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# Characterization of a novel glycerol-3-phosphate dehydrogenase (GPD2) in the alga *Chlamydomonas reinhardtii*

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# CHARACTERIZATION OF A NOVEL GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPD2) IN THE ALGA *CHLAMYDOMONAS REINHARDTII*

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The green alga *Chlamydomonas reinhardtii*, like many eukaryotic microalgae, accumulates triacylglycerol (TAG) under certain environmental stresses, such as nitrogen deprivation. TAG is of interest because it is an essential precursor for biofuel production. Canonical glycerol-3-phosphate dehydrogenases catalyze the synthesis of glycerol-3-phosphate (G3P), a key precursor for glycerolipid and TAG synthesis in eukaryotes. The *C. reinhardtii* genome encodes six GPD homologs. Interestingly, GPD2 is a novel multidomain enzyme, consisting of a phosphatase motif fused to a G3P dehydrogenase domain. GPD2 expression is significantly up-regulated under nutrient deprivation or high salinity, coincidental with the accumulation of TAG or glycerol. Conversely, RNA-mediated silencing of GPD2 reduces TAG and glycerol production under the same stresses. Based on these observations, we hypothesize that GPD2 contributes to the synthesis of both glycerol and TAG, depending on the environmental conditions. Thus, our goals are to decipher how the enzymatic activities (i.e., dehydrogenase and phosphatase) of GPD2 are regulated under different environmental conditions. Understanding how cells regulate GPD2 enzymatic activities, and what other components GPD2 may interact with, may contribute to broadening our biochemical and cytological understanding of algal TAG and glycerol metabolic pathways, with possible implications for biotechnological biofuel/biomaterial production.

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# TABLE OF CONTENTS

Page

# LIST OF FIGURES

**Chapter 1**



**Chapter 2**

# Chapter 1

# Literature Review

# LITERATURE REVIEW

## Introduction

High soil salinity and droughts are severe issues affecting plants and crops worldwide that humanity will have to face in the coming decades. Salinity already limits the productivity of crops, with adverse effects on germination, plant resilience, and crop yield (Shanker and Venkateswarlu, 2011; Flowers and Flowers, 2005). For example, drought and salinity affect about one-third of cultivated land and cause a loss of approximately 1,500,000 ha agricultural land per year (Shinwari *et al.*, 2020). High soil salinity and droughts hamper plant growth and reduce plant development and survival by inducing water stress, ion toxicity, oxidative stress, alteration of metabolic processes, reduction of cell division and expansion, genotoxicity, among other problems (Shanker and Venkateswarlu, 2011; Wang *et al.*, 2018; Flowers and Flowers, 2005). Additionally, major biochemical processes such as photosynthesis, protein synthesis, and energy and lipid metabolism are affected (Shanker and Venkateswarlu, 2011). An excess of sodium and chloride has the potential to affect plant enzymes by interfering with enzyme activity and disrupting protein synthesis. They can cause toxicity symptoms, such as chlorosis and/or necrosis, cell swelling, resulting in reduced energy production and other physiological changes (Shanker and Venkateswarlu, 2011).

Understanding the molecular mechanisms of osmotic stress and high salinity response in plants is essential to develop salt-tolerant crops that can withstand worsening soil conditions. However, studies in plants are slow, costly, and time-consuming. Thus, similar studies in model organisms such as *Chlamydomonas reinhardtii*, may contribute to quickly improve our knowledge in osmotic stress response in plants (Tietel *et al.*,

2020). This knowledge could lead to the faster development of salt-resistant crops (Wang *et al.*, 2018) and to the advance of our understanding of cellular pathways and adaptations triggered by different metabolic stresses.

**Utilizing *Chlamydomonas reinhardtii* to study cell response to high salinity stress**

*C. reinhardtii* has been already well established as a model organism because this species of microalgae is relatively adaptable and has fast growth (doubling time 8-10 hours) (Driver *et al.*, 2017; Goodenough *et al.*, 2014). Its nuclear, mitochondrial, and chloroplastic genomes have been sequenced and published, and it is comparatively easy to genetically manipulate (Hallmann, 2007; Yan *et al.*, 2016). *C. reinhardtii* can be found in soil or freshwater, and it can metabolize $CO_2$ through photosynthesis or growth heterotrophically by using organic compounds such as acetate as carbon sources. *C. reinhardtii* has been used to study photosynthetic, physiological, and metabolic processes, structure and function of flagella, and it is a useful model to understand stress responses of photosynthetic organisms (Driver *et al.*, 2017; Goodenough *et al.*, 2014; Morales-Sánchez *et al.*, 2017). *C. reinhardtii* response and adaptation to high salinity stresses are comparable with the response of other microalgae. For instance, when placed under osmotic stress, both Dunaliella -another green alga- and *C. reinhardtii* produce glycerol as the preferred osmoprotectant. Likewise, under osmotic stress, similar genes are up-regulated in both Dunaliella and *C. reinhardtii* (Cai *et al.*, 2013; Oren, 2017). The genus Dunaliella, especially, has an extraordinary ability to survive and thrive under a wide range of salinity concentrations (Chen and Jiang, 2009). Thus, this genus has been studied, to some extent, to understand the metabolic reactions and changes that allow cells to persist under osmotic stress conditions. However, due to our current inability to

generate stable genetically modified Dunaliella mutants, *C. reinhardtii* is a more suitable option for studies focusing on stress responses and the genes and pathways involved.

Finally, glycerol -the accumulated osmoprotectant found in *C. reinhardtii* undergoing osmotic stress- is also the osmoregulatory compound found in yeast and other fungi (Hohmann, 2002). Functioning as both an osmoregulator and an osmoprotectant of enzymes under salt-stressed condition. These similarities suggest that studying the stress response mechanisms in microalgae like *C. reinhardtii* can lead to the application of findings in plants and other species.

**Quick overview of how *C. reinhardtii* adapt to osmotic stress**

Studies have shown that when *C. reinhardtii* is placed under osmotic stress, there is a shift in the carbon metabolism from sugar phosphate and starch synthesis to glycerol synthesis, which leads to an accumulation of glycerol (Miyasaka and Ikeda, 1997). Similar to Dunaliella, starch is used as the carbon source for glycerol synthesis during the initial periods of photosynthesis inhibition (Miyasaka and Ikeda, 1997; Chen and Jiang, 2009). In *C. reinhardtii* -depending on the salt concentration- after the initial osmotic shock, photosynthetic activity is momentarily suppressed, followed by a decrease in cell size, and lower growth rates. Then, a palmelloid morphology appears, and flagella reabsorption occurs (Wang *et al.*, 2018). However, photosynthesis and growth rates are restored after the cells have acclimated to the stress (León and Galván, 1999).

As a possible explanation to *C. reinhardtii* 's initial response to osmotic stress, Wang *et al*., 2018 have suggested that the observed photosynthesis inhibition could also hinder the transport of carbohydrates and, thus, lead to an accumulation of starch or sucrose. In response, *C. reinhardtii* may enhance glycolysis metabolism to decrease carbohydrate

accumulation in cells. This reaction would promote glycerol synthesis, the respiratory metabolism, and mitochondrial electron transport. Glycerol synthesis is essential because glycerol is the osmoregulatory compound required to maintain proper cell and protein activity. In the same manner, efficient ATP generation, reduced reactive oxygen species and mitochondrial osmolytes are important for salinity tolerance (Jacoby *et al.*, 2011); thus, the stimulation of the respiratory metabolism and the mitochondrial electron transport would help reducing the effects of ionic toxicity and osmotic stress caused by high salt concentrations.

An additional observed effect in *C. reinhardtii* is that osmotic stress triggers several phospholipid signaling pathways (Meijer *et al.*, 2017) and triacylglycerol (TAG) accumulation. . It is a possibility that excess glycerol is converted back to glycerol-3-phosphate, which consequently can be used for TAG synthesis. However, the roles of fatty acids (such as TAG) in the cell response to osmotic stress are not well understood. The detected activation of phospholipid signaling pathways, which leads to the accumulation of phosphatidic acid as well as lysophosphatidic acid, is similar to stress response in other organisms. For example, phosphatidic acid and lysophosphatidic acid are well-known secondary messengers in plant and animal systems that respond to water deficit (Wang *et al.*, 2018; Tietel *et al.*, 2020; Meijer *et al.*, 2017).

Under osmotic stress conditions, there is also an increased expression of a Glycerol-3-phosphate dehydrogenase (GPD) homolog: GPD2. The gene coding for GPD2 has been found to have up-regulated expression in both *C. reinhardtii* and Dunaliella during high salinity treatments (Wang *et al.*, 2018; Lee *et al.*, 2012; He *et al.*, 2020; Wu *et al.*, 2019); and more recently, a similar homolog was also identified in two

Antarctic Chlamydomonas species from a saline lake (Raymond *et al.*, 2020). This evidence suggests a conserved role for this enzyme in the glycerolipid pathway of microalgae. GPD is considered the rate-limiting enzyme that regulates the glycerol level following osmotic shock (Cai *et al.*, 2013; Oren, 2017) and is also an important enzyme for the synthesis of Glycerol-3-phosphate in the TAG synthesis pathway. However, further studies need to be done to fully understand the role(s) of these homologs in the osmotic response and glycerolipid pathways of *C. reinhardtii.*

**Glycerol-3-phosphate dehydrogenase (GPD)**

GPD is an enzyme that catalyzes the reversible conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (Glycerol-3-P), a TAG and glycerol precursor.

GPDs are found across the domains of life, and they serve as a link between carbohydrate and lipid metabolisms. GPD has several known functions, such as the catalysis of glycerol-3-P, a glycerol precursor; roles in lipid metabolism as an important enzyme for the synthesis of TAG precursors; and the maintaining of redox ($NADH/NAD^+$) potential. Additionally, if GPDs are deregulated, cell damage can occur. For example, in mammals, deregulation or improper functioning of GPD can lead to diseases such as obesity and diabetes (Liu *et al.*, 2018).

*Evolution of the GPD gene*

Due to its importance and conservation across the domains of life several phylogenetic analyses have been performed to gain insight into the evolution, origin, and functions of the several GPD isoforms found in living organisms. Studies have shown that GPD isoforms can be classified into two big classes, $FAD^+$-dependent GPD ($FAD^+$-

GPD) and NAD(P)$^+$-dependent GPD (NAD(P)$^+$-GPD). FAD$^+$-GPDs are usually located on the outer surface of the mitochondrial inner membrane and are linked to the respiratory chain (Wu *et al.*, 2019; Liu *et al.*, 2018; Wang *et al.*, 2018). It has been proposed that FAD$^+$-GPDs can form a Glycerol-3-P shuttle with cytosolic (NAD(P)$^+$-GPD to maintain homeostasis of the NADH/NAD$^+$ ratio (Wu *et al.*, 2019; Liu *et al.*, 2018). NAD(P)$^+$-GPD are usually located in the cytosol and chloroplast of the cell and, based on differential expression, it has been proposed that these isoforms may be needed to protect the cell against environmental stresses such as osmotic and oxidative stresses, and they are also involved in the lipid metabolism (Wu *et al.*, 2019; Raymond *et al.*, 2020; He *et al.*, 2020; Morales-Sánchez *et al.*, 2017). In *C. reinhardtii*, there are six identified GPD genes (Morales-Sánchez *et al.*, 2017). Five genes code for NAD(P)$^+$-GPDs and one gene codes for FAD$^+$-GPD. In *D. salinas,* seven GPD genes have been identified, five genes code for NAD(P)$^+$-GPDs and two genes code for FAD$^+$-GPD (Wu *et al.*, 2019). In this work, we will focus on discussing the NAD(P)$^+$-GPDs isoforms.

Canonical structure of NAD(P)$^+$-GPDs consists of a NAD$^+$-binding domain in the N-terminal region and a C-terminal substrate binding domain. Morales-Sánchez *et al.* reported that, in Chlamydomonas, out of the five identified NAD(P)$^+$-GPDs, just two isoforms have the canonical structure. Likewise, Wu *et al.* reported that in *D. Salinas* just one out of the five identified NAD(P)$^+$-GPDs share this protein organization. The rest of the homologs have a unique structure consisting of the canonical configuration plus an additional motif in the N-terminal region (figure 1). This motif is a haloacid dehalogenase (HAD)-like superfamily domain, and it is most closely related to PSP (phosphoserine

phosphatase), which is commonly involved in L-serine biosynthesis (Morales-Sánchez *et al.*, 2017).

Although canonical GPD is well-conserved and it is found across eukaryote and prokaryotes, it seems that GPDs with these unique structures (PSP like-GPD) are limited to algae species and cannot be found in higher plants (Morales-Sánchez *et al.*, 2017; Wu *et al.*, 2019). PSP like-GPD have been found, thus far, in *D. salinas*, *C. reinhardtii*, *D. viridis*, and two other antartic Chlamydomonas species (Wu *et al.*, 2019; Raymond *et al.*, 2020). Furthermore, recent phylogenetic analysis showed that GPDs from *C. reinhardtii* share the same clade with characterized GPDs from Dunaliella (Herrera-Valencia *et al.*, 2012). Thus, we can speculate that GPDs in microalgae share a common evolutionary origin and a similar function.

Phylogenetic studies of PSP like-GPDs in *Chlamydomonas* showed these enzymes clustering with canonical GPD of diverse eukaryotes, including metazoans and fungi, suggesting a common ancestry (Morales-Sánchez *et al.*, 2017). However, in addition to plant and algal sequences, canonical GPDs from Chlamydomonas also clustered with eubacterial GPDs sequences (Morales-Sánchez *et al.*, 2017). Thus, it has been hypothesized that these canonical GPDs might have been obtained through an endosymbiotic event or horizontal gene transfer. On the other hand, homology-modeling studies suggested that the GPD domains of Chlamydomonas PSP like-GPDs had features similar to human GPD (Herrera-Valencia *et al.*, 2012; Morales-Sánchez *et al.*, 2017). Thus, it has been proposed that the PSP like-GPDs enzymes may have arisen by duplication of a gene encoding an ancestral PSP in an ancestral algal lineage, then one of

these duplicated copies may have been translationally fused to a gene encoding a eukaryotic-like GPD (Morales-Sánchez *et al.*, 2017).

When speculating why would PSP like-GPDs emerge in organisms like microalgae, one could argue that they arose as way for the cell to rapidly adapt to external stressors or environmental changes. For example, Glycerol production in higher photosynthetic organisms such as *Arabidopsis thaliana* is thought to be the product of Glycerol-3-phosphate phosphatases (Gly-3-PP). Usually, DHAP is converted to Glycerol-3-P by GPD; then, if it is needed, Gly-3-PP will catalyze the dephosphorylation of Glycerol-3-P to glycerol (figure 2). *A. thaliana* encode two Gly-3-PP. One of them is located in plastids, and the second one is most likely found in the cytosol (Caparrós-Martín *et al.*, 2007; Morales-Sánchez *et al.*, 2017). In contrast, in the genome of green microalgae a single Gly-3-PP has been detected, and it is more closely related to the cytosolic form from *A*. *thaliana* (Morales-Sánchez *et al.*, 2017). Given that these PSP like-GPDs isoforms has been predicted or found to be located in the chloroplast of several microalgal species (Morales-Sánchez *et al.*, 2017; Raymond *et al.*, 2020; Wu *et al.*, 2019; He *et al.*, 2020), we can speculate that, during evolution, microalgae may have lost (or perhaps never evolved) a chloroplastic Gly-3-PP, and that this activity may have been replaced by the divergent PSP domain of the PSP like-GPDs enzymes as a way to rapidly adapt and respond to environmental pressures such as osmotic or high salinity stresses.

In recent years, interesting studies have been done aiming to elucidate the evolution of both canonical and PSP like-GPDs. Studies performed in organisms such as Drosophila and *Danio rerio* indicate that purifying selection was a major force in the

evolution of canonical GPD genes (Spencer Wells, 1995; Barat *et al.*, 2012; Wu *et al.*, 2019). This purifying selection force can also be detected in the evolution of PSP like-GPD from *Dunaliella* and *C. reinhardtii* (Herrera-Valencia *et al.*, 2012). Additionally, since both canonical and PSP-like GPDs have been reported in several algae species, there has been an interest in understanding how these two forms of the GPD genes evolved.

The natural selection forces that lead to evolution are purifying selection that removes deleterious variations, and positive selection that fixes the beneficial variations in the population and promotes the emergence of new phenotypes (Choudhuri, 2014). Recently, Wu et al. conducted pairwise Ka/Ks ratio analysis as an indicator of the selective pressure and reported that canonical GPD and PSP like-GPDs are under these different evolutionary forces in *D. salinas*. Their results indicate that canonical GPDs are mainly under positive selection (contrary to what it has been observed in *Drosophila* and *Danio rerio)*, and PSP like-GPDs are under purifying selection forces (in agreement with previous results by Herrera-Valencia et al., 2012*)*. This study also determined that the evolution of the PSP-like domain in the N-terminal region and the canonical GPD structure in the C-terminal region are highly correlated. These findings indicating purifying selection constraining the evolution of PSP like-GPDs enzymes may imply that the catalytic functions of these enzymes are not easily changed (Wu *et al.*, 2019). Thus, suggesting that PSP like-*GPDs* roles may be critical for the rapidly response, adaptation, maintenance, and survival of green algae to environmental and abiotic stresses. On the contrary, results showing canonicals GPD being under positive selection suggests that they may undergo accelerated evolution (Wu *et al.*, 2019). Thus, one could

speculate that canonical GPD was necessary for different algae to adapt to different changeable living environments, such as oceans and freshwater (Wu *et al.*, 2019).

Although canonical and PSP like-GPDs isoforms are under different evolutionary pressures, and they are differentially expressed depending on the environmental conditions; they both have important roles in the cell. Either to ensure that the cell can survive and adapt to different environments and stresses, or just to maintain the normal function of the cell.

## PURPOSE OF THIS WORK

In biotechnology, GPD is of interest as a possible target for genetic engineering because it has been identified as a rate-limiting enzyme in the glycerolipid pathways of several organisms. For example, GPD enzymes catalyze the synthesis of Glycerol-3-P, a TAG precursor. Like other microalgae, *C. reinhardtii* shows a significant increase in nonpolar lipid production, such as TAG production, under nutrient deprivation conditions (i.e., nitrogen and phosphorus depletion) and osmotic stress (Morales-Sánchez *et al.*, 2017; Zienkiewicz *et al.*, 2016). TAG production is of industrial and economic interest because it is a primary precursor for biofuel, biodiesel, production; thus, one of the objectives of current research in the biofuel field is to increase TAG production in microalgae. Consequently, research efforts have been directed to study the lipid metabolism pathways of model organisms such as Chlamydomonas, as well as the enzymatic components (such as GPD) that may be involved in these pathways. The final goal is to find possible genetic engineering targets and to understand how these targets are regulated. Findings could be applied in microalgae and other photosynthetic organisms through genetic engineering.

Even though GPD has been studied and its function well characterized, there are multiple GPD isoforms and it has been proposed that they may have different functions within the cell (Morales-Sánchez *et al.*, 2017; Lee *et al.*, 2012; Wu *et al.*, 2019). For instance, in organisms like yeast, while some GPD homologs are expressed constitutively, the expression of other homologs varies depending on different abiotic stress such as high osmolarity(Hohmann, 2002). In addition, GPD homologs are localized

in several cell compartments, such as the cytoplasm, mitochondria, and chloroplast, suggesting different roles of these homologs within the cell.

In *C. reinhardtii,* five $NAD(P)^+$-dependent GPD homologs have been found located in different cellular regions. Like in other organisms, some homologs are expressed constitutively, but others, such as GPD2 homolog, are abundantly expressed under nutrient deprivation and osmotic stress conditions. Moreover, evidence suggests that these isoforms may have different roles under multiple abiotic stresses, such as high salinity or osmotic stress (Morales-Sánchez *et al.*, 2017; Wu *et al.*, 2019; He *et al.*, 2020; Raymond *et al.*, 2020). Further research is needed to completely understand the roles of GPD homologs in cellular homeostasis. For example, recent studies in a cytosolic GPD homolog in *Arabidopsis* suggested that these homologs may have a role in maintaining cellular redox and reactive oxygen species' (ROS) homeostasis during osmotic stress conditions(Zhao, Liu, Wang, *et al.*, 2019), which in turn can be used to help reduce oxidative stress in plans. Other studies in maize showed that cytosolic GPD homologs could provide plants tolerance to osmotic stress (Zhao, Liu, He, *et al.*, 2019). One can argue that chloroplastic homologs could also be utilized to increase salt tolerance in plants, but more research needs to be done before using this technique in crops. Additionally, high salinity stress treatment has been proposed as a strategy to induce TAG production in microalgae. However, the metabolic processes and the pathways involved in the responses to osmotic stress in the cell are not fully understood. Thus, to be able to take advantage of genes that could help develop salt-resistant crops, or to be able to improve TAG synthesis in microalgae under osmotic stress conditions, more research has to be done on the response pathways and the possible elements involved. For

instance, it is known that one or more GPD homologs may have roles in the osmotic stress response in microalgae, however exact roles of chloroplastic GPD homologs are barely understood. Thus, it is worth investigating such roles to fill the gap in the knowledge about the possibility of a plastidic pathway for glycerol production; the involvement of GPD2 homolog in this pathway; and how this pathway may help contribute to a rapid response to osmotic stresses in *C. reinhardtii*.

The research discussed here focuses on the Glycerol-3-phosphate dehydrogenase (GPD) homolog GPD2. GPD2 gene expression is up-regulated under both nitrogen deprivation and osmotic stress condition, suggesting this isoform plays a role in cellular stress response.

GPD2 differs from other homologs in that it has a phosphoserine phosphatase (PSP) domain(Morales-Sánchez *et al.*, 2017; He *et al.*, 2020; Raymond *et al.*, 2020). The function of this domain is currently unknown. However, it has been suggested that under certain conditions, this domain may allow the enzyme to catalyze the direct conversion of glycerol-3-P to glycerol (Morales-Sánchez *et al.*, 2017; He *et al.*, 2020; Casais-Molina *et al.*, 2016; Raymond *et al.*, 2020). In addition, while other GPD homologs are located in the cytoplasm of the cell, GPD2 is localized in the chloroplast, which suggests a novel pathway in the chloroplast that is needed for rapid cell acclimation under osmotic stress (figure 3b).

With this project, we aimed to find the role of the PSP domain in GPD2, and how cells regulate the activity of such domain. Additionally, GPD2 homolog is of interest because previous research has shown that when GPD2 expression is decreased in the cell, TAG production is significantly reduced(Morales-Sánchez *et al.*, 2017). Consequently,

GPD2 may be essential for cell acclimation to stresses like nutrient deprivation and osmotic stress. These results make this an enzyme of interest for genetic engineering.

The central hypothesis of this work is that GPD2 homolog is a bifunctional protein that catalyzes the conversion of dihydroxy acetone phosphate (DHAP) to G3P under nitrogen deprivation conditions and the direct conversion of DHAP to glycerol under high salinity conditions (figure 3a). If confirmed, this suggests a novel plastidic pathway is needed for rapid cell acclimation under osmotic stress (figure 3b).

Overall, the objective of the project is to better comprehend how the homolog GPD2 is regulated, and the specific functions of this homolog in the microalgae *Chlamydomonas reinhardtii.* This can improve our understanding of the glycerolipid metabolic pathway and stress response mechanisms of these organisms.

GPD characterization and functions under specific abiotic stresses need to be investigated to adequately understand the roles and importance of GPD in the metabolism of *C. reinhardtii*. Our findings will broaden our knowledge of the functions of these enzymes in the cells and may lead to biotechnological applications for economically important crops.

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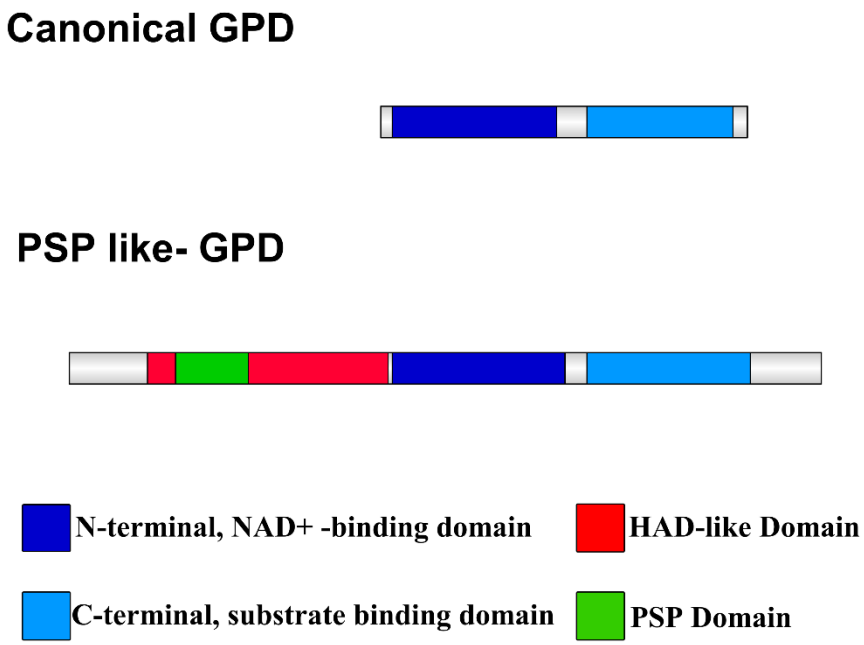



**Figure 1.** Schematic representation of GPD homologs structures based on GPDs found in Chlamydomonas. Modified from Morales-Sánchez *et al*. Color bars indicate conserved protein domains.

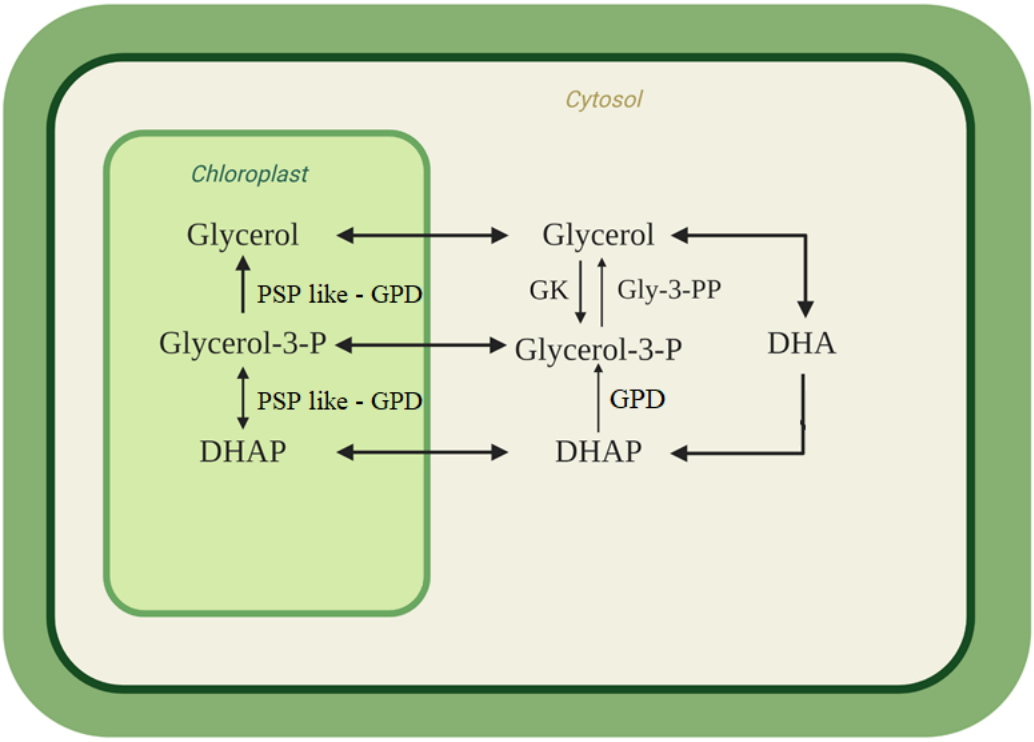
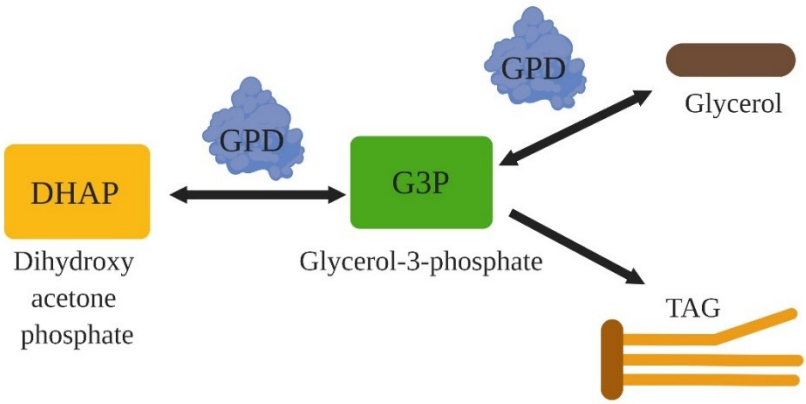



**Figure 2.** Hypothesized glycerol synthesis cycle in Chlamydomonas. In the cytosol, glycerol is synthesized from Glycerol-3-P (glycerol-3-phosphate) by Gly-3-PP (glycerol-3-phosphate phosphatases) when needed. However, since Gly-3-PP has not been identified in the chloroplast, it is speculated that PSP like-GPDs fulfills that role directly dephosphorylating Glycerol-3-P to Glycerol.

a)




b)

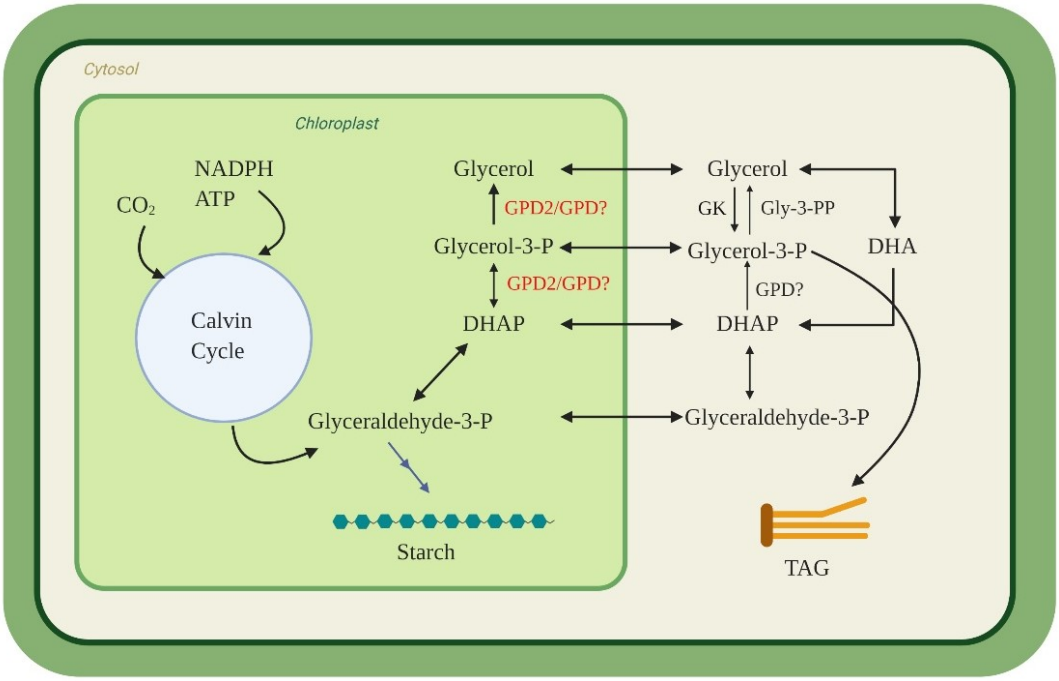



**Figure 3.** Illustration of the central hypothesis of this work. a) GPD2 homolog is a bifunctional protein that catalyzes G3P, a precursor for TAG synthesis, under nitrogen deprivation conditions, but also catalyzes the direct conversion of DHAP to glycerol under high salinity conditions. b) Glycerol metabolism in *C. reinhardtii.* Metabolites: DHAP, dihydroxy acetone phosphate; GK, glycerol kinase; Gly-3-PP, glycerol-3-phosphate phosphatase; DHA, dihydroxy acetone.

# CHAPTER 2

# CHARACTERIZATION OF A NOVEL GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPD2) IN THE ALGA *CHLAMYDOMONAS REINHARDTII*

# Introduction

*Chlamydomonas reinhardtii*, a widely used model organism, is a photosynthetic freshwater microalga with a fast growth rate. *C. reinhardtii* is relatively easy to culture, grow, and genetically modify(Yan *et al.*, 2016; Hallmann, 2007). Thus, it has been used extensively to study important biological processes, such as cell movement, cell-to-cell recognition, photosynthesis activities, and cell responses to light and abiotic stresses, among others(Driver *et al.*, 2017; Goodenough *et al.*, 2014; Morales-Sánchez *et al.*, 2017). Similar to other microalgae species, *C. reinhardtii* exhibits substantial accumulation of triacylglycerol (TAG) when placed under certain stresses like nutrient deprivation. TAG is an economically important carbon and energy storage glycerolipid**,** and it is of interest for industrial purposes because it is an essential precursor for biofuel production. Thus, recently research efforts in the field have been directed to study the mechanisms to increase TAG accumulation in microalgae. Glycerol-3-phosphate (G3P) is the indispensable precursor for TAG accumulation, and it is also the precursor to other relevant nutraceutical and biopharmaceutical products in the cell. Glycerol-3-phosphate dehydrogenase (GPD) is the enzyme that catalyzes the synthesis of G3P. In *C. reinhardtii,* five $NAD(P)^+$-dependent GPD homologs have been found. For the work here presented, we decided to focus on one of these homologs: GPD2.

GPD2 homolog is of interest because Morales-Sánchez *et al.*, 2017 found that it is abundantly expressed under nutrient deprivation and osmotic stress conditions; and RNA-mediated silencing of GPD2 leads to a significant decrease of TAG and glycerol accumulation in nitrogen-starvation and high-salinity conditions, respectively. Additionally, Morales-Sánchez *et al.*, 2017 also reported that *C. reinhardtii*'s GPD2

differs from other GPD homologs because it has a phosphoserine phosphatase-like domain (PSP) that is capable of catalyzing glycerol production *in vitro.* Similar homologs with unusual PSP-like domains have been also reported in Dunaliella (He *et al.*, 2020; Cai *et al.*, 2013; Wu *et al.*, 2019) and other species of Chlamydomonas (Raymond *et al.*, 2020). Moreover, the structure of the chimeric protein has been published for *Dunaliella salinas* (He *et al.*, 2020).

Based on these findings, GPD2 homolog may contribute to the catalysis of both compounds, glycerol and TAG, depending on the environmental condition. Intriguingly, GPD2 has been located in the chloroplast. This suggests a novel plastidic pathway for glycerol production under high salinity conditions (Morales-Sánchez *et al.*, 2017) (figure 1b). These findings suggest that GPD2 homolog may be important for the cellular response of *C. reinhardtii* to both stresses.

Understanding how cells regulate GPD2 enzymatic activities, what pathway(s) this enzyme plays a role in, and what other enzymatic components GPD2 may interact with, can help to broaden our biochemical and cytological understanding of TAG and glycerol synthesis and accumulation in the cell, with possible implications for biotechnology. Additionally, discerning the molecular mechanisms of osmotic stress response in model organisms such as *C. reinhardtii* is important because it could improve our knowledge in osmotic stress response in plants, knowledge that could lead to the development of salt-resistant crops (Wang *et al.*, 2018). For example, recent studies in a cytosolic GPD homolog in *Arabidopsis* suggested that these homologs may have a role in maintaining cellular redox and reactive oxygen species' (ROS) homeostasis during osmotic stress conditions (Zhao, Liu, Wang, *et al.*, 2019). Other studies in maize showed that cytosolic

GPD homologs could confer plants a tolerance to osmotic stress (Zhao, Liu, He, *et al.*, 2019). However, the roles of the GPD homologs in chloroplast are barely understood. Moreover, there is a need to investigate the possibility of a plastidic pathway for glycerol production, how this pathway is regulated by the cell, and how it contributes to a rapid response to osmotic stresses.

In the work here presented, we hypothesized that GPD2 homolog is a bifunctional protein with a phosphatase domain that allows the enzyme to not only catalyze TAG synthesis, but also to catalyze glycerol synthesis. The activity of this domain may be regulated through post-translational modifications (PTM) that depend on environmental conditions (figure 1a).

PTMs can alter the physiological and chemical properties of a protein, thus, altering its function. Therefore, a PTM can be a regulatory mechanism used by the cell to control protein activity. Recent developments of proteomic technologies and methods have revealed that a great number of chloroplast proteins are post-transcriptionally modified. Extensive research has been done regarding phosphorylation and redox regulation of proteins in the chloroplast (i.e. photosynthetic machinery), but also recent works have confirmed widespread acetylation, and instances of protein methylation, nitration and nitrosylation, glycosylation, sumoylation, and glutathionylation in chloroplasts (White-Gloria *et al.*, 2018; Grabsztunowicz *et al.*, 2017; Hartl *et al.*, 2017).

Due to the regulatory nature of PTMs, it is important to understand how such modifications affect the enzymatic activity of proteins in the chloroplast and their physiological significance in the cellular metabolic pathways. In this study, we aimed to determine what PTM is responsible for the glycerol synthesis activity of the PSP-like

domain of the GPD2 homolog in *C. reinhardtii*. Understanding how cells regulate GPD2 enzymatic activities can broaden our knowledge of *C. reinhardtii's* response to osmotic stresses.

# Materials and Methods

Strains and culture conditions

*C. reinhardtii* CC-124 (WT) (Harris, 1989) as well as transgenic strains (described below) were used in all reported experiments. If not otherwise specified, cultures were incubated on an orbital shaker under continuous illumination at 25°C and ambient level of $CO_2$ (Morales-Sánchez *et al.*, 2017; Msanne *et al.*, 2012). Strains were pre-cultured to middle logarithmic phase in minimal (HS) medium (Sueoka, 1960). As previously described (Morales-Sánchez *et al.*, 2017), cells were collected by centrifugation, washed twice, and resuspended in HS medium, HS medium containing 100 mM NaCl (HS+NaCl), or HS medium lacking nitrogen (HS+N-) at a density of ~$2.0x10^6$ cells/mL. Strains were then cultured during 6 hours under photoautotrophic conditions for the high salinity experiments, or during 2 days for the nitrogen deprivation analyses. At the end of the experimental period, cells were harvested by centrifugation at 2000 g for 5 min and the pellets frozen in liquid nitrogen and stored at -70°C for further analyses.

GPD2 overexpression cell line constructs

Overexpression lines were created as described in (Kim, 2018). Briefly, *GPD2* (Cre01.g053000) cDNA tagged with AcV5 was cloned from template (pIVEXGPD2) (Morales-Sánchez *et al.*, 2017) by PCR using the following primers GPD2_F (5'-GGTACCATGATGCTGTCAGGCCGCACCTGC) and GPD2-AcV5_R (5'-GAATTCtcagctccagccgctggcgtccttccagctCACGCTGTTGCTGGCAGC). Then, PCR

product was inserted into pSTBlue1, digested by *Kpn*I, blunted by T4 polymerase, and digested by *Eco*RI to generate the *GDP2-AcV5* fragment. Next, NE-589 was digested by *Nco*I, blunted by T4 polymerase, digested by *Eco*RI, and then ligated with the *GPD2-AcV5* fragment. The new construct NE-589-*GDP2-AcV5* was then digested by *Nsi*I, blunted by T4 polymerase, and ligated in-frame to the coding sequence. To generate the *PsaD* promoter-*GPD2-AcV5-PsaD* terminator fragment, *Kpn*I and *Xba*I were used. Fragment was then blunted by T4 polymerase. Finally, pSP124S, which contains the Zeocin-resistance gene (BleR), was digested by *Eco*RI, and blunted by T4 polymerase. Both constructs were ligated to make pSP124S-*GPD2-AcV5-BleR.* These constructs were transformed into CC-124 by electroporation, as previously described (Plucinak *et al.*, 2015) and colonies selected on TAP-agar plates containing 16 μg $mL^{-1}$ zeocin (Invitrogen, Carlsbad, CA). Expression of GPD2 recombinant proteins was verified in several independent transgenic strains by immunoblotting with an anti-AcV5 antibody.

Reverse transcriptase (RT-PCR) and Real time (RT-qPCR) assays

Total RNA extraction, reverse transcription, and qPCR procedures were followed as previously described (Morales-Sánchez *et al.*, 2017).For reverse transcription, oligo dT primer was used. The qPCR reaction was confirmed to have a single-sized product by electrophoresis agarose-gel running. qPCR was performed using RT² SYBR Green Fluor qPCR Mastermix (Qiagen, 330519) and CFX96 Touch™ Real-Time PCR Detection System (BioRad). For qPCR, the primers: *GPD2* (Cre01.g053000) GPD2-RT-F1 5'-AGAGCAAGTACCCGCTGTTCAC-3' and GPD2-RT-R1 5'-AAGGAACGTCCTCCTTTACACG-3'; for *GK* (Cre04.g224150) Glykin-F 5'-ATGTCTTCTAACGCCATCTCATAC-3' and Glykin-R 5'-

CACCGTGCGCCAAATCTCAA-3'; for *AKR3* (Cre10.g432900) AKR3-F 5'-TCACAGCAGCGGATATGGCTCTGG-3' and AKR3-R 5'-ATGACCTGCGGGTCCTTGATCATGG-3'; for heat shock experiments detecting *HSF1* transcripts as described by Zalutskaya *et al.*, 2015. HSF1F 5'-AACATCGTCTCATGGGGTGC-3' and HSF1R 5'- TCCATAGGTGTTGAGCTGGC-3' were used to amplify each transcript. For ACTIN, ACT-cod-F (5'-GACATCCGCAAGGACCTCTAC) and ACT-cod-R (5'-GATCCACATTTGCTGGAAGGT) were used.

Inhibitor Treatments

Unless stated otherwise, selected inhibitor was present in cell cultures for the duration of the experiment. Cell cultures for all the following experiments were grown as previously described.

*Cycloheximide and spectinomycin treatment:* WT and transgenic strains were exposed to either 150 µg/mL of cycloheximide or 100 µg/mL of spectinomycin for an hour. Then, cell lines were subjected to osmotic stress (100 mM NaCl for 6 hours). Glycerol production was estimated using free glycerol reagent.

*Okadaic acid treatment:* Cells were grown in HS media as previously described. In day 2 of cell growth, okadaic acid (10 nM) was added. When cultures were at the logarithmic state, ~day 3, cells were treated with 100 mM NaCl for 6 hours and glycerol accumulation was measured.

*Dark treatment:* WT cells were cultured in HS medium in the dark (no photosynthesis) for 3 days, and then treated with 100 mM NaCl for 6 hours. Half of the

controls and treated cultures were exposed to light during those 6 hours, and the other half were kept in the dark. Then, glycerol accumulation was determined.

*N-Acetyl Cysteine (NAC) treatment:* NAC (final concentration 1 mM) was added to WT and transgenic strain cultures for an hour. Then, cell lines were subjected to osmotic stress (100 mM NaCl for 6 hours). Glycerol production was estimated using free glycerol reagent.

*Staurosporine treatment:* Staurosporine (final concentration 1 mM) was added to WT and transgenic strain cultures for an hour. Then, cell lines were subjected to osmotic stress (100 mM NaCl for 4 and a half hours). Glycerol production was estimated using free glycerol reagent.

*Torin1 treatment:* Torin1 (final concentration 1 mM) was added to WT and transgenic strain cultures for an hour. Then, cell lines were subjected to osmotic stress (100 mM NaCl for 4 and a half hours). Glycerol production was estimated using free glycerol reagent.

Glycerol Content

Glycerol content was determined as previously described (Morales-Sánchez *et al.*, 2017). More specifically, *C. reinhardtii* cells were collected by centrifugation and the pellets frozen in liquid nitrogen and stored at -70 ºC until use. Pellets were resuspended in deionized water, briefly vortexed, and boiled for 10 minutes to release the glycerol from cells. Cell debris was pelleted by centrifugation at 2000 g for 5 minutes and the supernatant was used for measuring glycerol amounts with the free glycerol reagent (Sigma-Aldrich, St. Louis, MO).

Lipid Accumulation

WT and transgenic *C. reinhardtii* cell were grown photoautotrophically in HS medium for three days. Then equal concentration of cells (determined by absorbance at 750 nm) were transferred to fresh nitrogen depleted medium (HS + N-) and incubated for 2 days. Cells were collected by centrifugation and the pellets frozen in liquid nitrogen and stored at -70 ºC until use. Pellets were resuspended in media and nonpolar lipid content was estimated by staining with the lipophilic fluorophore Nile Red (Sigma, 72485). Nile Red was added to a final concentration of 1 μg/mL and fluorescence (excitation at 488 nm; emission at 565 nm) measured in a multi-well plate reader (Synergy H1, Biotek). Nile Red fluorescence was normalized to cell density (determined as absorbance at 750 nm) and expressed in arbitrary units (A.U.).

SDS-PAGE and Western Blot Assays

The protein levels of recombinant GPD2 under the different experimental conditions was assessed by immunoblotting using anti-AcV5 antibody (Thermo Fisher Scientific, eBioscience, 14-6995). Around ~$10^8$ *C. reinhardtii* cells were heated at 95 °C for 8 minutes to get crude extracts. Equal volumes of whole-cell protein extracts were loaded onto and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide gel). Proteins were then transferred to nitrocellulose membranes (Amersham™ Protran® Western blotting membranes, nitrocellulose); 7% skim milk in TBS-T buffer (Tris-buffered saline with 0.1% tween-20) was used to block the membranes overnight. The membrane was incubated with 1:5000 diluted mouse anti-AcV5 antibody in TBS-T buffer with 7% skim milk overnight at 4°C. Membranes were washed thrice: one time in TBS-T buffer for fifteen minutes and twice more in TBS-T

buffer for five minutes each. A peroxidase conjugated anti-mouse IgG antibody in TBS-T with 7% skim milk was used to incubate membranes for one hour at 4°C, then membranes were washed one time in TBS-T buffer for fifteen minutes and twice more in TBS-T buffer for five minutes each. Peroxidase substrates (WBKLS0500, ImmobilonTM, Millipore) were provided to illuminate chemiluminescent signals on membranes. The signals were visualized using films (28906845, Amersham Hyperfilm MP, Life Sciences).

Purification of GPD2 protein for Mass Spectrometry analysis.

GPD2 overexpressing cell lines were grown under control (HS medium) and treatment (HS+100 mM NaCl) were prepared and analyzed by mass spectrometry. GPD2 recombinant protein was purified under nutrient replete and osmotic stress conditions as described by (Kim, 2017) with some modifications. More specifically, for each treatment ~$10^{10}$ cells were resuspended in 10 ml of lysis buffer [20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 0.1 mM EDTA; 2.0 mM $CaCl_2$; 2.0 mM $Mg(CH_3COO)_2$; 2.0 mM Benzamidine; 0.2 mM PMSF; and 10% glycerol] containing 5 µl/ml protease inhibitor cocktail (Sigma P9599), and phosphatase inhibitor cocktail tablets (Sigma PHOSS-RO). Cells were broken by two passages through French-press at ~5,000 psi. The lysate was centrifuged at 16,000 *g* for 30 minutes at 4°C. Supernatant was centrifuged again at 80,000 *g* for 90 minutes at 4°C to separate out soluble proteins. The supernatant was incubated with 20 µg of anti-AcV5 antibody (Thermo Fisher Scientific, eBioscience, 14-6995) for one hour followed by an overnight incubation with beads (~200 µL of packed resin per 40 mL of original cell extract; beads are protein G immobilized on agarose; Sigma P7700) at 4°C. The beads were collected by centrifugation (5000 g for 5 min) and

washed five times with wash buffer [20 mM Tris-HCl (pH 7.5); 300 mM NaCl; 0.1 mM EDTA; 2.0 mM $CaCl_2$; 2.0 mM $Mg(CH_3COO)_2$; 2.0 mM Benzamidine; 0.2 mM PMSF; 0.1% Triton X-100; and 10% glycerol] containing 5 µl/ml protease inhibitor cocktail, and phosphatase inhibitor cocktail tablets. Polypeptides retained in beads were subsequently analyzed using mass spectrometry at the UNL Proteomic Core Facility. For 'gel experiments' cell extracts were processed as previously explained in SDS-PAGE and Western Blot Assays with the minor difference that phosphatase inhibitor cocktail tablets (Sigma PHOSS-RO) were added to the sample buffers**.** Equal volumes of whole-cell protein extracts were loaded onto and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide gel). Then to analyze our protein of interest, bands were excised (based on size) from the SDS-PAGE and subsequently analyzed using mass spectrometry at the UNL Proteomic Core Facility.

*Chlamydomonas reinhardtii's* GPD2 Structure Prediction

As described by Raymond *et al.*, 2020, a protein model of Cre01.g053000.t1.2 (GPD2) of *Chlamydomonas reinhardtii* was predicted with Swiss Model (https://swissmodel.expasy.org/) using as template the structure of the Dunaliella salina bidomain protein (6IUY, a homodimer) (He *et al.*, 2020). The resulting model was a monomeric unit with a QMEAN value of -1.53. In the 587-a.a. region of overlap, the two proteins had an identity of 56.26% and Overall RMSD: 0.389. Molecular graphics and analysis were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (http://www.rbvi.ucsf.edu/chimera/).

Statistical Analysis

For statistical analyses, we used the "stats" package in R environment (RStudio Team, 2020). <0.05 p-value in ANOVA was used to determine if there were statistically significant data. If data passed the ANOVA criteria, 0.05 p-value in Tukey's HSD was applied to identify the statistically different pairs in the data.

# Results

### *C. reinhardtii* GPD2 synthesizes different metabolites depending on environmental conditions

To verify the role of GPD2 during osmotic stress and nutrient starvation conditions, we performed stress experiments with wild-type and transgenic cell lines. This transgenic cell line (OX12) is constitutively expressing GPD2 (figure 2). Morales-Sánchez *et al.*, 2017 has shown that in *C. reinhardtii,* GPD2 gene expression was up-regulated under nitrogen starvation and osmotic stress conditions, suggesting a role of this enzyme in the cell response to both abiotic stresses. In contrast, when GPD2 gene expression is downregulated, lipid and glycerol accumulation decrease during nitrogen deprivation and osmotic stress respectively (Morales-Sánchez *et al.*, 2017).

Our results support this information showing that when wild-type *Chlamydomonas* cells are grown under nitrogen deplete conditions (N-) non-polar lipid accumulation is observed. Conversely, when cells are exposed to osmotic stress, glycerol production increases (figure 3). We also observed that for our OX12 cell lines (where GPD2 is constitutively expressed), there is no glycerol accumulation under control conditions despite GPD2 being present in the cell; it was just when cells were stressed with NaCl that glycerol accumulation occurred. Our stress experiments with transgenic cell lines

also confirm that even though this GPD2 protein is present under both treatments, the product accumulated changes depending on the stress (figure 3). Thus, the function of GPD2 may differ depending on the environmental cues, and its activity may be regulated through mechanisms such as post-translational modifications (PTM) that depend on the treatment's conditions.

**Cycloheximide treatment does not affect glycerol synthesis function of GPD2 homolog in the GPD2 overexpressing strain OX12**

In order to study the involvement of GPD2 in the osmotic stress response of *Chlamydomonas,* we inhibited *de novo* protein synthesis using cycloheximide. Cycloheximide is a protein synthesis inhibitor in cytoplasmic ribosomes. We treated wild-type and OX12 cell lines with 150 µg/mL of cycloheximide for an hour. Then, wild-type and OX12 cell lines were subjected to osmotic stress (100 mM NaCl for 6 hours). Glycerol production was estimated using free glycerol reagent. Results show that OX12 cell lines are producing glycerol even when ribosomal protein translation has been inhibited (Figure 4). This may indicate that already existing (constitutively expressed) GPD2 proteins are catalyzing glycerol production. However, it is not known how this function has been triggered. We have hypothesized that cells must regulate phosphatase activity of GPD2 by some type of regulatory mechanism (such as a PTM).

When GPD2 protein levels from OX12 lines were analyzed using Western Blot (Figure 5), results showed that GPD2 protein levels are relatively constant, even under control conditions. This indicates that although GPD2 is present in the cell under control and treatment conditions, there is no glycerol accumulation unless the cells are stressed with high salt. Thus, in control condition the phosphatase activity of GPD2 is not active,

this activation just occurs under osmotic stress. Surprisingly, our results also suggest a degradation of GPD2 under osmotic stress treatment. However, even though protein levels seem to be lower under NaCl stress, there is still glycerol accumulation. Thus, we hypothesized that under NaCl treatment, the phosphatase domain of GPD2 is activated to catalyze glycerol synthesis, but this same activation could decrease protein stability or target the protein for degradation.

We also evaluated GPD2 protein levels under conditions where the protein should be 'stable', that is when GPD2 has not been regulated or activated for glycerol production. Thus, GPD2 protein expression levels during nitrogen starvation conditions were determined using Western Blot. Our rationale was that under N- conditions, GPD2 is not catalyzing glycerol production but instead it is catalyzing G3P synthesis. Thus, the phosphatase domain responsible for glycerol synthesis should be 'inactive' and, therefore, protein stability should not change. Our results appear to support our hypothesis (figure 6). Relative GPD2 expression did not significantly changed after the 48-hour treatment. Suggesting protein is relatively stable when its phosphatase function is inactive.

**Post-translational modification inhibitors have different effects in the glycerol synthesis role of GPD2**

We performed experiments testing different inhibitors to determine what PTM was responsible for triggering the glycerol synthesis function of GPD2.

*Potential redox regulation:* We explored the possibility of GPD2 functions being regulated by reversible oxidized sites. GPD2 protein has been found to have several redox cysteine-containing peptides (McConnell *et al.*, 2018), so we wanted to evaluate if

glycerol production occurred when redox state of the chloroplast was changed. We had two methods; first, we performed experiments in the dark (when photosynthesis is not occurring, figure 7a-c). We found that wild-type Chlamydomonas kept in minimal (HS) media and in dark conditions shows a decrease in glycerol accumulation (Figure 7b). However, when this experiment was repeated with a minor change: cells were grown in TAP medium (acetate was provided as a carbon source); glycerol accumulation was observed even under dark conditions (Figure 7c). The provided extra carbon may explain this production. When combined, these results suggest that light is required to supply energy and carbon source to the system, but it is not needed to regulate the phosphatase function of GPD2 homolog. Our second approach used an antioxidant such as N-Acetyl Cysteine (Nac). Nac is a common cell-permeable ROS scavenger (Wang *et al.*, 2016) and change the normal chloroplast stromal redox status. Our results indicate that glycerol accumulation is not inhibited when cells are treated with Nac (figure 8), which suggests that reversible oxidized sites site may not be responsible for the regulation of GPD2 activity.

*Okadaic acid treatment:* We analyzed the possibility of dephosphorylation as the PTM mechanism responsible for the 'activation' of the glycerol synthesis function of GPD2; following procedures described by Sheremet *et al.*, 2012 we utilized okadaic acid, a potent phosphatase inhibitor. This inhibitor has been used previously in Chlamydomonas to enrich for and subsequently perform phosphoproteome analysis (Wagner *et al.*, 2006) and, when used at higher concentrations, it has been demonstrated to inhibit cell growth and photosynthetic electron transport in *Dunaliella tertiolecta* (Perreault *et al.*, 2012). In our cell growth experiments, however, it seems that the used concentrations did not affect

cell growth (figure 9). Additionally, our results indicated that the phosphatase inhibitor did not prevent Chlamydomonas from producing glycerol under osmotic stress conditions (figure 10).

*Kinase inhibitors treatment:* We analyzed the possibility of phosphorylation as the PTM responsible for the 'activation' of the GPD2 domain that synthesizes glycerol. Experiments were performed following procedures described by Sheremet *et al.*, 2012. First, we tested staurosporine, a potent non-selective inhibitor of protein kinases. Our results suggested that staurosporine is effectively inhibiting glycerol accumulation in both WT and OX12 cells (figure 11). To make sure staurosporine is successfully entering the cells, we performed a heat shock stress experiment following procedures by Zalutskaya *et al.*, 2015. Results showed that staurosporine is successfully inhibiting the transcriptional activation of the gene of interest suggesting that it is in fact penetrating the cell (figure 12). To further validate our results, experiment was repeated by treating cells with cycloheximide and staurosporine simultaneously (figure 13). Cycloheximide inhibits ribosomal protein translation, so no new GPD2 homolog was synthesized. However, pre-existing GPD2 homolog should have been able to catalyze glycerol synthesis, but staurosporine treatment seems to have inhibited this activity and, therefore, there was no glycerol accumulation.

We repeated this experiment using torin1, a more specific kinase inhibitor and known TOR (target of rapamycin) kinase inhibitor (figure 14) and (as previously performed) we also treated cells with cycloheximide and torin1 simultaneously (figure 15). Results showed that tornin1 does not inhibit glycerol accumulation in OX12 cells, but there is inhibition in WT cells, suggesting that there may be a TOR kinase involved in the gene

expression pathway of GPD2. However, unlike staurosporine, torin1 does not inhibit glycerol accumulation in OX12 cell lines (which have constitutively expressed GPD2), thus torin1 does not affect phosphatase activity of GPD2.

In conclusion, our experiments with staurosporine suggest that there is an unknown kinase involved in the 'de/activation' of the glycerol synthesis function of GPD2. However, the mechanisms by which this unknown kinase regulates GPD2 activity is unknown. Additionally, torin1 did not affect phosphatase activity of GPD2, but our results suggest that TOR kinase may be involved in the GPD2 gene expression pathway.

**Mass-Spectrometry analyses identify phosphorylation of GPD2 Ser145 as potentially induced by osmotic stress**

Chlamydomonas samples under regular and osmotic stress conditions were prepared and analyzed using mass spectrometry at the UNL Proteomic Core Facility as explained in materials and methods. The objective was to determine what post-translational modification, if any, was responsible for the 'activation' of the PSP-like domain of GPD2 and, thus, its glycerol synthesis activity. Three different experiments were performed, and samples were screened for PTMs. Our results indicate that there is an amino acid within the PSP-like domain that may be important for the glycerol synthesis function of the domain. This amino acid is Serine-145 (figure 16 and 17), and it appears to be phosphorylated under osmotic stress treatment, but no phosphorylation is found in this residue under control conditions. We speculate that the phosphorylation of Ser145 may be important to trigger the glycerol synthesis function of the PSP-like domain when cells are under high salinity stress.

# Discussion

Canonical glycerol-3-phosphate dehydrogenase (GPD) catalyzes the conversion of dihydroxy acetone phosphate (DHAP) to glycerol-3-phosphate (G3P), which is a precursor to triacylglycerol (TAG). Of the five GPD homologs *Chlamydomonas reinhardtii* has, GPD2 is of interest because its transcript level has been found to increase under osmotic stress and nitrogen deprivation conditions. Moreover, when GPD2 homolog was further studied it was found it also plays a role in glycerol production under certain stress conditions such as osmotic stress (figure 3) (Morales-Sánchez *et al.*, 2017). GPD2 homolog has an extra PSP-like domain, and it has been hypothesized, and shown in vitro, that such domain plays a role in glycerol synthesis for cell protection under stress (Morales-Sánchez *et al.*, 2017; He *et al.*, 2020). However, given the multiple roles of this enzyme (TAG synthesis under nutrient deprivation and glycerol synthesis under osmotic stress), we tested how Chlamydomonas cells regulate the phosphatase activity of GPD2 homolog depending on the environmental conditions. Our hypothesis was that cells undergo a post-translational modification of GPD2 homolog that somehow triggers glycerol synthesis activity of the enzyme when it is needed. To test our hypothesis multiple PTM inhibitors were tested in Chlamydomonas cell cultures: okadaic acid (phosphatase inhibitor), torin1 and Staurosporine (kinases inhibitors), dark treatments (potential altering redox regulation in the chloroplast) and N-acetyl cysteine (Nac, an antioxidant).

**GPD2 protein levels may be regulated by the cells under osmotic stress conditions**

Overall our results, such as product accumulation measurements and protein level analysis experiments (figure 3 and 5 respectively), indicate that although GPD2 is present

in the cell under control and treatment conditions, there is no glycerol accumulation unless the cells are stressed with high salt. Thus, in control condition the phosphatase activity of GPD2 is not active, this activation just occurs under osmotic stress.

Previous reports indicate that GPD2 homolog transcripts levels increase when cells are placed under both osmotic and nutrient deprivation stresses. However, when we performed western blot analyses, it was surprising to find out that, under osmotic stress, GPD2 protein levels seems to be decreasing in the constitutively expressed OX12 cell lines (figure 5). Although further research is needed to support our findings, we hypothesized that whatever cellular mechanism (or PTM the cell is performing on GPD2 to activate the PSP-like domain to trigger glycerol synthesis) is also affecting protein stability and thus protein levels decrease. This observation seemed to be supported by our western blot analyses of GPD2 protein levels of cells under nitrogen deprivation (figure 6). In those experiments, protein levels remained similar throughout multiple conditions. This suggests that when the PSP-like domain is not ‘activated’ or ‘modified’, it is not synthesizing glycerol and protein levels are more stable. While further experimentation is needed to establish a reliable conclusion, one can consider several explanations on why GPD2 protein level changes depending on the environmental stress. It may also be possible that cells regulate protein levels through a negative feedback loop when placed under osmotic stress. For example, when cells are stressed with high NaCl levels, GPD2 gene is induced, and transcripts levels increase. Then, GPD2 protein is translated. Next, the PSP-domain of GPD2 homolog is modified with a PTM triggering phosphatase activity of this domain and, thus, glycerol synthesis occurs. This regulation is necessary to facilitate the acclimation of the cells to the changes in their environment. However,

after an acclimation time, it is plausible that the excess of glycerol accumulation triggers a negative feedback loop that results in the degradation of GPD2 protein; such response seemingly is not happening under nitrogen deprivation conditions.

More in-depth research is needed to test these possibilities. However, it may be worthwhile exploring how cells regulate proteins, such as GPD2 homolog, under the various stresses because it can broaden our understanding of cellular response and adaptation to environmental changes.

**A protein kinase may be involved in the glycerol synthesis activity of GPD2 homolog**

To find what PTM or cellular mechanism is responsible for activating the PSP-like domain of GPD2 and, perhaps destabilizing protein, we tested different PTM inhibitors. GPD2 was predicted to have reversible oxidized sites, therefore cells were treated with Nac or cultured under dark conditions, as previously described, to change the redox state of the chloroplast. However, our results suggested that GPD2 was working properly under the treatments and thus, glycerol synthesis was not affected by these stresses (figure 7b, 7c, 8). Then, we evaluated the effect of okadaic acid, a phosphatase inhibitor. Our results indicated that glycerol synthesis was not inhibited under this treatment (figure 10), thus okadaic acid did not affect phosphatase activity of GPD2. Next, the effects of kinases inhibitors on GPD2 function were assessed. When cells cultures were treated with Staurosporine, a potent and broad kinase inhibitor, glycerol synthesis was successfully inhibited in both WT and OX12 cell lines (figure 11). We also tried a more specific kinase inhibitor, torin1, which did inhibit glycerol synthesis in WT cells but not in OX12 cell lines, where GPD2 is constitutively expressed (figure 14). This could suggest that there are kinases involved in both processes: the expression of GPD2 protein,

and the activation of the PSP-like domain that triggers glycerol synthesis. For example, recent studies in *D. salina* have identified an alternative splicing form of a mitogen-activated protein kinase (MAPK) that is involved in the regulation of GPD expression and thus glycerol synthesis under salt stress. They found that when the expression of the kinase increased, GPD expression increased likewise. This suggested a role of the MAPK cascade pathway in the cell response to osmotic stress and regulation of GPD expression (Tang *et al.*, 2020). Studies in *D. tertiolecta* have also demonstrated that MAPK-mediated signaling pathway may be involved in the cellular response to osmotic stress (Zhao *et al.*, 2016).

For GPD2, we consider that there could be three possibilities on how the PSP-like domain of the GPD2 protein is being activated for glycerol synthesis (figure 18). The first is a direct effect of the kinase on GPD2 (figure 18a). In this scenario, an unknown kinase is directly phosphorylating the PSP-like domain of the GPD2 protein, and thus, triggering glycerol synthesis. The second scenario is an indirect effect in which a kinase phosphorylates an unknown protein that then activates PSP-like domain with another PTM (figure 18b). The third possibility is that GPD2 is associated with an unknown protein in its native state (figure 18c). In this configuration, the PSP-like domain is not functioning. Then, an unknown kinase phosphorylates the associated protein, which disassociates from GPD2, leaving the PSP-like domain free to synthesize glycerol from G3P. To investigate which scenario is responsible for the glycerol synthesis activity of GPD2, protein analysis by mass spectrometry was performed.

**Phosphorylation of GPD2 Ser145 may be responsible for the glycerol synthesis function of GPD2**

Recent developments and improvements of PTMs detection methods have revealed a great number of these modification in the chloroplast. The most characterized PTMs in the chloroplast are, perhaps, (de)phosphorylation, and oxidation-reduction. However other PTMs such as methylation, acetylation, glycosylation, nitration, sumoylation, etc. have also been identified (Grabsztunowicz *et al.*, 2017). For this reason, we wanted to identify what PTMs in the PSP-like domain of GPD2. Samples were analyzed with mass spectrometry techniques and a region of interest was found. Our results indicate that, within this region, there is an amino acid residue (Ser145) that appears to be phosphorylated when the cells are under high salinity stress, but this residue is not phosphorylated under regular (control) conditions. These results, combined with our findings that a protein kinase may be involved in the glycerol synthesis activity of GPD2 homolog (from our kinase inhibitor experiments), allow us to speculate that phosphorylation of Ser145 may be required for the activation of the glycerol synthesis activity of GPD2, and that this activation happens when cells are under osmotic or high salinity stress. On the contrary, when cells are not under osmotic treatment, Ser145 is not phosphorylated, the 'PSP-like' domain of the enzyme is inactive and, thus, GPD2 catalyzes G3P synthesis instead. Furthermore, our findings seem to support model A for activation of the 'PSP-like' domain of the GPD2 protein (figure 18). A protein kinase may directly phosphorylate the 'PSP-like' domain of GPD2 and triggers its catalytic activation, which leads to the rapid synthesis of glycerol by dephosphorylation of G3P (figure 18a). In order to assess our hypothesis, we would have to substitute Ser145, to

avoid phosphorylation, and test if this affects the glycerol synthesis activity of the protein. Additionally, it is also necessary to perform studies to determine what kinase is responsible for the activation of the PSP domain of GPD2.

Studies have confirmed that phosphorylation is a common mechanism to regulate protein function in the chloroplast. Multiple protein kinases and phosphatases have been localized to the chloroplast, additionally to those participating in the photosynthetic machinery (White-Gloria *et al.*, 2018). Although this field has achieved numerous findings, it is still a challenging task to identify PTMs in the chloroplast. In addition to identify these modifications, we will also need to understand how these PTMs regulate the chloroplastic metabolic pathways. For example, a chloroplastic protein may have several PTMs at a given time with either antagonistic or cooperative effects, or the cell may be able to target and modify a specific amino acid residue with different PTMs, triggering different protein activities (Grabsztunowicz *et al.*, 2017). In this study, we identified Ser145 within the 'PSP-like' domain of the GPD2 enzyme that is phosphorylated under osmotic stress. Modification of Ser145 may have a role in the bifunctional characteristic of this enzyme, rendering it important for the survival of the cell under stress conditions. Thus, this highlights the importance of eventually revealing the entire PTM code of a protein in order to understand the physiological significance of PTM-mediated regulation in a given metabolic pathway (Grabsztunowicz *et al.*, 2017).

GPD2 homolog is a novel multidomain enzyme, consisting of a phosphatase motif fused to a G3P dehydrogenase domain. GPD2 transcripts expression increases when the cell is under osmotic stress and nitrogen deprivation conditions. Suggesting this homolog has an important role for cell acclimation under these stresses. Conversely, RNA-

mediated silencing of GPD2 resulted in decrease of the glycerol and TAG accumulation. The observed metabolites accumulated under osmotic stress and nitrogen deprivation conditions, respectively. Additionally, GPD2 has been located in the chloroplast, which suggests the possibility of a plastidic pathway for glycerol production, which could significantly contribute to the rapid acclimation of the cell under osmotic stress.

However, further research is needed to completely understand the roles of GPD homologs in cellular homeostasis. Especially the functions of chloroplastic GPD homologs (such as GPD2) are poorly understood. Thus, it is worth investigating such roles to fill the gap in the knowledge about the possibility of a plastidic pathway for glycerol production, the involvement of GPD2 homolog in this pathway, and how this pathway may help contribute to a rapid response to osmotic stresses in *C. reinhardtii*.

Chloroplastic GPD homologs could be important for biotechnology. They could be expressed in higher plants to increase salt tolerance. Additionally, high salinity stress treatment could be applied to induce TAG production in microalgae for industrial purposes . However, the metabolic processes and the pathways involved in the responses to osmotic stress in the cell are not fully understood. Thus, to implement such applications, further research is necessary.

Here we investigated the GPD2 homolog. A bifunctional protein with a phosphatase domain that allows the enzyme to not only catalyze TAG synthesis, but also to catalyze glycerol synthesis; and how the activity of this domain is regulated by the cell. We hypothesized that the cell triggers the phosphatase domain activity of GPD2 by phosphorylation of Ser145, in the PSP-like domain of the GPD2 enzyme. Further studies

need to target this amino acid residue to determine its role in the glycerol synthesis activity of GPD2.

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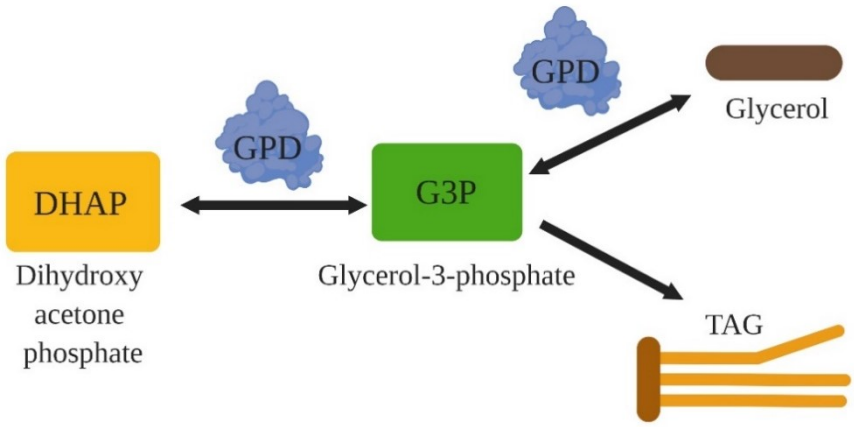
a)

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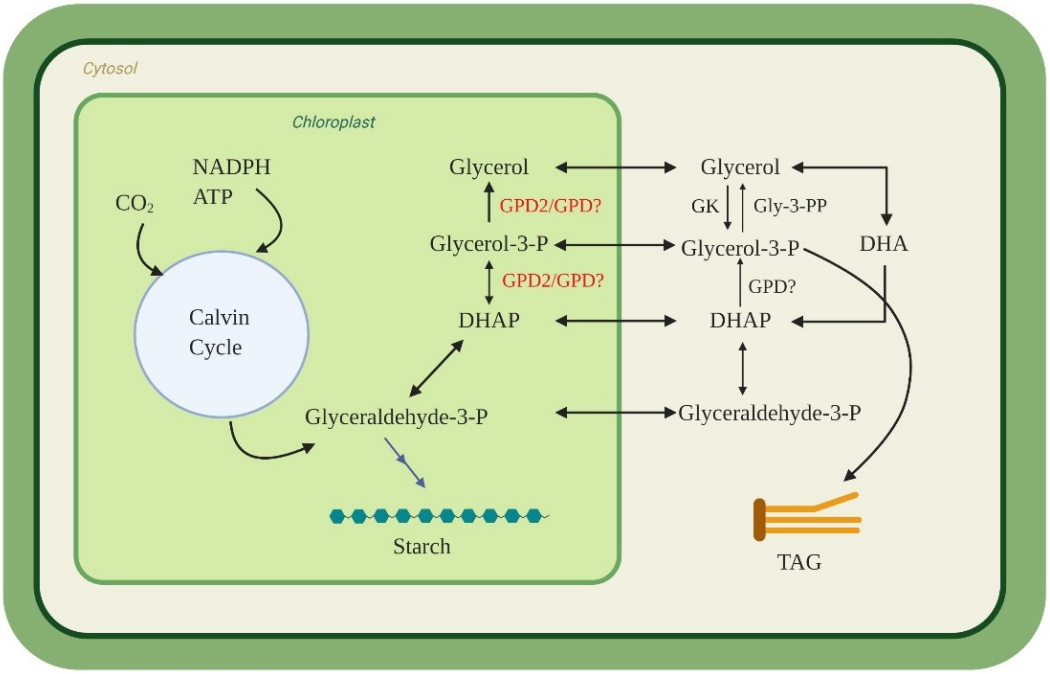
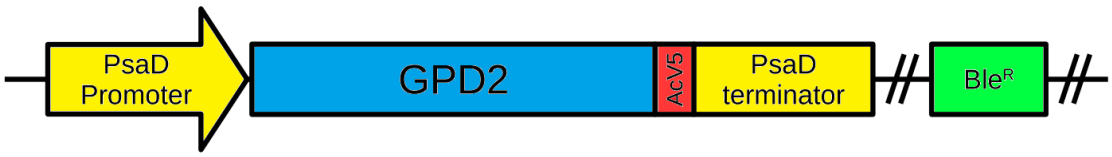



**Figure 1.** Illustration of the central hypothesis of this work. a) GPD2 homolog is a bifunctional protein that catalyzes G3P, a precursor for TAG synthesis, under nitrogen deprivation conditions, but also catalyzes the direct conversion of DHAP to glycerol under high salinity conditions. b) Proposed glycerol metabolism in *C. reinhardtii.* Metabolites: DHAP, dihydroxy acetone phosphate; GK, glycerol kinase; Gly-3-PP, glycerol-3-phosphate phosphatase; DHA, dihydroxy acetone.

a)

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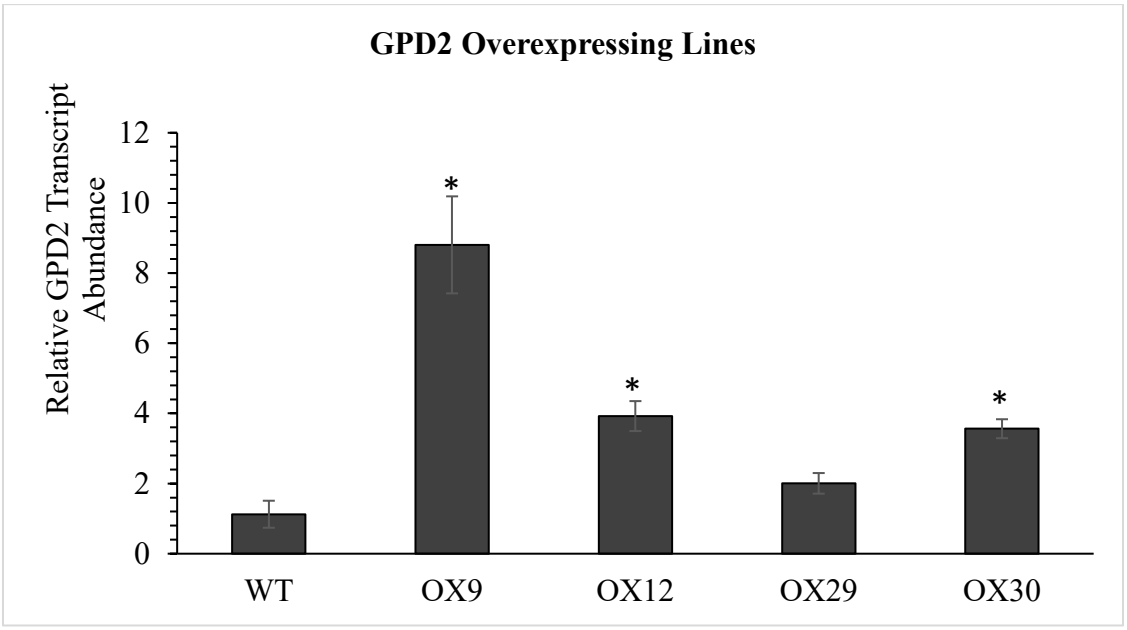


**Figure 2**. GPD2 construct and relative GPD2 transcript abundance analyzed by quantitative RT-PCR. a) Diagram of the construct used to develop GPD2 overexpressing cell lines. b) The graph shows wild-type (WT) and GPD2 overexpressing lines subjected to High-salt minimal medium (HS). Values shown, normalized to those in WT grown in standard HS medium, are the mean ± standard error of three biological replicates. Samples indicated with an asterisk are significantly different from the controls ($P < 0.05$) in a two-tailed Student's t-test.

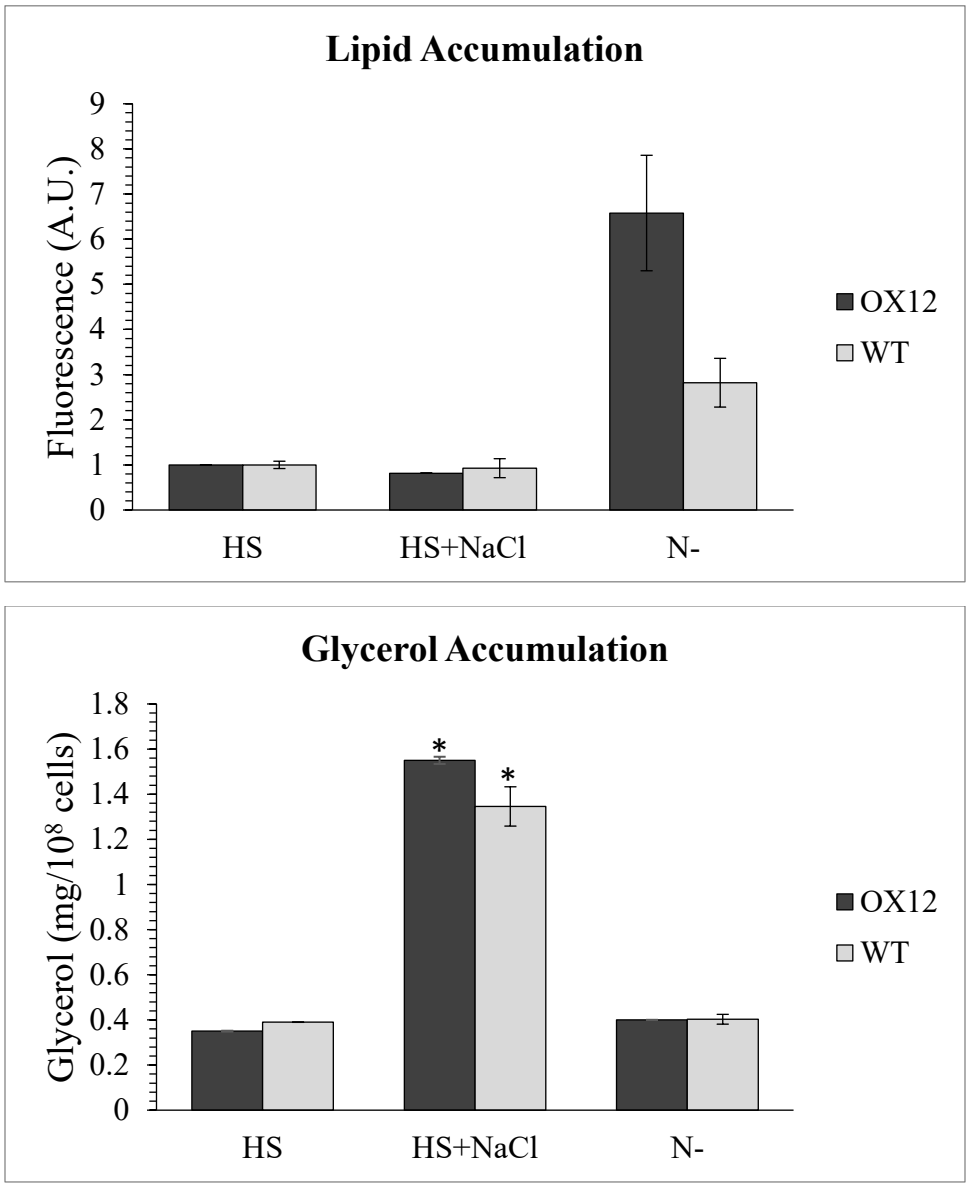


**Figure 3**. Product accumulation under different environmental conditions. When wild-type (WT) and transgenic (OX12) *Chlamydomonas* cells are grown under nitrogen deplete conditions (N-), non-polar lipid accumulation, determined by Nile Red fluorescence, is observed. Conversely, when cells are exposed to osmotic stress, glycerol production increases. Statistical differences were tested by the Student t-test relative to HS samples (* indicates a p-value <0.05).

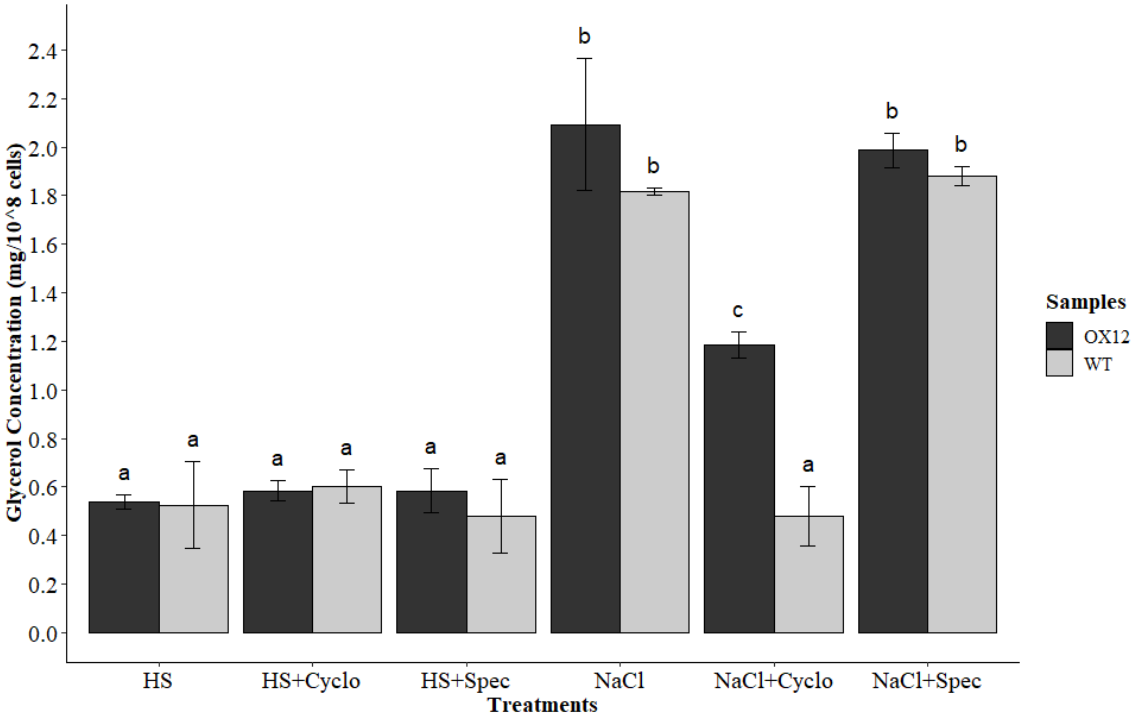


**Figure 4**. Glycerol production by *Chlamydomonas* increases under osmotic stress (+NaCl) conditions. Cycloheximide inhibits protein synthesis from cytosolic ribosomes and prevents accumulation of glycerol in wild-type (WT) cells. Overexpressing GPD2 lines (OX12) maintain higher glycerol production even when cytosolic protein translation has been inhibited. This may indicate that already-existing GPD2 proteins are post-translationally activated to synthesize glycerol.

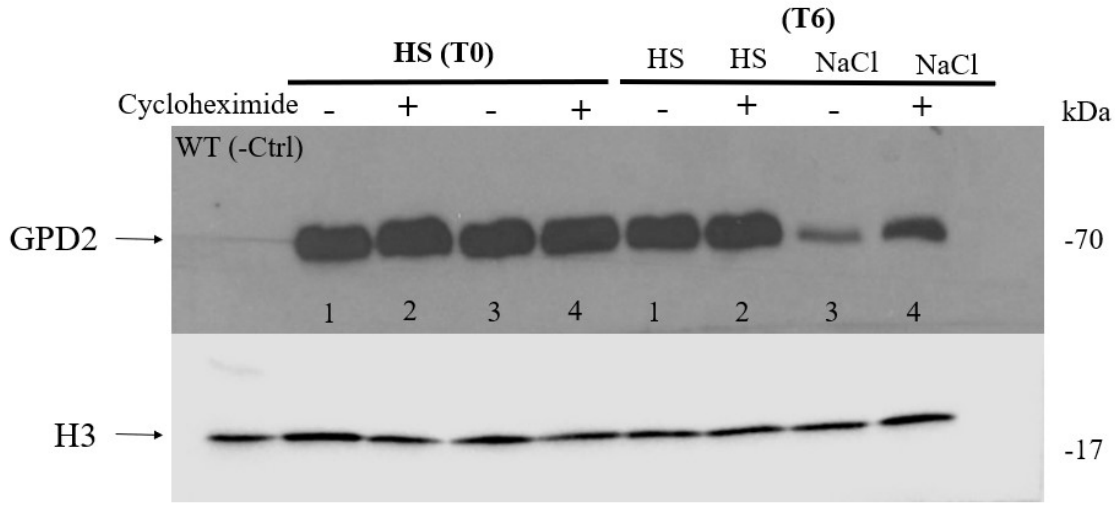


**Figure 5**. SDS-PAGE image to verify the expression level of GPD2 under different treatments. Whole protein extracts from samples with ~$10^8$ cells were loaded into SDS-PAGE gels. The transgenic proteins were epitope-tagged. Time zero (T0) are samples taken after inhibition of ribosomal protein translation in the cytosol of OX12 cell lines with 150 µg/mL of cycloheximide for an hour. Time six (T6) are samples taken after treatment with osmotic stress (100 mM NaCl for 6 hours). The Western blot for histone H3 served as loading control.

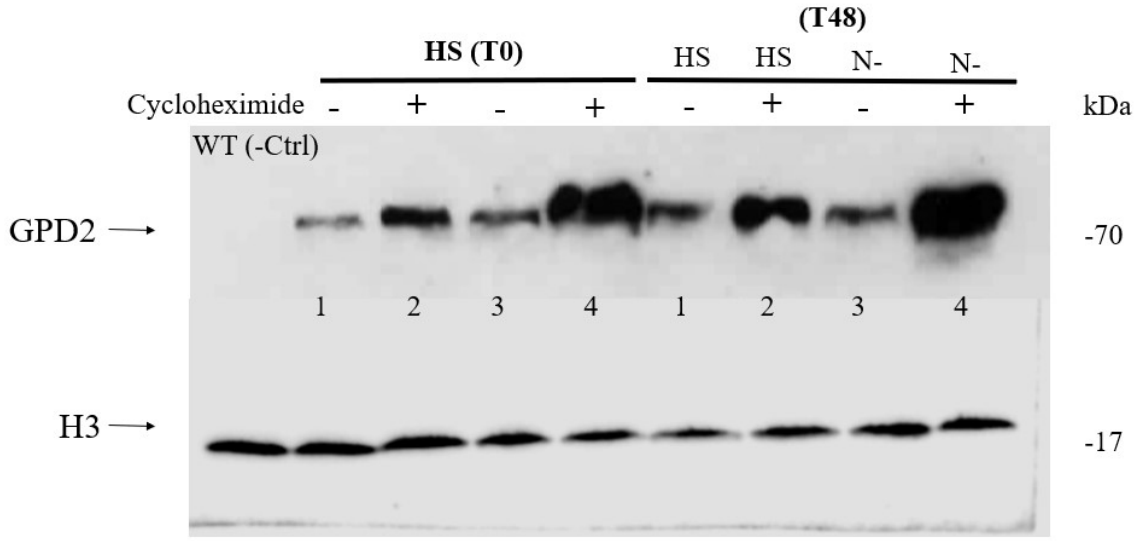


**Figure 6**. SDS-PAGE image to verify the expression level of GPD2 under different treatments. Whole protein extracts from samples with ~$10^8$ cells were loaded into SDS-PAGE gels. The transgenic proteins were epitope-tagged. Time zero (T0) are samples taken after inhibition of ribosomal protein translation in the cytosol of OX12 cell lines with 150 µg/mL of cycloheximide for an hour. Time forty-eight (T48) are samples taken 48 hours after nitrogen deprivation treatment. The Western blot for histone H3 served as loading control.

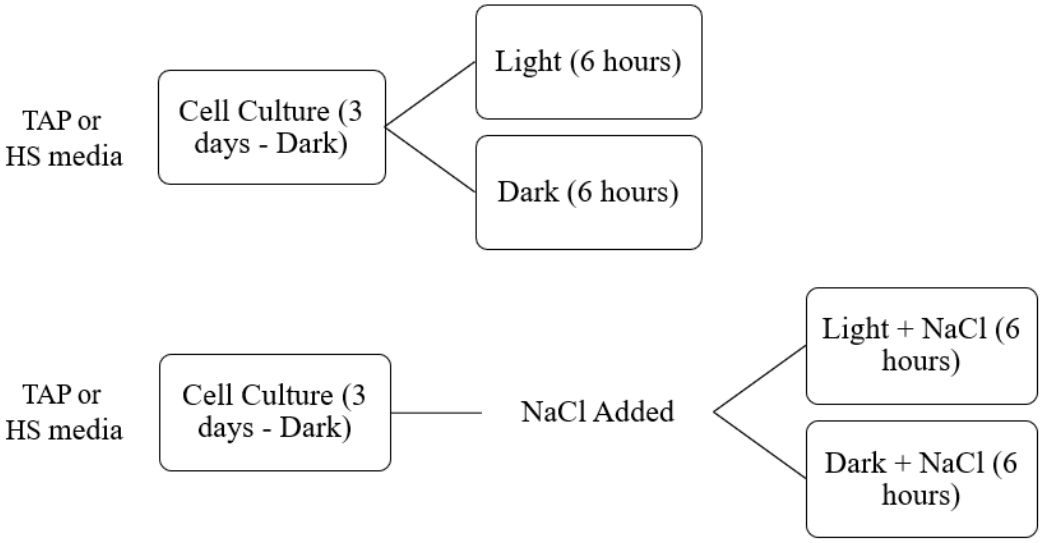


**Figure 7a**. Experiment design. We measured glycerol synthesis when photosynthesis is not occurring (experiments were conducted in the dark). WT cells were grown in HS or TAP medium in the dark (no photosynthesis) for 3 days, and then treated with NaCl for 6 hours. Some cultures were exposed to light during those 6 hours, and some others were kept in the dark. Then, glycerol accumulation was determined. Results shown are average of biological and technical replicates.

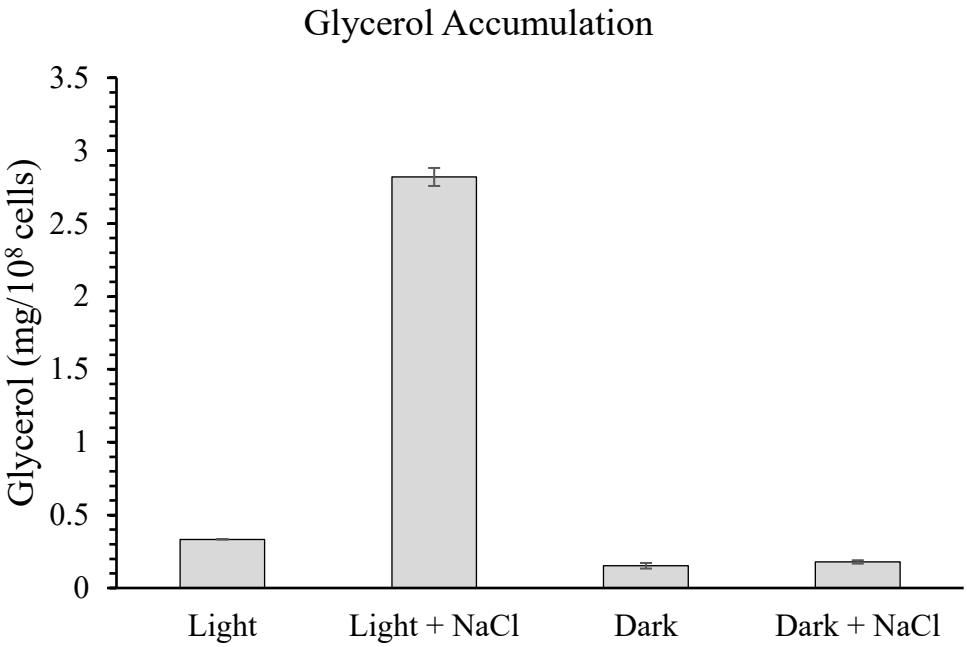


**Figure 7b**. Glycerol accumulation of cells grown in HS media under dark conditions and then exposed to osmotic stress.

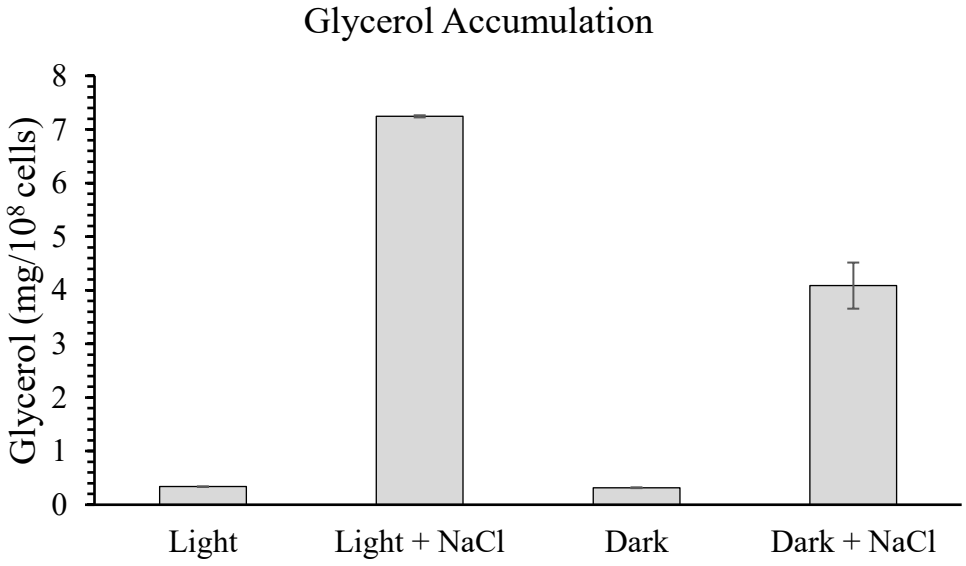


**Figure 7c**. Glycerol accumulation of cells grown in TAP media under dark conditions and then exposed to osmotic stress.

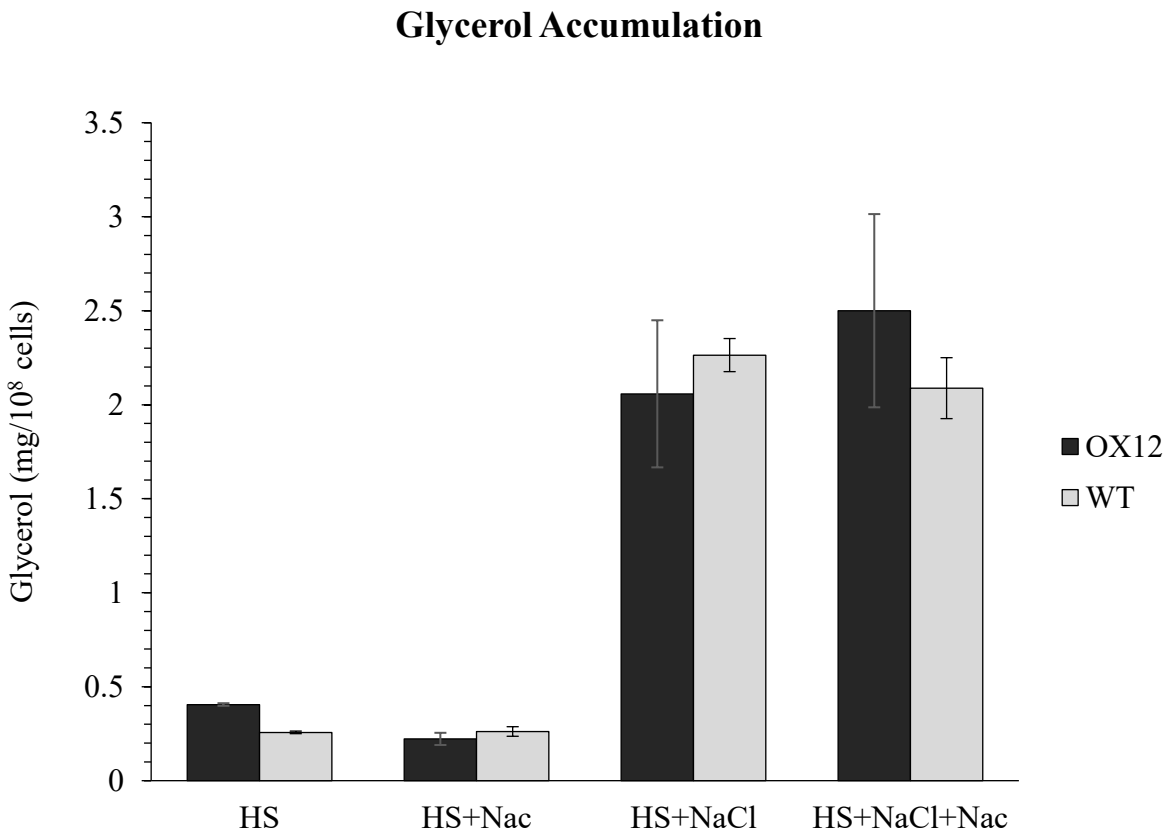


**Figure 8**. Glycerol accumulation of cells treated with 1 μM of Nac (permeable ROS scavenger) was not affected.

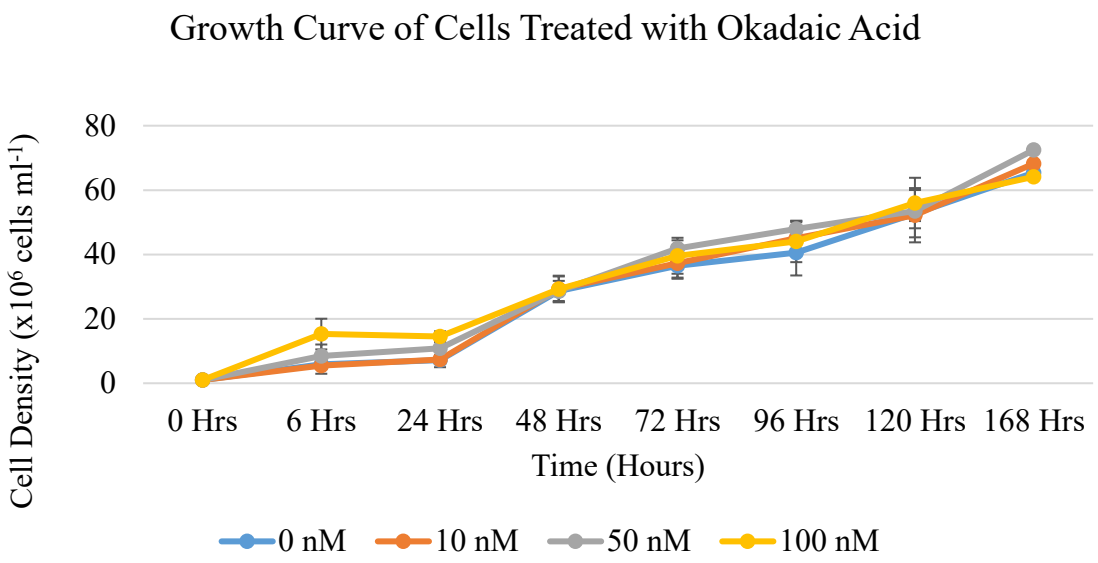


**Figure 9**. Cell density of wild-type (WT) Chlamydomonas cultures grown in High-Salt (HS) minimum nutrient media exposed to different inhibitor concentrations and under continuous light.

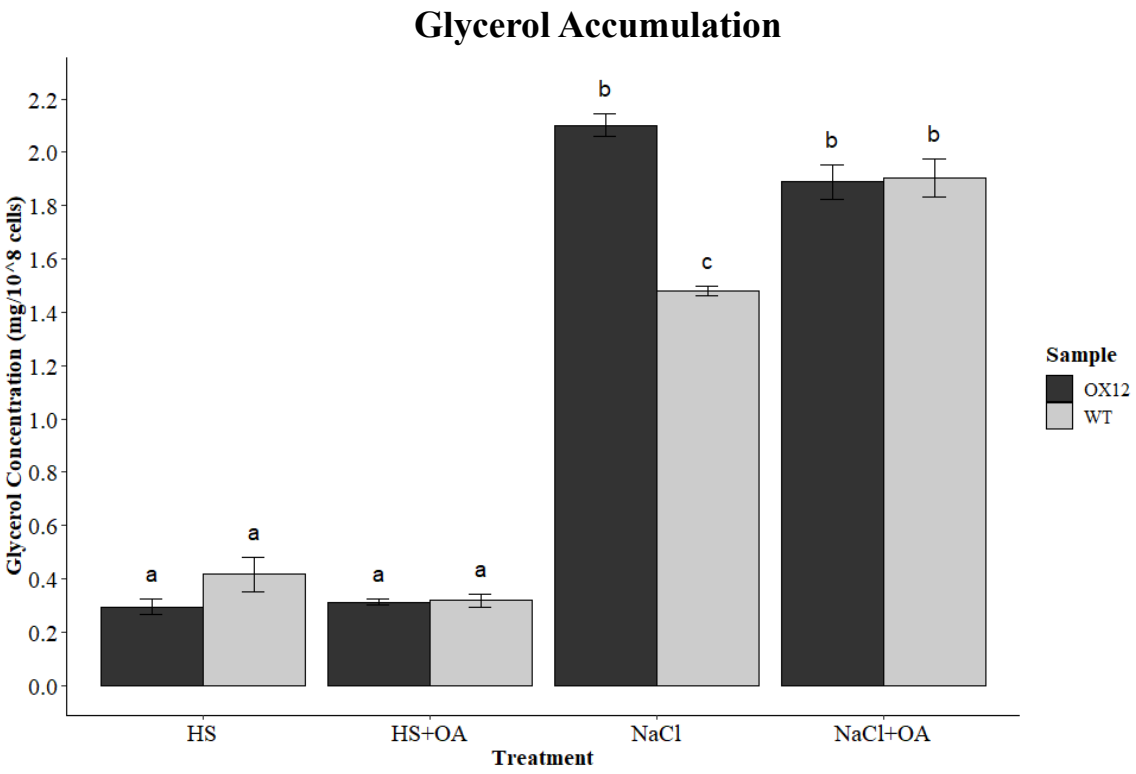


**Figure 10**. Glycerol accumulation of WT and OX12 cells cultures treated with okadaic acid (OA) and exposed to osmotic stress. Cells were grown in HS media for 2 days, then inhibitor was added. On day 3, when cultures were at the logarithmic state, cells were treated with NaCl for 6 hours and glycerol accumulation was measured. OA does not inhibit glycerol accumulation.

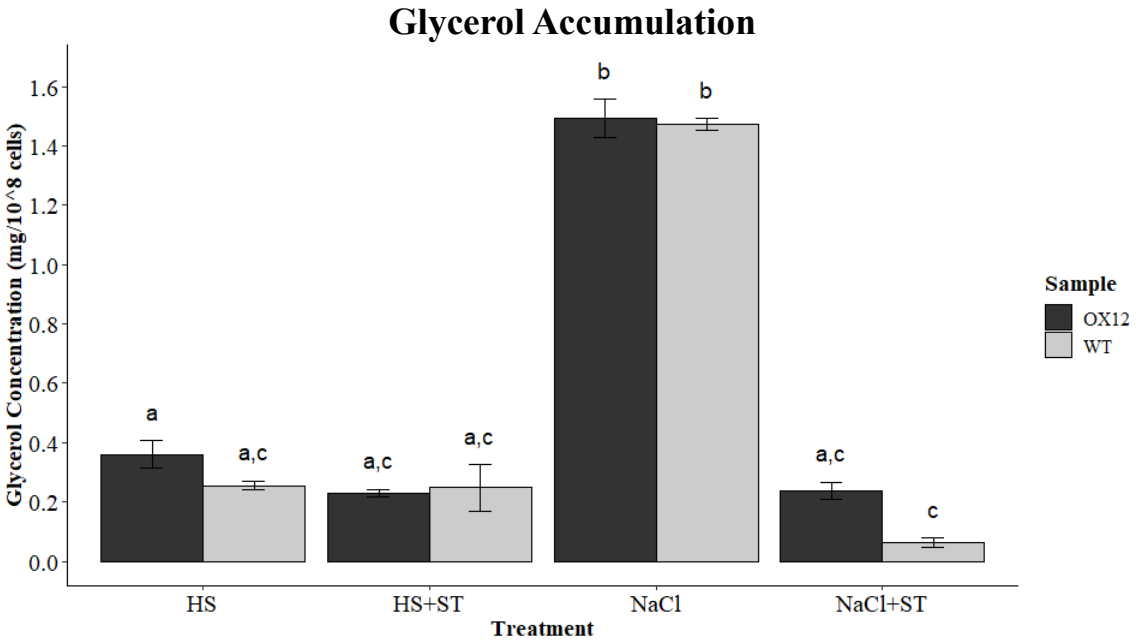


**Figure 11**. Glycerol accumulation of cells treated with 1 µM of staurosporine (ST). Glycerol accumulation was inhibited when cells were under high salinity treatment. The presence of ST inhibits glycerol accumulation in both WT and OX12 cell lines.

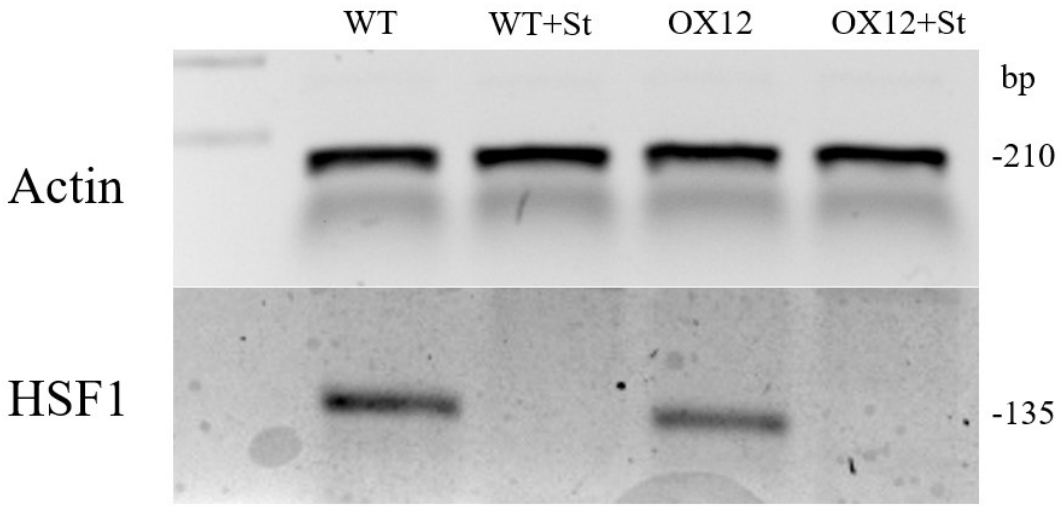


**Figure 12**. RT-PCR of cells treated with staurosporine. Staurosporine inhibits the expression of *HSF1* gene, which is expressed under heat shock stress, as demonstrated by (Zalutskaya *et al.*, 2015).

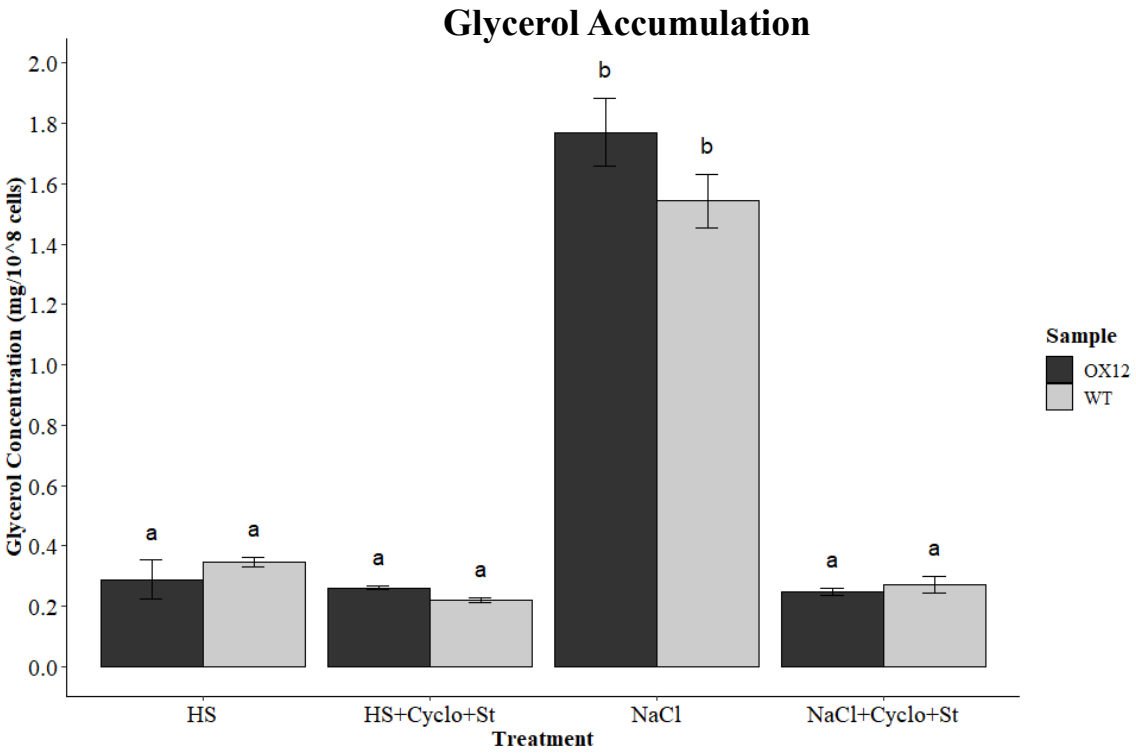


**Figure 13**. Glycerol accumulation of cells treated with staurosporine and cycloheximide. Cells were treated with 1 µM of staurosporine (ST) and 150 ug/mL of cycloheximide as previously described. No new proteins were being synthesized, and pre-existing GPD2 was inhibited and glycerol accumulation was not observed.

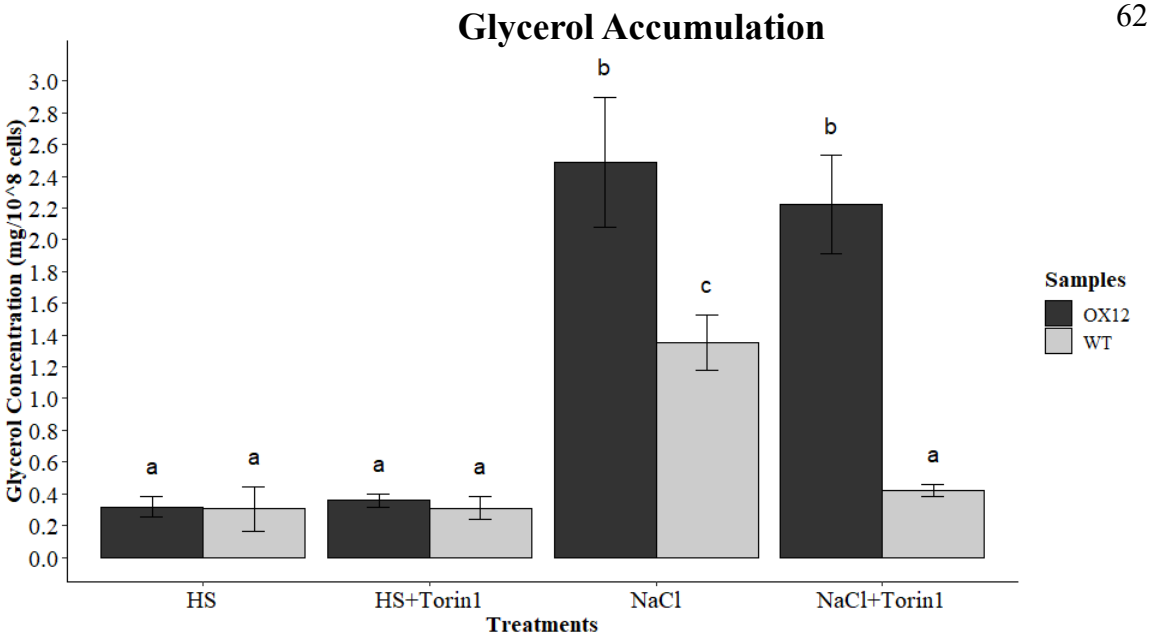


**Figure 14**. Glycerol accumulation of cells treated with 1 µM of Torin1. Torin1 did not inhibited glycerol accumulation of OX12 cells under high salinity stress conditions.

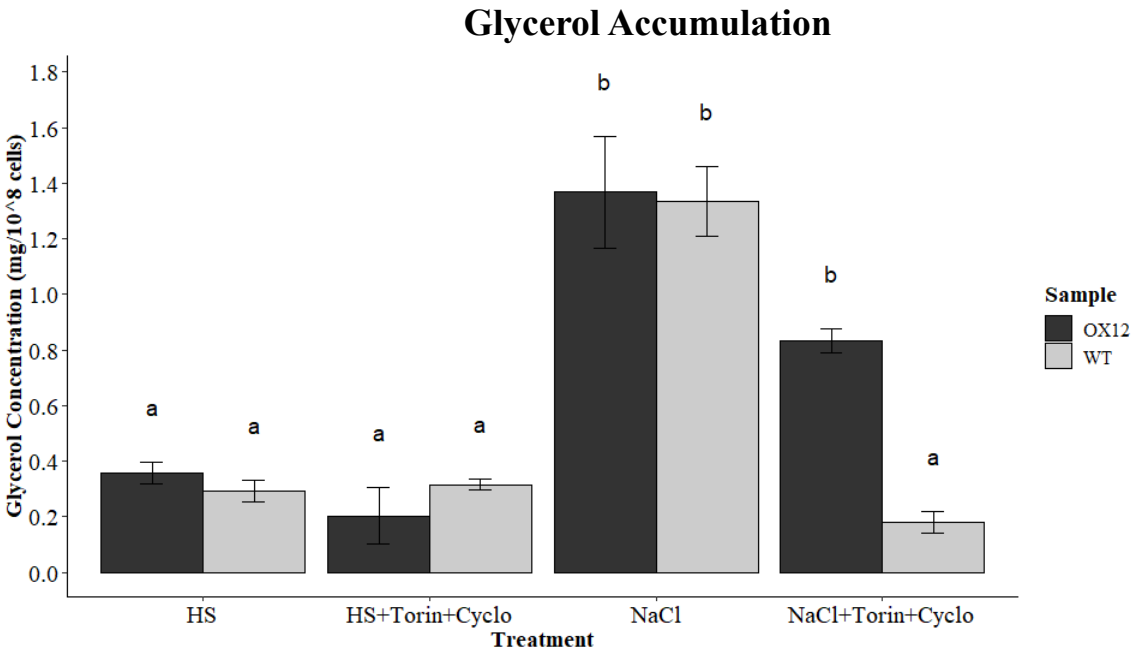


**Figure 15**. Glycerol accumulation of cells treated with 1 µM of Torin1 and 150 ug/mL of cycloheximide. Torin1 did not inhibited glycerol accumulation of OX12 cells under high salinity stress conditions.

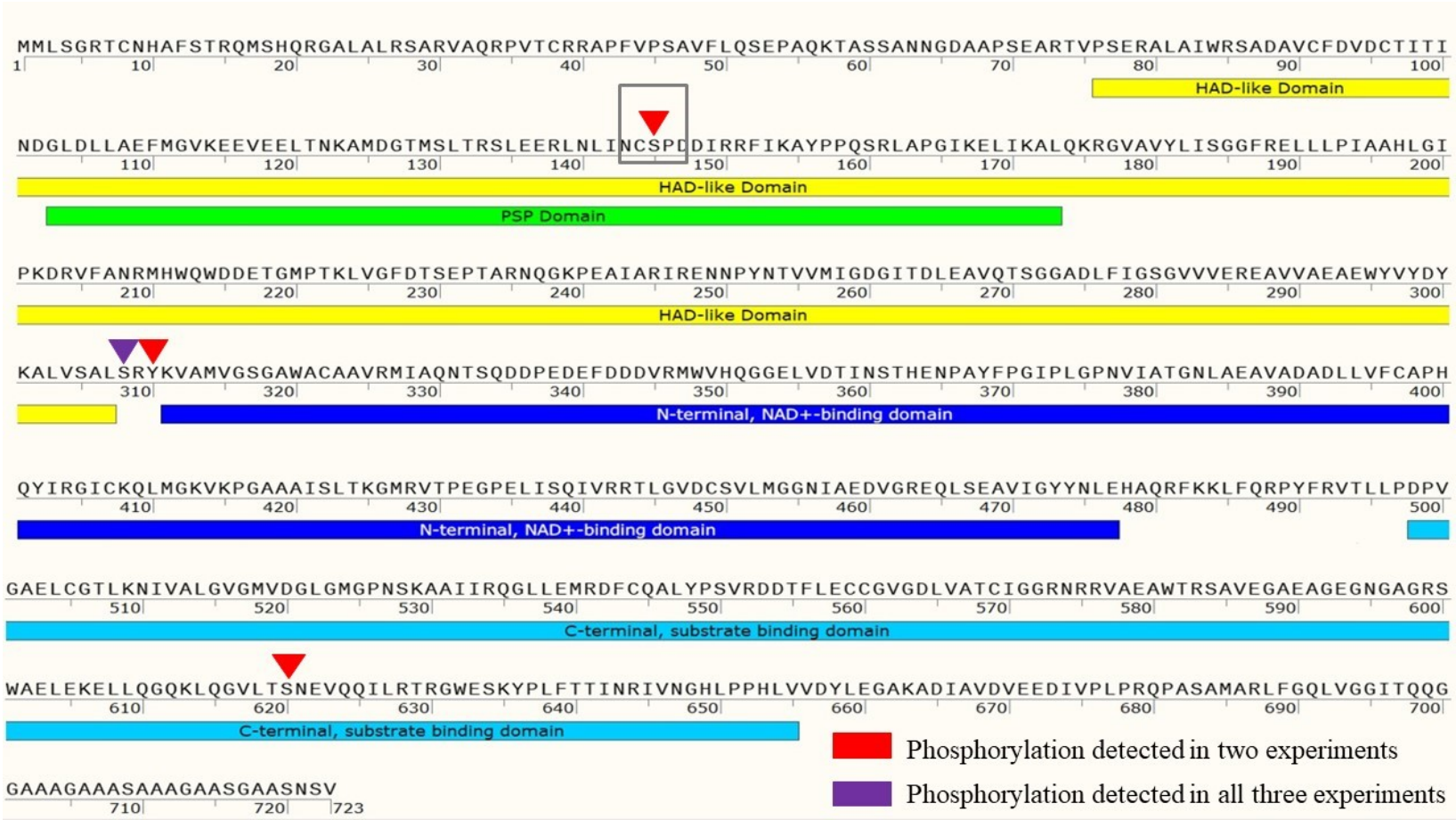


**Figure 16.** Mapping of phosphorylation sites. Phosphorylation sites detected by mass spectrometry analysis of GPD2 overexpressing cell lines grown under control (HS medium) and treatment conditions (HS+ 100 Mm NaCl). Phosphorylation in Ser308, Tyr310, and Ser620 were detected in protein isolated under both control and treatment conditions. Phosphorylation in the Ser145 site (boxed) was detected only under NaCl (treatment) conditions.

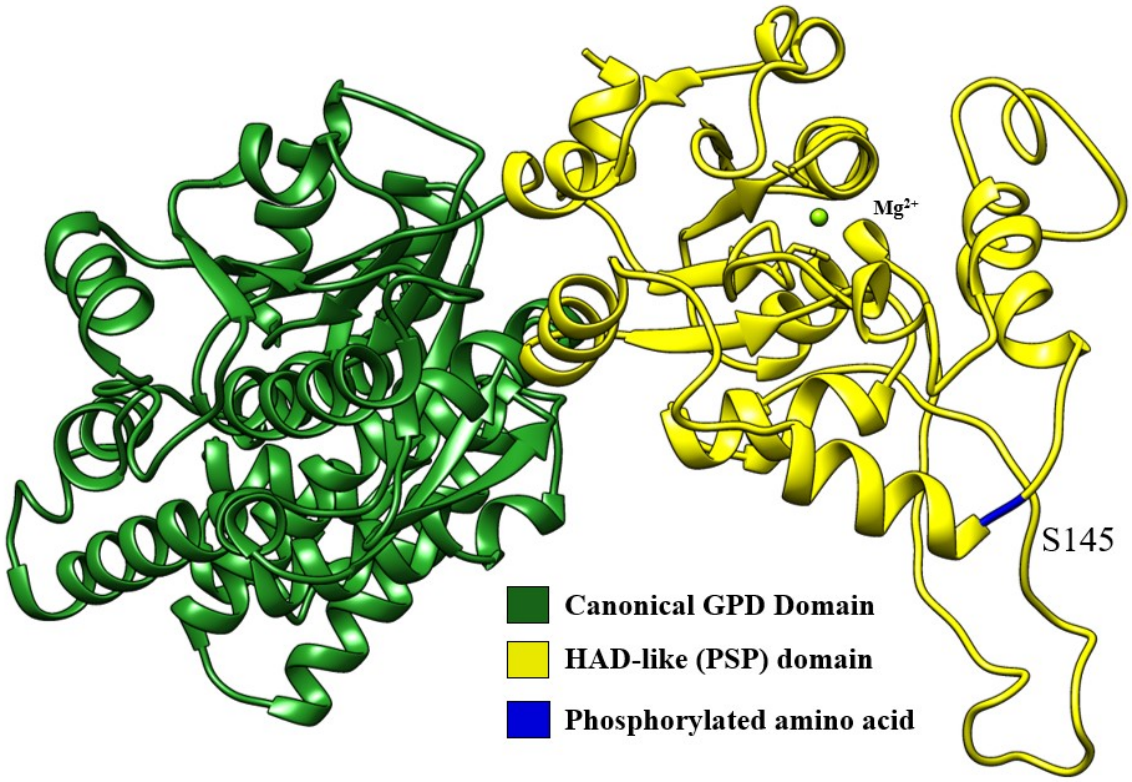


**Figure 17**. Structure of *Chlamydomonas reinhardtii* GPD2 homolog highlighting phosphorylated amino acid. Molecule generated by Swiss-Model using the PSP/GPDH of *D. salina* as template. Green region shows the canonical GPD domain. Yellow region indicates the PSP-like domain with an embedded magnesium ion (light green). Blue shows the amino acid of interest within the PSP-like domain. The residue Serine-145 appears to be phosphorylated under osmotic stress but not under control conditions.

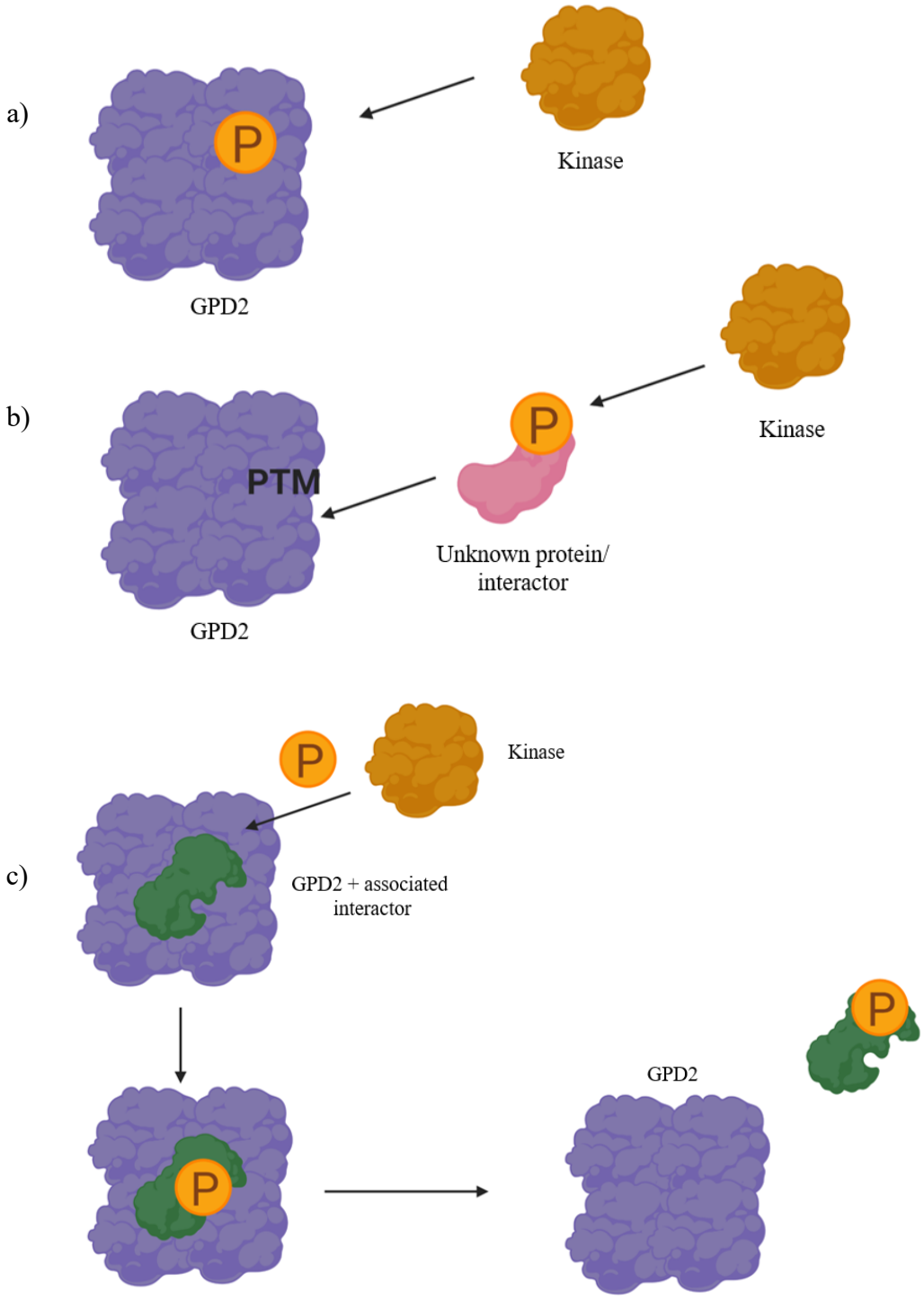


**Figure 18**. Illustration of how cells may regulate PSP-like domain activity of GPD2 homolog in *C. reinhardtii* based on our kinase inhibitor experiments. a) direct phosphorylation of PSP-like domain on GPD2 homolog. b) Indirect effect in which a

kinase phosphorylates an unknown protein or interactor that in turns ‘activates’ PSP-like domain with another PTM or other mechanism. c) PSP-like domain is associated with an unknown protein forming a configuration that renders it non-functional. An unknown kinase phosphorylates the associated protein, which disassociates from GPD2. PSP-like domain becomes functional and is capable of glycerol synthesis.