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Genetic Modification of the *HSP90* Gene Using CRISPR-Cas9 to Enhance Thermotolerance in *T. Suecica*

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Abstract: Phytoplankton are marine microorganisms that play a key role in the production of oxygen and serve as the foundation of the marine food chain. Over the past century, the population of phytoplankton has declined significantly with the onset of climate change. Although phytoplankton have the capacity to adapt to rising ocean temperatures, rapid environmental changes, including increased top-down control and thermal stratification, reduce populations before adaptations are incorporated into the genome. To enhance survival rates, thermotolerance in common algal strains can be enhanced through increased expression of the conserved Heat Shock Protein 90 (*HSP90*). Trials will be conducted on the common algal species, *Tetraselmis suecica (T. suecica)*, for its considerable size, photosynthetic rate, and nutrient-rich properties. Thermotolerance will be augmented by splicing the *HSP90* gene into the *T. suecica* metallothionein (*Mt*) promoter using CRISPR-Cas9. A period of incubation in a copper sulphate solution ensures *Mt* promoter stimulation, thereby increasing *HSP90* expression. The efficacy of the proposed methods will be measured by comparing HSP90 protein production between transgenic and wild-type *T. suecica* cultures. The genomic incorporation of the modified *HSP90* gene enables future populations to exhibit thermotolerance in the presence of heavy metals in the ocean beyond its basal level of expression. By accelerating the adaptation of thermotolerance, the overall fitness of *T. suecica* can be increased to re-establish its population under warmer oceanic conditions. By applying similar methods to other phytoplankton, the repopulation of various species can increase biodiversity and global net primary productivity.

Keywords: Heat Shock Protein 90 (*HSP90*); metallothionein (*Mt*); phytoplankton; climate change; CRISPR-Cas 9; thermotolerance

Introduction

Phytoplankton produce 70% of the world's oxygen, despite accounting for 1% of global photosynthetic biomass [1-3]. As a food source for numerous aquatic species, this producer serves a foundational role in the marine food web [4]. Although phytoplankton are capable of mitigating the effects of climate change by fixing between 30 to 50 billion metric tons of carbon each year, climate change has increased oceanic temperatures beyond the typical living conditions of phytoplankton [1,5]. This has resulted in a 40% population decline since 1950 [6-7]. In response to climate change, species are able to modify their reproductive habits and geographical dispersion. Thus, phytoplankton can withstand incremental increases in temperature [8]. However, it is predicted that organisms would not be able to adapt rapidly enough to resist the effects of climate change, notably elevated levels of top-down control and thermal stratification [8]. Besides rising oceanic temperatures, increased top-down control further amplifies the predation of primary producers, resulting in the decreased accumulation of biomass. This effect may significantly decreasing the phytoplankton population, preventing the inheritance of genetic mutations that allow for thermal resistance [9]. These marine organisms must also overcome the environmental challenge of thermal stratification, which involves the division of lakes into horizontal layers based on their temperature. Increased thermal stratification has been linked to a decrease in phytoplankton population [8,10].

Within 80 years, the average global ocean surface temperature is estimated to increase by 4°C [11-13]. Certain species of phytoplankton, such as *Desmodesmus armatus*, are capable of tolerating warmer water temperatures due to an intrinsically high expression of the Heat Shock Protein 90 (*HSP90*) gene [14]. Under stressful conditions, such as increases in temperature, HSP90 chaperone proteins are produced to assist in protein folding [15-17]. This prevents the thermal denaturation of proteins to maintain homeostasis, which improves the thermotolerance of the organism [15-16]. Other common species, notably *Tetraselmis suecica*, optimally function in temperatures of approximately 18.0°C [18-20]. This phytoplankton will need

to adapt to the onset of warmer conditions in the upcoming decades by becoming more thermotolerant [21]. Since the *HSP90* gene is naturally expressed in *T. suecica*, the adaptation of heat tolerance can be accelerated by genetically modifying this species to increase *HSP90* expression [21-22]. With potential mutation rates between 1.1 to 2.5%, CRISPR-Cas9 is an efficient and cost-effective gene-editing tool, which has been used multiple times to edit algal genes in the past [4,23-25]. CRISPR-Cas9 has two major components that allow it to splice genes: single guide RNA (sgRNA), which directs the Cas-9 enzyme to the correct location on the genome and the Cas-9 enzyme, which splices the gene to allow foreign DNA to be inserted [23].

Methods

To enhance the thermotolerance of *T. suecica*, CRISPR-Cas9 will be used to cut the *HSP90* gene and place it next to the *Mt* promoter [26]. The *HSP90* gene is a part of a plasmid, called *HSP90 HA*, which will be purchased from a commercial vendor. Under normal conditions, the *Mt* promoter transcribes the conserved metallothionein 1A (*Mt1A*) gene [27-28]. However, CRISPR-Cas9 will be used to replace the *Mt1A* gene with the *HSP90 HA* plasmid, thereby activating the *Mt* promoter to produce *HSP90* [29].

Due to the toxicity of Cas9 to microalgae, Cas9 ribonucleoproteins (RNPs) will be used to prevent cell death, as they eventually degrade within the cell [30-31]. As this complex is comprised of the Cas9 protein and sgRNA without a vector, gradual degradation is enabled [32]. A Cas-9 RNP is created through the incubation of the Cas-9 protein with sgRNA. The sgRNA that targets the *Mt1A* gene and the Cas9 protein will be purchased from manufacturers, such as ToolGen, Inc [32]. The Cas-9 RNPs will be created through the incubation of 10 µg of sgRNA and 7.5 µg of Cas9 protein at 37°C for 30 minutes [32].

To ensure the successful incorporation of the *HSP90* gene into microalgal DNA, a traceable marker will be inserted. By splicing *HSP90* into a plasmid that codes for antibiotic resistance, the insertion of this plasmid can be tested by treating the host with antibiotics [33]. This is accomplished with a plasmid that contains the code for zeocin antibiotic resistance, *pMOD-zeo*, which will be ligated to the *HSP90 HA* plasmid using the restriction endonucleases NcoI-Styl and BseRI [34-35]. Using polymerase chain reaction (PCR) at 96°C, this mutant plasmid will be linearized to create free ends that can bind to *T. suecica* DNA.

The *T. suecica* cells will be placed in 6-well plates and 250 V will be applied for electroporation [31]. This technique will increase the permeability of cell membranes by placing cells in an electric field [36]. The cells will be transfected with 2.0 µg Cas-9 RNP and 0.4 µg of the linear *HSP90 HA* plasmid [37].

These cells will then be moved onto agar plates where they will be incubated in 100 µg/ml of zeocin solution for 16 hours [38]. The surviving cells will contain the *pMOD-zeo* gene and thus the *HSP90* gene. These transgenic cells will be referred to as *Mt90* cells, as they contain the *HSP90* gene under the control of the *Mt* promoter. *Mt90* cells express the *HSP90* gene when initiated by the *Mt* promoter.

To test the increased thermotolerance of the transgenic phytoplankton, 50 µL samples of the modified cells and wild-type cells will be distributed to 96-well microplates as the experimental and control groups [39]. These wells will be incubated with a 200 µM solution of copper sulphate for two hours, followed by a heat-shock of 36°C to allow for the distribution of *HSP90* [40].

Thermogravimetric analysis (TGA) will test the effects of genetic modification on thermotolerance by measuring sample mass difference over a temperature range of 25°C to 45°C. As increasing mass loss corresponds to population reduction, temperatures with minimal mass loss indicate the optimal temperature range for *Mt90 T. suecica* strains [41].

Results

The insertion of the metallothionein-controlled *HSP90* gene into *T. suecica* cells is expected to increase *HSP90* protein production to induce an increase of thermotolerance up to 4°C. As one of the most photosynthetically productive organisms, phytoplankton play a vital role in greenhouse gas uptake and oxygen production [42]. Hence, phytoplankton populations must be maintained at an appropriate size for the long-term amelioration of climate change [43].

Discussion

In a lab setting, experimenters have exquisite control over phytoplankton development with minimal ethical and financial challenges [44]. As a result, laboratory testing will be both feasible and accessible for researchers.

T. suecica was chosen as a model organism for its nutritional content, photosynthetic, and reproduction rates [45]. As most organisms possess the conserved *HSP90* gene, similar results may also be recreated in other phytoplankton species.

The thermotolerance of *T. suecica* will be increased by inserting the *HSP90* gene in the place of the *Mt1A* gene to be inducible by the *Mt* promoter [40]. This technique will initiate gene expression in the presence of heavy metals. CRISPR-Cas9 genetic modification techniques will be used to splice the *HSP90* and *Mt1A* genes together, as this process is able to target specific eukaryotic DNA sequences with a low error rate [40].

Although no studies to date have performed CRISPR-Cas9 gene editing on *T. suecica*, researchers have used this technology on similar microalgae, including *Phaeodactylum tricornutum* [46]. Researchers were able to locate target genes using guide RNAs and insert foreign DNA using the Cas9 complex. As well, the investigators successfully tested for the uptake of the inserted genes, demonstrating that this genetic editing technique can be employed in microalgae [46]. Subsequently, genetic editing in *T. Suecica* is expected to

have similar results to the aforementioned studies, allowing for the increased expression of HSP90 in this species.

Copper is an effective inducer of the *Mt* gene with high levels of soft-tissue uptake. Therefore, this metal will be used to initiate *Mt90* gene expression [47]. However, other heavy metals, including arsenic, cadmium, and lead are also able to induce the expression of *Mt90* [48].

Research has shown that the aquatic copper concentration required to induce the expression of the *Mt* gene is 5.00 μg/L and several studies have measured concentrations above this level in the Pacific Ocean [49-51]. Thus, the recombinant *Mt90* will be expressed in transgenic *T. suecica* in their natural habitat.

The conducted trials may potentially induce thermotolerance by genetically incorporating the modified DNA in future *T. suecica* populations. However, the proliferation of modified phytoplankton must be measured in a laboratory setting prior to their introduction in ecosystems. For these trials, algal species will be incubated through photobioreactors to grow sample colonies [49]. As well, statistical analyses should be performed to estimate the effects of introducing this modified strain to oceanic environments [52]. After comprehensive studies and appropriate alterations, closed-system segments within smaller bodies of water must be designated to simulate the performance of modified *T. suecica* in marine environments [52-53]. Each trial must be performed with caution to minimize biological consequences in ecosystems, such as competition with native species [49]. Depending on the stage of testing, adverse effects resulting from the introduction of the modified *T. suecica* strain must be alleviated through the consideration of additional variables [52]. With the introduction of thermotolerance into the gene pool, algae reproductivity can gradually be re-established to increase oceanic photosynthetic rates.

Conclusion

The proposed study will determine the thermotolerant capability of genetically modified *T. suecica*. Additional lab testing will be required to observe the impact of these genetically modified phytoplankton on the marine biosphere. With the development of thermoresistant phytoplankton, this study has the potential to initiate a feasible and effective solution to mitigate the consequences of climate change.

List of Abbreviations used

Mt: Metallothionein
HSP90: Heat Shock Protein 90
sgRNA: Single guide ribonucleic acid
DNA: Deoxyribonucleic acid
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
URNCST: Undergraduate Research in Natural and Clinical Science and Technology
Mt1A: Metallothionein 1A
PCR: Polymerase chain reaction
TGA: Thermogravimetric analysis

Conflicts of Interest

All article types: The author(s) declare that they have no conflict of interests

Ethics Approval and/or Participant Consent

This research protocol did not require ethics approval and/or participant consent.

Authors' Contributions

JX: Made substantial contributions to the design of the study, collected and analysed data, drafted the manuscript, revised the manuscript critically and gave final approval of the version to be published.
VS: Contributed to study design and planning, created the methodology and analysis of data, revised the manuscript critically and gave final approval of the version to be published.
MC: Made contributions to the design of the study, the collection of data and an analysis of the data, revised the manuscript critically, and gave final approval of the version to be published.
OC: Contributed to study design and planning, the interpretation and analysis of collected data, revised the manuscript critically and gave final approval of the version to be published.

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