

Study of Functional Verification to Abiotic Stress through Antioxidant Gene Transformation of *Pyropia yezoensis* (Bangiales, Rhodophyta) APX and MnSOD in *Chlamydomonas*

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Seaweeds produce antioxidants to counteract environmental stresses, and these antioxidant genes are regarded as important defense strategies for marine algae. In this study, the expression of *Pyropia yezoensis* (Bangiales, Rhodophyta) ascorbate peroxidase (*PyAPX*) and manganese-superoxide dismutase (*PyMnSOD*) was examined by qRT-PCR in *P. yezoensis* blades under abiotic stress conditions. Furthermore, the functional relevance of these genes was explored by overexpressing them in *Chlamydomonas*. A comparison of the different expression levels of *PyAPX* and *PyMnSOD* after exposure to each stress revealed that both genes were induced by high salt and UVB exposure, being increased approximately 3-fold after 12 h. The expression of the *PyAPX* and *PyMnSOD* genes also increased following exposure to H₂O₂. When these two genes were overexpressed in *Chlamydomonas*, the cells had a higher growth rate than control cells under conditions of hydrogen peroxide-induced oxidative stress, increased salinity, and UV exposure. These data suggest that *Chlamydomonas* is a suitable model for studying the function of stress genes, and that *PyAPX* and *PyMnSOD* genes are involved in the adaptation and defense against stresses that alter metabolism.

Keywords: *Pyropia yezoensis*, ascorbate peroxidase, MnSOD, *Chlamydomonas reinhardtii*, transformation, abiotic stress tolerance

Introduction

Red algae (Rhodophyta), including the genus *Pyropia*, are the oldest lineage of photosynthetic eukaryotes [1]. *Pyropia* grows in the intertidal zone, an environment that undergoes dynamic changes, and so accordingly *Pyropia* is exposed to wide variations in temperature, osmotic strength, and light [2]. These environmental stresses result in the accumulation of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), singlet oxygen (O₂[·]), and hydroxyl radicals (OH[·]) [3]. The ROS generated through these environmental stresses inhibit the growth of algae by interfering with integral metabolic processes.

In both algae and higher plants, antioxidant systems, such as the enzymes ascorbate peroxidase (APX) and

superoxide dismutase (SOD), are known to be the principal protective mechanisms against oxidative stress [3–6]. In order to scavenge ROS, APX catalyzes the conversion of H₂O₂ to H₂O and O₂ within the chloroplast and cytosol of photosynthetic cells using ascorbate as the specific electron donor [7, 8]. The SOD enzyme family converts the superoxide radicals to H₂O₂ and oxygen. The SOD family members are classified according to the metal species that is present in their redox active centers. Generally, SODs are divided into three classes: MnSOD is found in the cytosol and the thylakoid membrane, Fe- and NiSODs are found in the cytosol, and Cu/ZnSODs are found in the periplasm [9, 10]. APX and SODs are therefore important enzymes in protecting against oxidative damage.

Studies examining the relationship between environmental

stress resistance and increased antioxidants have been conducted in higher plants [11–14] as well as in algae [15–21]. In addition, the mechanisms of action of antioxidant enzymes have been elucidated following treatment with various abiotic stresses in *Pyropia* [22–24]. The search for stress-related genes is crucial to elucidating adaptive mechanisms under the stresses of plants or algae.

The unicellular green alga *Chlamydomonas reinhardtii* has been used extensively as a model system for understanding numerous biological processes in eukaryotes at the molecular level [25–30]. Moreover, the full sequences of the *Chlamydomonas* genome, its plasmids, and its mitochondrial genome are available and the organism is amenable to genetic manipulation by transformation [31–33].

Recently, studies using transformation have been conducted in *Chlamydomonas* to characterize the function of the heat shock proteins (HSPs) from *Porphyra seriata* and *Pyropia tenera*, [34–36]. Similarly, the role of cyclophilins in protein folding, symbiosis, and plant responses to abiotic stress have also been examined following their transformation into *Chlamydomonas* [37]. *Chlamydomonas* species are photoautotrophs that have advantages over yeast for elucidating the functional relevance of genes that respond to plant and alga stress [38]. However, little research has been performed aimed at characterizing the biological and molecular mechanisms of stress responses following the transformation of *Pyropia yezoensis* antioxidant genes into *Chlamydomonas*.

In this study, the expression pattern of antioxidant genes of *P. yezoensis* (*PyAPX* and *PyMnSOD*) was analyzed following abiotic stress treatment. In addition, we examined the functional role of *PyAPX* and *PyMnSOD* following their expression in *Chlamydomonas*.

Materials and Methods

Sample Culture and Experimental Conditions

Gametophytes of *P. yezoensis* were provided by the Seaweed Research Center at Mokpo, Korea. Blades were cultured in modified Grund medium under light intensity of 80 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 10 h light/14 h dark cycle at 12°C in a growth chamber. These cultures were continuously aerated with filter-sterilized air, and the medium was renewed weekly. Subsequent experiments were performed using the gametophyte blade cultured at 1 month.

H₂O₂ and NaCl were used to examine the effect of stress on gene expression using a slight modification of the method described by Jin et al. [36]. *P. yezoensis* blades were placed in a 200-ml flask and treated with 1 mM H₂O₂ or 100 mM NaCl, and each treated flask was maintained under stationary culture conditions at 12°C for 0 to 12 h. To induce UV stress, healthy blades were selected and

transferred to a 200-ml flask and irradiated with UVB (285 nm; 0.26 W/m²) at 12°C for 6 or 12 h. All samples were extracted immediately before or after treatment with the abiotic stresses.

The GeneArt *Chlamydomonas* TOPO Engineering Kit contains the components of the *C. reinhardtii* c137 strain (Invitrogen, USA). Cells were grown in modified Tris-acetate-phosphate (TAP) medium (15.4 mM Tris-HCl, 7.0 mM NH₄Cl, 830 mM MgSO₄, 337 mM CaCl₂, 490 mM K₂HPO₄, 400 mM KH₂PO₄, 17.4 mM acetic acid, and Hutner's trace elements, pH 7.0). *Chlamydomonas* cells were cultured at 25°C in liquid TAP medium, with shaking at 120 rpm under a continuous cool fluorescent light (50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Gene Expression Analysis

Total RNA extraction from leafy gametophyte thalli was performed using an RNeasy Plant Mini kit (Qiagen, USA). First-strand cDNA was constructed from 2 μg of total RNA via reverse transcription in a 20 μl reaction volume using an oligo(dT)17 primer, and Superscript II reverse transcriptase, in accordance with the manufacturer's instructions (BRL Life Technologies, USA). The reaction was carried out for 60 min at 52°C, followed by 5 min of heating at 70°C. The first-strand cDNA reaction mixture was diluted 5-fold, and then 2 μl of the diluted cDNA was added to a 50- μl PCR mixture containing 5 μl of 10 \times PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1 μl of 10 mM dNTPs, 1 μl of each gene-specific primer (10 μM), and 2.5 units of ExTaq DNA polymerase (Takara, Japan). PCRs were conducted for 35 cycles, each consisting of 30 sec at 95°C, 30 sec at 60–64°C, and 90 sec at 72°C, and 7 min of termination at 72°C. The APX and MnSOD gene-specific primers from NCBI were as follows: *PyAPX* (Accession No. AY282755.1); *PyMnSOD* (Accession No. DQ146477.2); *Py18S* (sAccession No. D79976.2); and transgenic primers were prepared and used in the experiment (Table 1). The PCR products were separated by electrophoresis on 1% agarose gels.

All qRT-PCRs were performed using the following amplification program: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C, 58°C, and 95°C for 15 sec. All real-time PCRs were performed under the following conditions: 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, and 30 sec at 60°C. The specificity of the PCR products was confirmed by analyzing their dissociation curves at the end of each reaction (15 sec at 95°C, 1 min at 60°C, and 15 sec at 95°C). The reaction mixture (20 μl) contained 10 μl of SYBR Premix Ex Taq GC (TaKaRa Bio, Japan), 0.8 μl of each forward and reverse primer (5 μM), 0.4 μl of ROX Reference Dye, and 4 μl of cDNA template (50-fold dilution). The actin gene was used as an internal control to standardize the amount of mRNA in each reaction. The levels of the *Py18S* mRNA were calculated on the basis of a standard curve. All experiments were conducted in triplicate. The relative expression level was calculated as a ratio to the transcription level at 0 h after stress treatment.

Genomic DNA was extracted using a PureLink Genomic DNA Mini Kit (Invitrogen, USA) using 100 ml of cultured *Chlamydomonas*. The extracted genomic DNA was used to confirm the insertion of *PyAPX* and *PyMnSOD* into the *Chlamydomonas* genome using

Table 1. Primers used in the present study.

Primer name	(5'-3') Nucleotide sequence	Target
<i>PyAPX</i> qRT-PCR F	ATCAGCTACGGGACCTCTA	qRT-PCR
<i>PyAPX</i> qRT-PCR R	AAGCCCATCCGGTAAAAGAT	
<i>PyMnSOD</i> qRT-PCR F	GCTGTTTCATCAGCAGCACTC	
<i>PyMnSOD</i> qRT-PCR R	ACGTCAAAGTCGACGGTCTT	
18S rRNA F	CGACCGTTTACTGTGAAG	
18S rRNA R	GACAATGAAATACGAATGCC	
TOPO- <i>PyAPX</i> F	CACCGTGTCTGACCTGGAGAAGGCGGTCCGCGCC	Transformation
TOPO- <i>PyAPX</i> R	TCACGCCCAAACCGCGCCCAACTCACTCA	
TOPO- <i>PyMnSOD</i> F	CACCGCGTTTGTCTGCCCCCCTGCCCTATGCG	
TOPO- <i>PyMnSOD</i> R	TTACACCAGCGCGGTGTCAAAGGCAGGCAG	
<i>CrActin</i> F	TGTGCATACGTGGATAGCTTG	
<i>CrActin</i> R	ATGACCCGCTCCTCATATCTT	

specific PCR primers. *Chlamydomonas* actin (Accession No. D50838.1) was used as an internal control gene.

Plasmid Construction and *Chlamydomonas* Transformation

The PCR products were subcloned into the pChlamy_1/D-TOPO vector (Invitrogen, USA) using the GeneArt *Chlamydomonas* TOPO Engineering Kit according to the manufacturer's protocol. The pChlamy_1/D-TOPO vector harbors regulatory sequences with the *psaD* promoter and a terminator driven by the *Chlamydomonas* c137 genome for transgene expression. Additionally, it possesses a hygromycin resistance gene (*aph7*) under the control of the *tubB2* promoter and the Hsp70A-RbcS2 terminator for the selection of transformants. The open reading frames of the *PyAPX* and *PyMnSOD* genes were amplified by PCR and subcloned into the pChlamy_1/D-TOPO vector using the KpnI and BglII sites. To transform the DNA into *Chlamydomonas* c137, the *PyAPX* and *PyMnSOD* plasmids were linearized with ScaI, and the cells were electroporated using the Gene Pulser Xcell (Bio-Rad, USA). The electroporation parameters were as follows: 600 V, 50 μ F. After 2 weeks of growth on 24-well plates, the transformed colonies were selected on TAP agar medium containing 10 μ g/ml hygromycin (Invitrogen, USA).

Abiotic Stress and Irradiation Tolerance in Transformed *Chlamydomonas*

Transformed *Chlamydomonas* cells were grown in TAP medium containing hygromycin at 25°C under a 14 h light/10 h dark cycle. To investigate oxidative and osmotic tolerances, agar plates supplemented with 0, 2.5, 5, 7, and 10 mM H₂O₂ or 0, 10, 25, 50, and 100 mM NaCl were prepared. We then diluted 2–4 $\times 10^6$ of the initial cell concentration with fresh medium to reach a final concentration of 10¹–10⁵. Ten microliters of the diluted cells was inoculated onto the agar plates and cultured for 1 week. *C. reinhardtii* c137 and cells transformed with the pChlamy_1/D-TOPO vector were used as controls.

In order to measure the UV resistance of the transformed *Chlamydomonas*, cells were diluted to 10¹–10⁵ in fresh medium, and 10 μ l of the diluted cell suspension was inoculated onto agar plates, after which they were exposed to UV doses of 500, 1,000, 1,500, and 2,000 J (Ultraviolet Crosslinker CL-1000L; LLC, USA). The survival rate was then measured after transfer of the cells to a 25°C growth chamber and culture for 1 week with a 14 h light/10 h dark photocycle.

Results

Expression of Antioxidant Genes in Response to Abiotic Stresses

We synthesized *PyAPX* and *PyMnSOD* gene-specific primers (Table 1) and analyzed the expression of these antioxidant genes after exposure of *P. yezoensis* blades to abiotic stress. As shown in Fig. 1A, the expression of these two antioxidant genes increased following exposure to oxidative stress. We found that within 3 h of H₂O₂ treatment, the expression levels of *PyAPX* were significantly increased, whereas the expression levels of *PyMnSOD* were only slightly increased. However, after 6 h of treatment, the expression of *PyMnSOD* had increased to levels similar to those seen for *PyAPX*; however the expression levels of both genes declined by 12 h.

Following NaCl treatment, the expression of these two antioxidant genes showed a similar pattern to that seen for H₂O₂ treatment. Both the *PyAPX* and *PyMnSOD* genes showed a gradual increase in their expression following NaCl treatment up to 3 h, with *PyMnSOD* expression being maintained at this level for up to 12 h, while the levels of *PyAPX* declined slightly (Fig. 1B).

Fig. 1C shows that the expression levels of the *PyAPX*

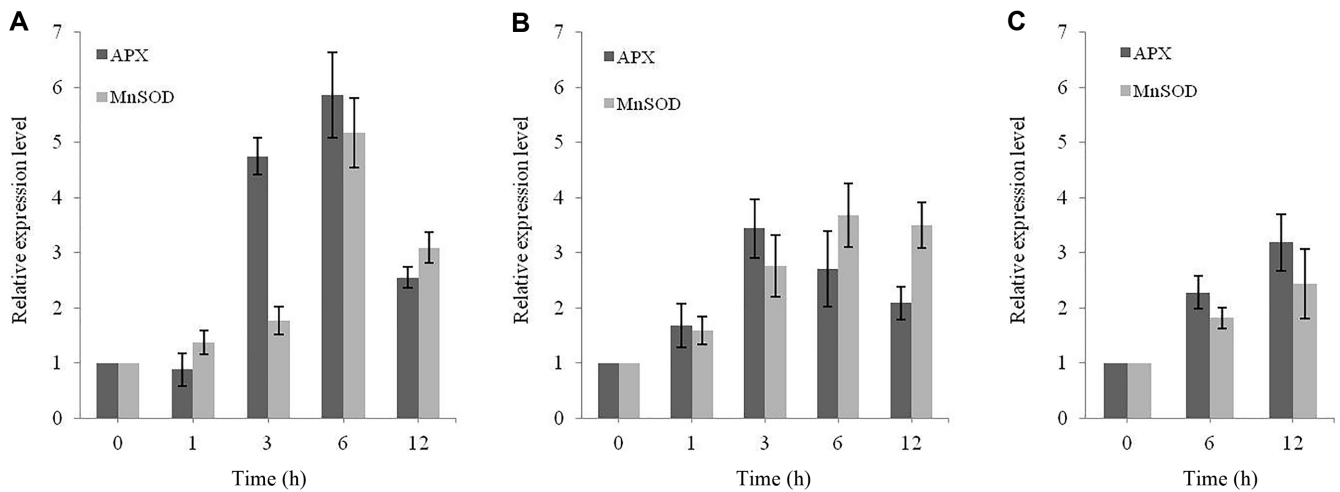


Fig. 1. Relative expression levels of *PyAPX* and *PyMnSOD* under abiotic stresses in *P. yezoensis*.

Gametophyte blades were harvested at 0, 1, 3, 6, and 12 h to determine the transcript levels of *PyAPX* and *PyMnSOD* following exposure to either H₂O₂ and increased salinity. (A) Relative expression levels of *PyAPX* and *PyMnSOD* following H₂O₂ stress. (B) Relative expression levels of *PyAPX* and *PyMnSOD* following exposure to increased salinity. (C) Relative expression levels of *PyAPX* and *PyMnSOD* following exposure to UVB. *Py18S* RNA was used as the internal control gene. Bars represent the mean \pm standard deviation.

and *PyMnSOD* genes were elevated following UVB exposure. The expression of both genes increased immediately following exposure to UVB stress, with an approximately 2-fold increase in expression occurring at 6 h. The levels of *PyAPX* and *PyMnSOD* expression further increased to approximately 3-fold after 12 h of UVB exposure.

This consumption by antioxidant genes could presumably minimize oxidative damage of the cells by removing ROS when exposed to stress conditions.

Oxidative and Osmotic Tolerance of Transformed *Chlamydomonas*

Chlamydomonas transformed with vectors encoding *PyAPX* and *PyMnSOD* were selected on a hygromycin-containing medium, and the successful introduction of the *PyAPX* and *PyMnSOD* genes was confirmed by RT-PCR and qRT-PCR (Fig. 2). We isolated one hygromycin-resistant *Chlamydomonas* clone following transformation with a plasmid encoding the *PyAPX* gene, and confirmed the successful expression of the *PyAPX* gene by RT-PCR (Figs. 2A and 2B). In the case of *PyMnSOD*, we obtained four independent hygromycin-resistant clones. The clone *PyMnSOD_2* showed the highest level of *PyMnSOD* expression compared with the other three transformed lines (Figs. 2C and 2D). For this reason, we elected to study the effect of abiotic stress on this *PyMnSOD_2* transformed *Chlamydomonas* line. Fig. 2E shows PCR confirmation of the successful insertion of the *PyAPX* and *PyMnSOD* genes

into the *Chlamydomonas* genome.

To assess salt tolerance, the *Chlamydomonas* strains expressing *PyAPX* or *PyMnSOD* were cultured on agar plates treated with different concentrations of NaCl, with *Chlamydomonas* strain c137 and vector-only transformed *Chlamydomonas* as controls. All *Chlamydomonas* cells were observed to grow at similar levels on agar plates at 10 and 25 mM NaCl. However, when cells were grown on plates at 50 mM NaCl, the *Chlamydomonas* expressing either *PyAPX* or *PyMnSOD* exhibited better growth than *Chlamydomonas* c137 or *Chlamydomonas* transfected with an empty vector (Fig. 3A).

Growth was also compared on plates supplemented with 1–10 mM H₂O₂ to assess tolerance to oxidative stress. All cells exhibited similar growth at 2.5 and 5 mM H₂O₂, but interestingly, at a concentration of 7.5 mM H₂O₂, only the transformed *Chlamydomonas* cells survived. Based on these data, the transformed *Chlamydomonas* cells proved to be more resistant to salt or oxidative stress than the control *Chlamydomonas* cells (Fig. 3B). In order to measure the UV resistance of the transformed *Chlamydomonas* following UV treatment, the cells were inoculated onto agar plates and exposed to various UV doses. The control and transformed *Chlamydomonas* cells grew at similar rates after exposure to UV doses of 0 and 500 J/plate, but at higher UV intensities (1,000 and 1,500 J), the transformed *Chlamydomonas* showed greater survival than the control cells (Fig. 3C). However, neither the control nor the transformed *Chlamydomonas* cell

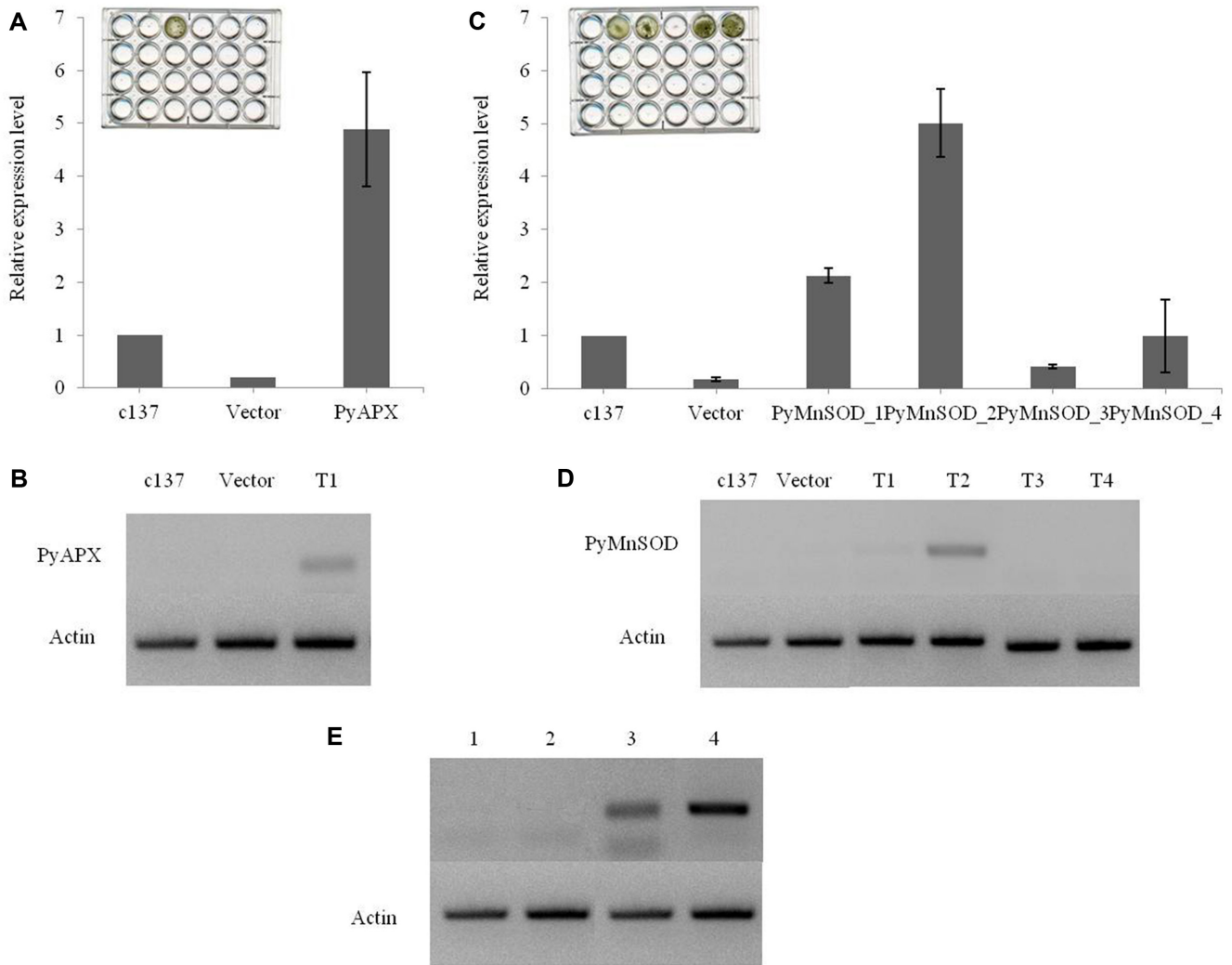


Fig. 2. Verification of the introduction and expression of the *PyAPX* and *PyMnSOD* genes in *Chlamydomonas*. cDNA was extracted from the indicated transformed *Chlamydomonas* cells and amplified with either *PyAPX* or *PyMnSOD* specific primers, as appropriate. (A, B) Confirmation of the introduction of the *PyAPX* gene in one clone by RT-PCR. (C, D) Confirmation of the introduction of the *PyMnSOD* gene in four clones by RT-PCR. (E) PCR confirmation of gene introduction into transformed *Chlamydomonas* cells, using genomic DNA. 1. *Chlamydomonas* c137; 2. Confirmation of empty TOPO_vector introduced into *Chlamydomonas*; 3. Confirmation of *PyAPX* gene introduced into *Chlamydomonas*; 4. Confirmation of *PyMnSOD* gene introduced into *Chlamydomonas*.

lines were detected on the culture plates after exposure to 2,000 J of UV radiation.

These results suggest that the *PyAPX* and *PyMnSOD* genes contribute to abiotic stress tolerance in *Chlamydomonas*.

Discussion

Pyropia species inhabiting the intertidal zone are inevitably exposed to high temperatures, UV radiation, high salinity, and oxidative stress. These stressors can inhibit growth, photosynthesis, and pigment synthesis, and

cause DNA damage. They also cause the accumulation of intracellular ROS, leading to mitochondrial changes and damage to chloroplasts and phycobilisomes [39–42]. In the case of macroalgae, they respond to stress by increasing the expression of antioxidant defense enzymes such as the ROS scavenging enzymes APX and SOD, and these enzymes have an important role in effectively eliminating the stress-induced ROS [43–45]. In *Ulva fasciata*, antioxidant genes and enzymes have been found to be overexpressed under high salt stress conditions, suggesting that they are important in stress response [46]. In addition, it has been

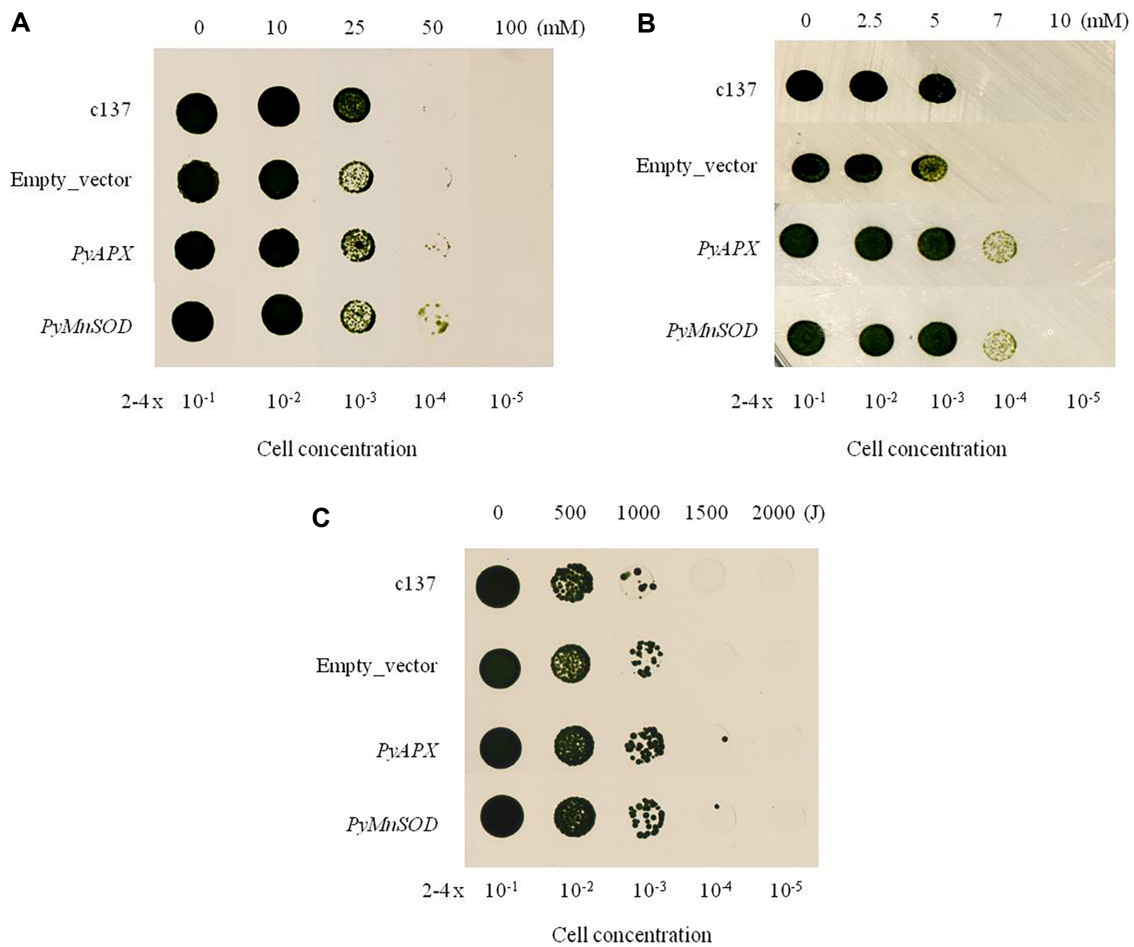


Fig. 3. Survival and growth pattern of transformed *Chlamydomonas* cells under H₂O₂ or salt stress conditions.

(A) Cells were inoculated on agar plates prepared by supplementation with 0, 10, 25, 50, or 100 mM NaCl, and cultured for 1 week. (B) Cells were inoculated on agar plates prepared by supplementation with 0, 2.5, 5, 7, or 10 mM H₂O₂, and cultured for 1 week. (C) Survival and growth pattern of transformed *Chlamydomonas* cells under UV stress conditions. Cells were inoculated on the agar plate and irradiated with UV at doses of 0, 500, 1,000, 1,500, or 2,000 J UVB, followed by incubation for 1 week. *Chlamydomonas* strain c137 and empty TOPO_vector-transformed cells were used as controls.

reported that treatment of cells with H₂O₂ can upregulate the expression of genes involved in antioxidative defense [47, 48]. Our study showed that the *P. yezoensis* antioxidant genes *PyAPX* and *PyMnSOD* are overexpressed when exposed to H₂O₂, salinity, and UVB stress.

Other stress studies have shown that the activities of antioxidant enzymes are significantly increased in response to H₂O₂, superoxide anions, and carbonyl content under desiccation stress condition in *Porphyra columbina* [22]. We suggest that overexpression of these antioxidant genes occurs in response to various stresses, and this presumably is a defense mechanism that effectively reduces the intracellular ROS levels.

Chlamydomonas is a suitable model system to study the

abiotic stress responses following transformation [38]. For *Pyropia* sp., previous studies have been conducted to identify and characterize genes using *Chlamydomonas* [34, 35, 39, 50]. Among these, it has been shown that transformation of *Chlamydomonas* with the HSP chaperone protein increased both resistance and survival rates under a variety of abiotic stress conditions.

The *APX* and *MnSOD* genes are important key components that contribute to cell homeostasis under a variety of stress conditions. Our results confirm that *Chlamydomonas* cells transformed with *PyAPX* and *PyMnSOD* genes had higher resistance than normal cells to abiotic stresses. It is thought that these genes are heterogeneously expressed in the *Chlamydomonas* cells and act as an additional stress defense

factor.

Our study has shown the response of *P. yezoensis* antioxidant genes under abiotic stress conditions and their biological functions following their transformation into *Chlamydomonas*. We conclude that the overexpression of the *PyAPX* and *PyMnSOD* genes that occurs following abiotic stress is an important defense mechanism in *P. yezoensis*. Furthermore, the introduction of genes encoding *PyAPX* and *PyMnSOD* into *Chlamydomonas* may play a role in the constitutive expression in cells and supporting essential resistance factors for abiotic stresses. We have demonstrated that the *PyAPX* and *PyMnSOD* genes are closely related to stress tolerance and suggest that the transformation system can be used to identify various physiological and molecular functions of *Pyropia*.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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