes (*Djrboh1-4*) have been identified from transcriptome data; the four *Djrboh* genes showed different expression patterns depending on the pathogen and the date of infection (Fig. 6). On day 4 of infection, *Djrboh1-2* was increased in *O. heterosiphoniae* infection, whereas it was decreased or unchanged in *O. dasysiphoniae* infection, which does not induce cell death in the host post-infection. On day 8 of infection, *Djrboh* genes was not significantly changed in *O. heterosiphoniae* infection, whereas *Djrboh1*, *Djrboh3*, and *Djrboh4* genes were increased in *O. dasysiphoniae* infection, which induce cell death in the host.

In this study, we propose the existence of whole plant immune resistance, systemic acquired resistance (SAR), in the red alga *D. japonica* to *O. heterosiphoniae* infection, but that this resistance is not seen in *D. japonica* when infected with *O. dasysiphoniae*. In SAR process, the signal induced by the pathogen is transmitted to the whole plant by the appropriate concentration of ROS produced by NADPH-oxidase in the membrane without cell death response. This is not seen in *O. dasysiphoniae*,

the whole plant is completely consumed by the pathogen with cell death, suggesting that cell death is not necessary for an effective immune response, and also that *D. japonica* does not produce a SAR response against *O. dasyisiphoniae*. Future research should focus on the presence of SAR in other red algae diseases and the function of NADPH-oxidase generated ROS as a signaling molecule in disease defense.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained within this article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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