# INVESTIGATION OF CELL WALL INTEGRITY SIGNALLING IN *CHLAMYDOMONAS REINHARDTII*

by

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## Abstract

A fundamental aspect of plant survival is the development of a resilient cell wall. Throughout the life cycle of the unicellular alga *Chlamydomonas reinhardtii*, the cell wall is continuously synthesized and remodelled. When cells are starved of nitrogen, they differentiate into gametes and enter the sexual (meiotic) phase. In doing this, mating gamete cells shed their cell walls via gametolysin, fuse their naked protoplasts, and build an exceptionally hardy zygote cell wall. Previous research on the sexual life cycle has focused on regulatory events upstream of the mating reaction, i.e. gametogenesis, and downstream of cell fusion during zygote development. As such, we don't yet understand how cells are responding to wall shedding at the onset of the mating reaction. A recent transcriptome analysis has reported a set of genes that are specifically regulated by gametolysin-mediated wall shedding, consisting of two major functional categories – protein processing-related and cell wall-related. The research presented here attempts to understand the level at which these genes are regulated and establishes three hypotheses for what the 'trigger' of their regulation might be. Using a suite of target genes as representative factors of the gametolysin-mediated event, we performed promoter-reporter and mRNA expression analyses in several treatment conditions and cell lines to examine this regulation. We found both target genes and their respective promoters are strongly induced by gametolysin, indicating transcription-level regulation. When cells were pre-treated with a protein synthesis inhibitor prior to gametolysin, we observed differential effects on the transcription of our target genes, suggesting multiple regulatory proteins control this event. We also observed that an ER stress response likely helps to regulate cell wall recovery, as a stress response-impaired mutant showed diminished transcript expression following gametolysin treatment. Cell

wall-defective mutants were found to have a negligible response to gametolysin, but expressed our target genes constitutively, indicating the need for cells to maintain a cell wall. Early investigations of our hypotheses for the 'trigger' of the gametolysin-mediated response suggests a minor role for osmotic stress signalling and that sensing the condition of the cell wall is likely the primary 'trigger.'

## Lay Summary

A defining feature of plant cells is the presence of a cell wall. In the single-celled alga *Chlamydomonas reinhardtii* this wall is modified or built new many times throughout its life. Occasionally, when two nitrogen-starved algae cells meet, they initiate a 'mating reaction' and remove their cell walls. In doing this, two things happen: 1) the cells become 'naked' and exposed to their environment, and 2) the cells immediately rebuild their cell walls. This research aims to investigate these events; how cells sense their 'nakedness,' and how this leads to the rebuilding of the wall. By studying several genes involved with these events, we found that physically removing the cell wall turns these genes and their regulators 'on' and that there may be multiple elements controlling this regulation. We also found that cells lacking normal walls have their genes always turned 'on' in attempts to build a normal cell wall.

## Preface

A portion of the work presented in Chapter 2 has been submitted and accepted for publication as part of a larger study about global transcriptional change and regulation in *C. reinhardtii* during zygote development in Joo et al. (2017). Gene regulatory networks for the haploid-to-diploid transition of Chlamydomonas reinhardtii. Plant Physiology. 175(1), 314-332. In this work, I was responsible for designing and engineering promoter-reporter plasmids and transforming these constructs into *C. reinhardtii* cells. I also conducted promoter activity assays in mating cells, zygote cells, and cells treated with gametolysin.

All experiments and research questions were designed by Evan Cronmiller and Dr. Jae-Hyeok Lee with additional advising and supervision by Dr. Sunjoo Joo. All experiments were carried out by Evan Cronmiller, apart from engineering the *proRHM1* promoter-luciferase construct, which was done by Michelle Wang.

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## List of Symbols and Abbreviations

| | |
|---|---|
| AP | 2-acetylpyridine |
| bp | Base pair |
| bZIP | Basic-region leucine zipper |
| cgLUC | *Gaussian* luciferase |
| CHX | Cycloheximide |
| °C | Degrees Celsius |
| Cq | Quantification cycle |
| CV | Contractile vacuole |
| CW | Cell wall |
| CWD | Cell wall defective |
| DNA | Deoxyribonucleic acid |
| E | Einstein unit (measurement of photon flux; 1 E = 1 μmol $m^{-2}$ $s^{-1}$) |
| *E. coli* | *Escherichia coli* |
| e.g. | *exempli gratia* |
| EDTA | Ethylenediaminetetraacetic acid |
| ER | Endoplasmic reticulum |
| ERAD | Endoplasmic reticulum-associated degradation |
| EZ(Y) | Early zygote-specific |
| GLE | Gamete lytic enzyme; gametolysin |
| GOI | Genes of interest |
| HRGP | Hydroxyproline-rich glycoprotein |

| | |
|---|---|
| i.e. | *id est* |
| NF-TAP | Nitrogen-free Tris-acetate-phosphate media |
| NIC | Nicotinamide |
| Pa | Pascal unit (measurement of pressure; 1 Pa = 1 kg $m^{-1}$ $s^{-2}$) |
| PCR | Polymerase chain reaction |
| pDNA | Plasmid DNA |
| PM | Plasma membrane |
| pro | promoter |
| PS | Periplasmic space |
| qPCR | Quantitative polymerase chain reaction |
| RLU | Relative light unit |
| RNA | Ribonucleic acid |
| RPM | Rotations per minute |
| RPKM | Reads per kilobase (of transcript) per million (mapped reads) |
| RT | Room temperature (21°C) |
| TAP | Tris-acetate-phosphate media |
| TM | Tunicamycin |
| UPR | Unfolded protein response |

## Acknowledgments

First and foremost, I would like to thank Dr. Jae-Hyeok Lee, without whom I would not have succeeded in this project. His sage advice and realistic approaches to problems – big or small – kept my eyes open and mind stimulated, and allowed me to grow and learn as a research professional. As well, Dr. Lee's patience and understanding as a supervisor were intangibles I appreciated so much; qualities I especially appreciated when the "going got tough."

I also would not have successfully navigated my graduate studies without the help and support of the other Lee lab members. Dr. Sunjoo Joo's patience, time, and training were essential to my development as a scientist and her demeanor made working in the lab a pleasure. My fellow graduate student colleagues, Thamali Kariyawasam, Jacob Munz, Yuan Xiong, and Michelle Wang were always of great help and support, and provided oft-needed conversation and distraction. A number of transient undergraduate students should also be thanked for numerous reasons, from their hard work completing menial laboratory tasks to allowing me the opportunity to teach and supervise.

Part of my success I owe to the guidance of my committee members: Dr. Ljerka Kunst, Dr. Abel Rosado, and the late Dr. Carl Douglas. Their thoughtful ideas and constructive criticisms were helpful in guiding the direction of my thesis project. I am also very thankful for their realistic attitudes during meetings and in conversation, which kept me level-headed and grounded.

Finally, I owe so much to my friends and family. Without the continuous support of the amazing and loving people in my life, I would not have been able to achieve such challenging goals.

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## Dedication

I would like to dedicate this work to those who believed in, and supported me throughout the many years of my education.

I would also like to dedicate this research to the late Carl Douglas, whose brilliance and character were both inspirational and aspirational.

# Chapter 1: Introduction

## 1.1 The significance of the structurally resilient *Chlamydomonas reinhardtii* cell wall

A fundamental aspect of plant survival in nature is the ability of a plant to synthesize a resilient cell wall. This wall provides protection from the environment, predators, and pathogens, helps maintain water balance, and gives structure to the cell. The morphology and composition of plant cell walls varies across the Viridiplantae, but the differences are more distinct between aquatic and land dwelling species. The colonization of land is thought to have driven cell wall evolution including a dramatic change in composition (reviewed in Popper et al., 2011). For example, the land plant *A. thaliana* has a primary cell wall comprised mostly of complex polysaccharides and ~10% protein, whereas most Volvocine algae have cell walls that are mostly proteinaceous (Miller et al., 1974; van de Meene et al., 2017). The cell wall of the Volvocale *Chlamydomonas reinhardtii* is made almost entirely of glycoproteins and its unique layered arrangement make it both hardy and flexible (Goodenough & Heuser, 1985). The rapid growth of *C. reinhardtii* cells during the asexual (mitotic) life cycle requires flexibility and continuous building of the cell wall to accommodate quickly expanding cells. Not only is the cell dynamic during mitotic growth, but it is significantly remodelled during the sexual life cycle. In poor environmental conditions, two gamete cells may undergo sexual mating wherein the cell wall is completely shed to allow gamete fusion and is then rebuilt entirely to form an exceptionally tough zygote cell wall (Grief et al., 1987). This zygote wall is nearly impenetrable and dessication resistant, providing a safe environment for the cells to lay dormant until conditions are once again favourable.

## 1.2 The *C. reinhardtii* life cycle - in brief

The life cycle of *C. reinhardtii* is remarkably simple. This simplicity reflects the evolutionary history of the species, as its primarily haploid and aquatic lifestyle is indicative of an early divergent plant species (Cross & Umen, 2015). *Chlamydomonas* spend the large majority of their life in a haploid vegetative state. Provided ideal growth conditions – light, nutrients, etc. – cells will happily undergo successive mitotic growth and fission cycles every 8-10 hours, producing anywhere between 2-8 daughter cells (Harris, 1989). When environmental nitrogen sources are depleted, cells will initiate gametogenesis and enter a sexual life cycle. The gametes have 'sexes', either mating type plus (mt+) or mating type minus (mt-), depending on which mating-type locus the gamete carries, *MT*+ or *MT*- (reviewed in Goodenough et al., 2007). When in close proximity, gametes of opposite mating types will recognize one another via sex-specific agglutinin proteins displayed on their flagella and will begin the mating reaction (Ferris et al., 2005). During the mating reaction in *C. reinhardtii* gametes shed their cell walls, naked gamete protoplasts fuse, and cytosolic contents mix, all within a matter of minutes. Minus and plus gametes each possess a key cytosolic protein, GSM1 (Gamete-Specific Minus 1) and GSP1 (Gamete-Specific Plus 1), transcription factors that heterodimerize following gamete fusion and translocate to the nucleus to initiate zygote development (Lee et al., 2008). A hallmark of zygote development is the synthesis of an extremely hardy cell wall, which has a composition and geometry different than that of vegetative and gamete cells (Minami & Goodenough, 1978). This zygote, or 'zygospore', will now remain dormant until environmental conditions are favourable enough to promote meiosis and hatching of daughter cells.

## 1.3 Cell wall composition

The ultrastructure and composition of the *C. reinhardtii* cell wall has been researched extensively over the past several decades. Early electron microscopy and biochemical studies found the vegetative and gamete wall to be comprised of 5 discrete layers with highly ordered chemical and geometric arrangements (Goodenough & Heuser, 1985). Conversely, the zygote cell wall may contain 4 – 8 layers and lacks much of the order and crystallinity of the vegetative and gamete cell walls (Grief et al., 1987). The cell wall is known to be composed almost entirely of hydroxyproline-rich glycoproteins (HRGPs) and lacks matrix polysaccharides (Miller et al., 1974). This proteinaceous wall is unique to some aquatic algae, compared to the more common polysaccharide and cellulosic walls of most higher plants (Popper et al., 2011). A recent bioinformatics study found the *C. reinhardtii* genome to contain approximately 182 genes encoding an extensin-like domain signature - two or more serine/proline SPPP repeats - far more than any other plant species tested in the study (Liu et al., 2016). These SPPP motifs are glycosylated regions of HRGPs, which contributes to the strength and geometric arrangement of these proteins in the cell wall.

Curiously, no extracellular receptor proteins that directly detect the cell wall have been identified in *C. reinhardtii* (Liu et al., 2016). However, possible candidates could resemble the WSC (cell *w*all integrity and *s*tress response *c*omponent) protein family found in *S. cerevisiae* or the WAK (*w*all *a*ssociated *k*inase) family in *A. thaliana,* which detect perturbations in the cell wall and activate signal transduction cascades (Verna et al., 1997; reviewed in Humphrey et al., 2007).

## 1.4 Modification and regulation of the cell wall

During the life cycle of *C. reinhardtii*, there is a continuous building and rebuilding of the cell wall. Whether it is expansion of the cell during mitotic growth or complete removal of the wall during sexual mating there is a need for constant synthesis of wall components. While we understand to some extent what genes and proteins are involved in cell wall regeneration following wall removal, much of the genetic regulation has not been elucidated.

### 1.4.1 Cell wall lytic enzymes

There are two known enzymes that facilitate the removal of the *Chlamydomona*s cell wall, gametolysin and sporangin. The first identified, gametolysin or gamete lytic enzyme (GLE), is essential in the removal of the gamete cell wall during sexual mating/fertilization. GLE is a metalloprotease and is stored in the periplasm of both vegetative and gamete cells in the form of an inactive proenzyme "s-lysin" (Buchanan et al., 1989; Kubo et al., 2001). Following flagellar agglutination of gametes, another enzyme, p-lysinase, is synthesized and post-translationally modifies s-lysin to form active GLE (Snell et al., 1989). The now active enzyme will digest the gamete cell wall, allowing naked gamete protoplasts to fuse and initiate zygote development. It is hypothesized that GLE acts specifically on the "W2" layer, the second innermost layer of the *C. reinhardtii* cell wall, as it is readily degraded by GLE (Goodenough & Heuser, 1985). The other key cell wall removal enzyme, sporangin (vegetative lytic enzyme), is specific to the degradation of the sporangial (mother) cell wall and facilitates the hatching of daughter cells from the mother cell during mitotic fission (Matsuda et al., 1995; Kubo et al.,

2009). In contrast to the continuous transcription of s-lysin, sporangin was found to be transcribed only during the S/M phases of the mitotic cycle. The sporangin is then secreted and localized – by an unknown mechanism – to the flagella of the daughter cells in a clever manner to degrade only the sporangial cell wall and not their own (Kubo et al, 2009).

### 1.4.2 Regulation of cell wall regeneration - clues from early studies

A considerable amount of research has been dedicated to understanding the genetics associated with the haploid-to-diploid transition and zygote development in *C. reinhardtii*. Early studies generated cDNA libraries of zygote cells and found many genes expressed only during early zygote development (< 3 hours after mating). Uchida et al. (1993) identified 5 zygote-specific (*ZYS*) genes, one of which, *ZYS3,* was later found to encode an ER-resident protein putatively involved with the synthesis and processing of proteins (Kuriyama et al., 1999). Using a similar approach, Ferris and Goodenough (1987) found a number of zygote-specific (*ZSP*) mRNAs highly expressed within 3 hours of mating. Two of these genes, *ZSP1* and *ZSP2,* were sequenced and found to encode putative cell wall HRGPs similar to extensins and lectins of higher plants, respectively (Woessner & Goodenough, 1989; Suzuki et al., 2000).

von Gromoff and Beck (1993) generated cDNA libraries to compare vegetative and 9-hour nitrogen starved gamete cells and identified 4 gamete-specific (*GAS)* genes expressed at high levels only in gametes. Further analysis of these *GAS* genes found 3 out of 4 to be highly expressed in early zygotes as well, indicating the presence of a zygote-specific factor that acts to suppress a subset of gamete-specific genes. Only one of these genes, *GAS28,* was functionally characterized and found to encode a cell wall HRGP and has been used for analysis in our

present studies (von Gromoff & Beck, 1993). Other *GAS* genes, *GAS29, -30, -31* were later found by Rodriguez et al. (1999) and Hoffmann and Beck (2005), all of which encode cell wall HRGPs, suggesting that cells up-regulate cell wall transcripts in anticipation of the cell wall shedding during the mating reaction.

A study by Kubo et al. (2008) used cDNA macroarrays and identified 21 novel *EZY* (early zygote expressed) genes up-regulated within 1 hour of mating. Unlike earlier research, many of the *EZY* genes they annotated did not encode cell wall proteins, but rather were predicted to be involved with sugar metabolism, secretion, and gene expression (Kubo et al., 2008). They also observed that a few *EZY* mRNA were only highly expressed towards the end of the 1 hour time course, suggesting that there are multiple regulatory mechanisms involved with zygote development. Furthermore, they mated a gamete fusion-defective mutant *fus* with wildtype, and observed 9 of 21 *EZY* genes were still up-regulated. Of these 9 genes expressed following wall shedding, *EZY23,* which encodes a putative ER translocon subunit, was picked up in our transcriptome analysis and is used in the current study under the name *SEC61G*. The conclusion of their study suggested that some genes are up-regulated following cell wall shedding and others require cell fusion or events further downstream.

The key to this differential regulation during mating and zygote development was uncovered by Lee et al. (2008) who found the homeoproteins GSM1 and GSP1 to be the 'master regulators' of zygote development. Following fusion of gametes, GSM1 and GSP1 were found to dimerize and initiate a global transcriptional change inducing >200 zygote-associated genes (Lee et al., 2008; Joo et al., 2017). The requirement of GSM1:GSP1 as a zygote activator was further confirmed by Nishimura et al. (2012) who used a *GSP1* null mutant in mating reactions and found zygote-specific gene expression to be inhibited.

### 1.4.3 Regulation of cell wall regeneration – clues from transcriptome data

Over the past decade since the publication of the sequenced *C. reinhardtii* genome, there has been a tremendous amount of high-throughput data generated by the *Chlamydomonas* community. An important transcriptome analysis by Miller et al. (2010) compared total transcript expression of nitrogen-replete and nitrogen-starved cells. Their analysis indicated a global shift in gene expression in nitrogen-starved cells as >4000 genes were differentially expressed. This included the up-regulation of gamete-specific factors (sex factors, etc.) and transcription factors, the down-regulation of photosynthesis-related genes, and a metabolic shift leading to the accumulation of triacylglycerols (TAGs) (Miller et al., 2010).

Another group attempting to identify factors essential to sexual development analyzed transcriptome data from 8 experimental conditions, including both plus and minus gametes treated with GLE (Ning et al., 2013). They used a clustering analysis to group genes based on their expression patterns and observed 42 distinct gene clusters. Of note was Cluster 42 (C42), which consisted of 143 genes that were strongly induced by GLE treatment in both mating types. This cluster was highly enriched in genes encoding cell wall HRGPs and matrix metalloproteinases, indicating the requirement for a cell to rebuild its wall shortly after removal.

Both transcriptome data sets described in Miller et al (2010) and Ning et al. (2013) were used in a meta-analysis by the Lee Lab with the intent of identifying factors associated with the haploid to diploid transition (Joo et al., 2017). Transcriptome data from 13 independent conditions were subject to a clustering analysis using a method similar to that of Ning et al. (2013) producing 50 gene clusters based on expression profile. Of these clusters, three showed

early zygote specific expression (C33, C43, C50; labelled “EZ-core”), one showed early zygote and GLE-specific expression (C44; labelled “gL+EZ”), and one showed only GLE-specific expression (C24; labelled “gL-EZ”) (Joo et al., 2017). Functional annotation of the genes within these groups show that of the 113 analyzed from the lysin-induced set “gL-EZ”, 36 (32%) are predicted to encode HRGP cell wall proteins and a large number are involved with glycosylation and protein processing (Figure 1.1).

Knowing the number and identity of genes induced by GLE is one matter, but understanding their regulation is another. The existing transcriptome data provide clues and targets to examine further, but as gene regulation is complex, we need to observe the lysin-induced genetic response at a deeper level.

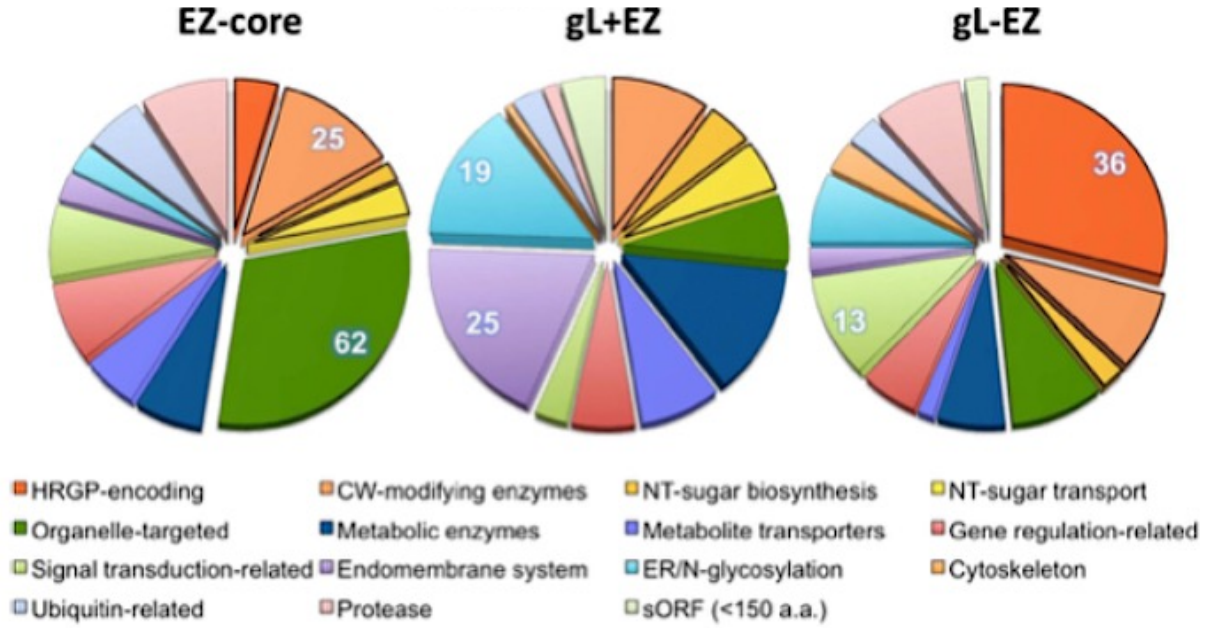


**Figure 1.1: Annotations of EZ and lysin-induced genes by functional category.** Charts are based on expression pattern, EZ-core/gL+EZ/gL-EZ and labelled accordingly. Functional categories are colour-coded and start from 12 o’clock moving clockwise, corresponding to the legend moving left to right. The number of genes for the functional categories with the two highest numbers for each set is labelled on the charts. EZ-core, n = 204; gL+EZ, n = 129; gL-EZ, n = 113. Reproduced with permission from Joo et al., 2017; Copyright American Association of Plant Biologists © 2017 (www.plantphysiol.org).

## 1.5 Three hypotheses for the trigger of the GLE-induced response

While we have a good understanding of which genes are up-regulated by GLE, we don't yet know *how* these genes are regulated. Based on existing literature and a general knowledge of *Chlamydomonas* cellular physiology, we have generated three hypotheses for what the 'trigger' of the GLE response might be and have illustrated these scenarios in Figure 1.2.

1) Osmotic stress. Osmotic stress as a trigger for the GLE response has only been discussed by Hoffmann and Beck (2005), who used Northern blot analyses to study three HRGP-encoding genes: *GAS28, GAS30*, and *GAS31.* Their results were inconclusive, suggesting that while varying osmotic conditions affected gene expression, GLE-mediated removal of the cell wall in iso-osmotic conditions also induced expression (Hoffmann & Beck, 2005). They also noted – importantly – that the removal of the cell wall could itself be a trigger. In a review of sexual differentiation and zygote development in *C. reinhardtii*, Nishimura includes osmotic stress as a potential regulator of the GLE-induced response in his comprehensive model of mating and zygote development, but does not address this inclusion directly (see Figure 8 in Nishimura, 2010). Based on fundamental cellular physiology, it is logical that osmotic stress could be a signal for cell wall regeneration. In a normal cell in liquid culture, the complete removal of the wall by GLE will suddenly expose the cell to greater hydrostatic pressure from the surrounding environment. In most plant cells, we expect some degree of turgor pressure generated in the periplasmic space between the plasma membrane and cell wall, which helps maintain water balance. Logic then suggests that the absence of a cell wall should disrupt this

water balance, which could elicit an osmotic stress response. A detailed explanation of osmoregulation in *C. reinhardtii* is discussed in section 4.1.

2) Pherophorin signalling. This hypothesis requires an extrapolation of knowledge from one of *Chlamydomonas*' close relatives, *Volvox.* One class of proteins within the HRGP superfamily is known as the pherophorins. These were originally described in *Volvox* to be biochemical 'pheromones' secreted during sexual mating (Godl et al., 1997). Recognition of these pheromones in *Volvox* stimulates both sexual differentiation of gonidial cells into gametes and remodelling of the extracellular matrix to allow for fertilization and zygote development (reviewed in Frenkel et al., 2014). Several pherophorins have been identified in *C. reinhardtii* based on sequence homology and protein domain prediction analyses, yet no evidence of a pheromone-based signalling system has been demonstrated (reviewed in Hallmann, 2006). Despite the lack of evidence for pherophorin signalling in *C. reinhardtii*, it may be possible that when cell wall proteins (of which many are pherophorin-like) are fragmented by GLE the fragments are recognized by some plasma membrane receptor protein(s).

3) Cell wall detachment. This is perhaps the simplest hypothesis. We posit that when the cell wall is removed following GLE activity, this loss is recognized by the cell. This mechanism would likely be mediated by a cell wall/membrane protein-receptor complex that, when forcibly disassociated from each other, will initiate a signal cascade.

A complication for both the pherophorin and cell wall detachment hypotheses is that no extracellular-sensing membrane proteins have been identified. This does not, however, preclude this hypothesis from being a valid explanation for the trigger of the GLE response. It is also important to note that more than one of these hypotheses could be involved in this complex regulatory mechanism.

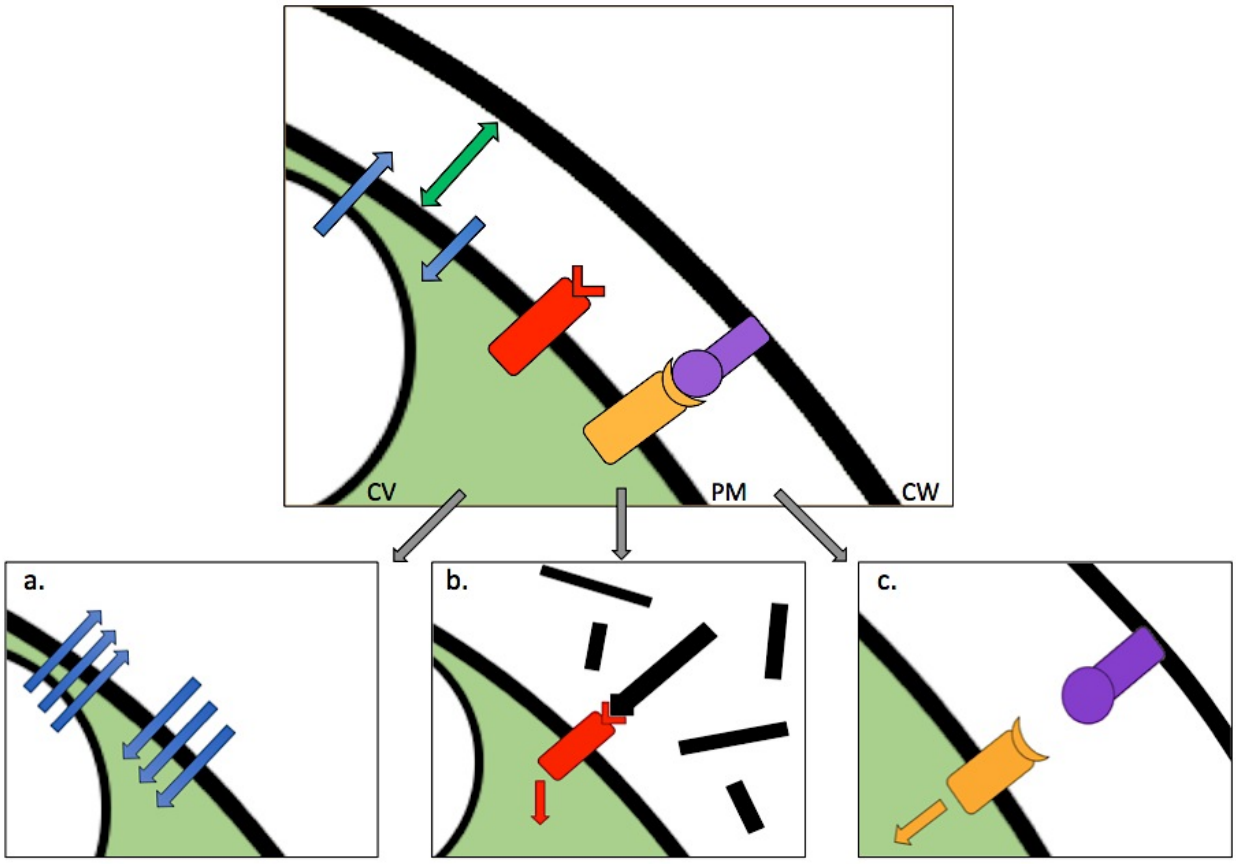


**Figure 1.2: Hypothetical mechanisms for the gametolysin-induced regulatory response.** Illustrations represent a magnified region of the cell near the periphery. Top panel shows the possible elements of the lysin-induced response in an inactive (non-lysin) condition including: receptor proteins, yellow and red shapes; water flow via contractile vacuole, blue arrows; hydrostatic pressure generated in periplasmic space, green double-headed arrow. Elements are arranged spatially as denoted by CV, contractile vacuole; PM, plasma membrane; CW, cell wall. Bottom panels show mechanisms by which cellular elements might be stimulated and drive the GLE-induced response: a. increased water flow into and out of the cell (increased flux rate); b. receptor-mediated detection of fragmented wall components; c. receptor-mediated detection of physical wall removal.

## 1.6 Research questions and project objectives

While prior research and existing transcriptome data have provided insights as to what genes are expressed following GLE activation, there remain many questions about how they are regulated and what gene networks that are involved with this process. There is also a lack of understanding about what exactly is the trigger of this transcriptional event.

These gaps in knowledge lead us to our first two research questions: 1) what gene regulatory networks control cell wall regeneration after lysin-facilitated removal, and 2) at what level (i.e. transcriptional or post-transcriptional) are these genes regulated? To address these questions, we first studied transcriptional activation following GLE-mediated cell wall removal, using a suite of target transcripts as representative factors. Then, based on evidence suggesting a role for an ER stress response in this transcriptional event, we studied transcript accumulation in response to artificially-induced ER stress. To investigate the level(s) at which these transcripts are regulated, we studied promoter dynamics and transcription for several genes of interest. As well, we quantified transcription in cells treated with a protein synthesis inhibitor prior to GLE treatment to investigate the hierarchical regulation of these transcripts. From these studies, we have been able to draw conclusions and propose hypotheses about how these transcripts are regulated and can use this information to address the regulation of post-GLE cell wall recovery as a whole.

Lastly, we addressed one final question, 3) what is/are the trigger(s) that initiate transcription following cell wall removal? The three hypotheses we have generated to answer this question are still being actively investigated. In this study, we explore the possibility of the

GLE response being triggered by: a) osmotic stress, b) pherophorin signalling, and/or c) cell wall detachment, by quantifying transcript accumulation in various cell wall defective mutants.

# Chapter 2: The transcriptional response to lysin-mediated cell wall removal

## 2.1 Introduction

An early event in the sexual mating of *C. reinhardtii* is the lysin-mediated shedding of the cell wall. In anticipation of mating, nitrogen-starved gametes will synthesize and store large amounts of GLE proenzyme, which, upon flagellar agglutination of mating partners, will become active and remove the cell wall. Then, in rapid succession, naked gametes will fuse and sex-specific transcription factors GSM1 and GSP1 will dimerize, initiating a global transcription event and 'turning on' the zygote development program. Previous genetic studies investigating the transition from haploid to diploid cells have determined that two sets of genes are expressed during this event; the first, in response to cell wall shedding, and second much larger set, in response to GSM1:GSP1 activation (Kubo et al., 2008; Joo et al, 2017). The cell wall shedding or 'lysin-induced' gene set consists of 162 genes, enriched in putative cell wall and protein processing components. How these genes are regulated remains unclear; no major regulatory proteins have been isolated and no 'trigger' inducing expression of these genes has yet been identified.

In this study, we selected a suite of transcripts, our 'genes of interest' (GOI), to observe the genetic response to GLE-mediated cell wall removal. First, we treated cells with exogenous GLE and measured transcript accumulation for our GOI to confirm the expression patterns indicated by the transcriptome data and to generate an expression baseline in the GLE condition. We then generated promoter-reporter cell lines to observe the promoter activity for four of our GOI to gain an understanding of the level at which these genes are regulated. To further our

understanding of this regulation, we pretreated cells with the protein synthesis inhibitor cycloheximide (CHX) prior to GLE treatment and observed whether transcription is affected. If transcript levels are differentially affected by CHX pretreatment, it suggests multiple regulatory proteins are involved with the regulation of the GLE-induced genetic response. Another tool we used to study the regulation of the lysin-induced response was the use of cell wall-defective (CWD) mutant lines. To investigate the hypotheses for the 'trigger' of the GLE-mediated response, the CWD cells can help tease apart the correct explanation(s). For example, if the detachment of the cell wall is a trigger for lysin-induced gene expression, then cells completely lacking a cell wall might exhibit constitutive expression of these genes. Another possibility is that CWD cells might be more susceptible to the osmolarity of the environment and similarly exhibit constitutive gene expression in an attempt to build a normal cell wall. We have presented here the first of our ongoing CWD investigations, where we quantified expression of our GOI in CWD cells treated with fresh GLE.

## 2.2 Results

### 2.2.1 Selecting genes of interest for investigation

To study the genetic response to lysin-mediated cell wall removal, it was essential to select a suite of target genes to use as markers. These genes of interest (GOI) were identified from the compiled transcriptome data sets discussed in Joo et al. (2017), which grouped transcripts based on their expression patterns using clustering analyses. Two gene clusters, C24 and C44, consist of 197 and 162 genes, respectively, exhibited high inducibility when treated

with GLE. However, C44 genes were also found to be highly expressed in early zygotes, unlike C24, which are found to be specifically regulated by the GLE-mediated response and not by the zygote-specific factor GSM1:GSP1 (Joo et al., 2017).

A majority of the GLE-induced transcripts from the C24 "gL-EZ" and C44 "gL+EZ" sets fell into two putative functional groups; protein processing-related (i.e. related to translation or glycosylation) and cell wall-related. As such, we selected three transcripts related to protein processing; *SEC61G, AraGT1*, and *RHM1*, and four related to the cell wall; *GAS28, GAS30, GAS31*, and *PHC19* (Table 1). *SEC61G* is a subunit of the ER translocon complex, which provides a channel across which growing polypeptides move into the ER during translation. *AraGT1* and *RHM1* are both likely to be involved with protein glycosylation, as the former encodes a putative glycosyltransferase and the latter a putative rhamnose synthase. While rhamnose is largely used as a pectin precursor in higher plants, the absence of pectins in the *C. reinhardtii* cell wall lead us to hypothesize that rhamnose is primarily incorporated into glycan chains of glycoproteins (Ridley et al., 2001). *PHC19* encodes a putative HRGP related to the pherophorins in *Volvox* and is associated with the cell wall or extracellular matrix. *GAS28*, *-30,* and *-31* are also cell wall HRGPs and were selected, in part, because of their previous use in a study by Hoffmann and Beck (2005) regarding osmotic stress responses in *C. reinhardtii*. All of the selected genes exhibit high expression in cells treated with GLE, according to transcriptome data provided by Ning et al. (2013). Using the reads per million kilobases mapped (RPKM) values from the Ning et al. (2013) dataset, we calculated the difference in RPKM values for a given transcript between untreated and GLE-treated cell conditions (ΔRPKM) (Table 1). The ΔRPKM values for our GOI all indicate a large increase in gene expression when cells were

treated with GLE. Table 1 also shows which genes we have generated promoter-luciferase transgenic lines for studying promoter activity.

Note that while *GAS28* was found to be gamete-specific in a study by von Gromoff and Beck (1993), the mRNA levels of *GAS28* did not increase until after 7 hours in nitrogen-free medium. In the present study, gametes were only subject to nitrogen-deplete conditions for 4 hours prior to experimental treatments to avoid background *GAS28* expression.

**Table 2.1: Genes of interest selected from curated transcriptome data**

| Transcript name | Gene locus[1] | Putative function[1] | Cluster [2] | ΔRPKM[3] | Promoter line? |
|---|---|---|---|---|---|
| SEC61G | Cre16.g680230 | ER protein translocase | 24 | +1445 | yes |
| AraGT1 | Cre14.g629000 | ER glycosyltransferase | 44 | +80 | yes |
| RHM1 | Cre02.g083800 | Rhamnose synthase | 44 | +169 | yes |
| GAS28 | Cre11.g481600 | Cell wall HRGP | 24 | +1351 | no |
| GAS30 | Cre11.g481750 | Cell wall HRGP | 24 | +73 | no |
| GAS31 | Cre11.g468359 | Cell wall HRGP | 24 | +2656 | no |
| PHC19 | Cre17.g696500 | Cell wall pherophorin | 24 | +594 | yes |

[1]phytozome.jgi.doe.gov; [2]From Joo et al., 2017; [3]Calculated using data from Ning et al., 2013

### 2.2.2 Increased expression of target transcripts is in agreement with transcriptome data

To generate a baseline expression pattern for our genes of interest following cell wall removal, wildtype gamete cells were with treated with exogenous GLE and the resultant mRNA levels were quantified. We used gamete cells (not vegetative cells) for all mRNA analyses because *C. reinhardtii* cells undergoing gamete differentiation will halt mitotic growth. Previous research has indicated that during mitotic growth, genes related to secretion and the cell wall are

constitutively expressed, so to reduce the background expression of our GOI we used gamete cells (Zones et al., 2015). Using gametes also mimics the natural state of cells (ie. nitrogen starved) during sexual mating and GLE-mediated cell wall removal.

Following treatment of wildtype cells with GLE, there was a noticeable increase in expression for all GOI. This significant change in activity is especially apparent for genes related to the cell wall: *PHC19*, *GAS28*, *GAS30*, and *GAS31*, all of which showed between a 100 to 500-fold increase in expression (Figure 3). The transcripts related to protein processing, *SEC61G*, *AraGT1*, and *RHM1* also increased, but by more modest levels of 4.9, 4.3, and 33.4-fold, respectively. This trend of increased expression following GLE treatment confirms the pattern identified by the transcriptome analyses. It is also clear from these results that genes encoding cell wall proteins are up-regulated to a much greater extent than those related to protein processing.

Table 2.1 has been included to demonstrate how the qPCR data presented in Figure 2.1 was analyzed. In each experiment, the expression of a constitutively expressed transcript *RCK1,* encoding a component of the 40S ribosomal subunit, was used as a baseline to compare the expression of other transcripts. Raw threshold cycle (Ct) values obtained from real-time PCR runs can be converted simply to cycle quantitation (ΔCq) by subtracting the Ct value of *RCK1* from the Ct value of a target transcript (in this case, our GOI). These values can be further transformed to give a relative quantitation expressed in terms of a target transcript's fold change from the control, termed ΔΔCq (see Pfaffl, 2001). For calculating ΔΔCq, we used the method described in Pfaffl (2001) because it corrects for differences in qPCR primer efficiency, unlike other common calculation methods. The ΔΔCq values in Table 2.2 with corresponding standard deviations were then plotted as bar graphs as seen in Figure 2.1.

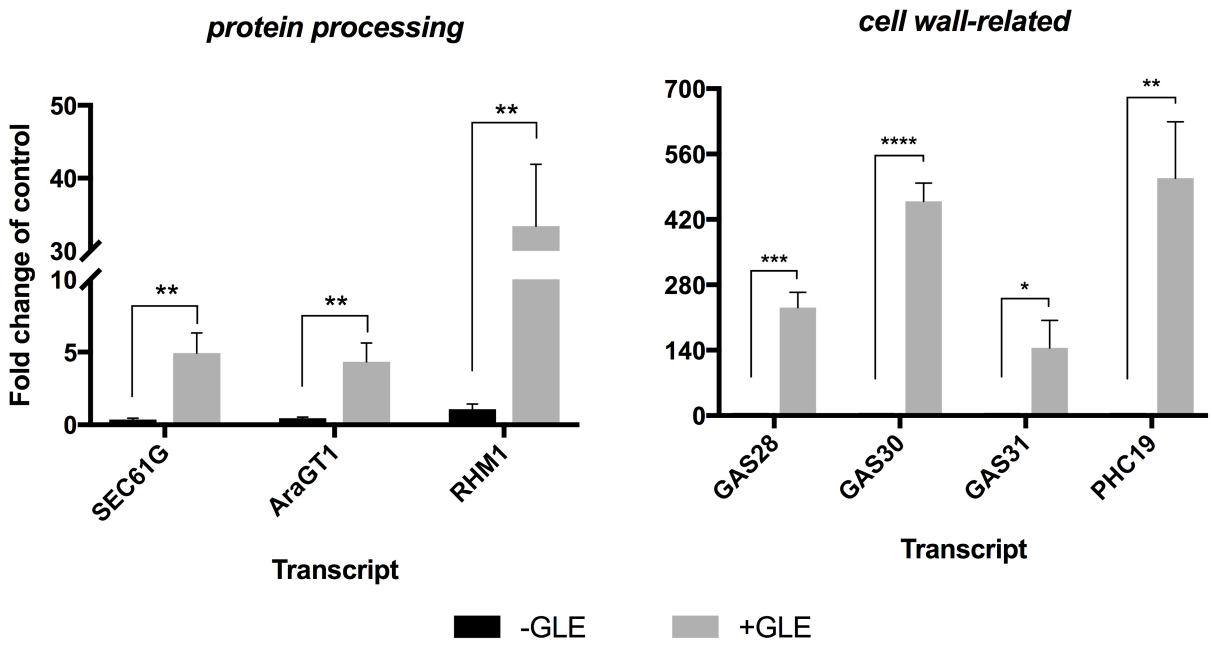


**Figure 2.1: Transcript levels of GOI in cells treated with gametolysin show global up-regulation.** Bar graphs represent the change in gene expression for protein processing-related and cell wall-related transcripts in wildtype (CC-125) cells. Untreated control samples are represented by black bars, grey bars represent cells treated with 50% GLE. Gene expression is quantified in terms of fold change compared to control condition (ΔΔCq). Error bars represent one standard deviation from the mean of biological triplicate samples. Welch's t-test indicates statistical significance at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****); $\alpha = 0.05$.

**Table 2.2: Raw and calculated transcript expression data for control vs. gametolysin treatment in wildtype cells.**

| Transcript | Ct value (RCK1 Ct value) | | ΔCq target – RCK1 | | ΔΔCq (1 SD) | |
|---|---|---|---|---|---|---|
| | GAM | GLE | GAM | GLE | GAM | GLE |
| SEC61G | 23.77 (20.00) | 19.88 (19.67) | 3.77 | 0.21 | 0.36 (0.10) | 4.93 (1.39) |
| AraGT1 | 34.30 (20.00) | 30.53 (19.67) | 14.30 | 10.86 | 0.45 (0.09) | 4.33 (1.30) |
| RHM1 | 32.10 (19.89) | 25.80 (19.83) | 12.21 | 5.97 | 1.07 (0.36) | 33.4 (8.47) |
| GAS28 | 27.77 (19.89) | 16.70 (19.71) | 7.88 | -3.01 | 0.87 (0.17) | 231 (33.0) |
| GAS30 | 34.14 (20.49) | 23.87 (20.76) | 13.62 | 3.11 | 0.76 (0.21) | 459 (38.9) |
| GAS31 | 30.07 (19.60) | 21.11 (19.33) | 10.47 | 1.78 | 0.98 (0.11) | 145 (58.6) |
| PHC19 | 28.78 (19.37) | 18.41 (19.63) | 9.41 | -1.22 | 0.66 (0.10) | 508 (121) |

GAM, gamete cells; GLE, GLE-treated cells

### 2.2.3 Genes of interest are regulated at the transcriptional level

To gain greater insight into the regulation of cell wall development, we used a promoter-reporter approach to observe the promoter activity for several genes of interest following cell wall removal. By engineering and transforming cells with promoter-luciferase transgenes, promoter activity can be inferred by quantifying the level of luminescence generated by the luciferase enzyme.

Following transformation of nicotinamide-requiring (*nic7*; mt-) cells with promoter-luciferase constructs, 36 auxotrophically-selected transformants were chosen for luciferase activity screens. Working with subsets of 8 transformants per screen, we subjected cells to test conditions (either GLE treatment or mating) and quantified luciferase levels to screen for transgenic lines with desirable promoter activity. Transformants for *proPHC19*, *proAraGT1*, and *proRHM1* were incubated in nitrogen-deplete media to induce gametogenesis (GAM), then gametes were treated with GLE for 1 hour (+GLE). As evident in Figure 2.2, many lines showed

increased luciferase activity when treated with GLE, indicated by an increase in relative luminescence (RLUs). Transformants with the greatest difference in luminescence between untreated and treated cells, as denoted by black arrows, were chosen for further experimentation. The screening for *proSEC61G* transformants was originally designed for another project, thus the comparison of cells in vegetative (VEG), gamete (GAM), and early zygote (ZYG) cell states. In this instance, early zygotes are analogous to GLE-treated cells and so transformants that showed the greatest difference in RLUs between VEG/GAM and ZYG conditions were selected for further analysis (Figure 2.2).

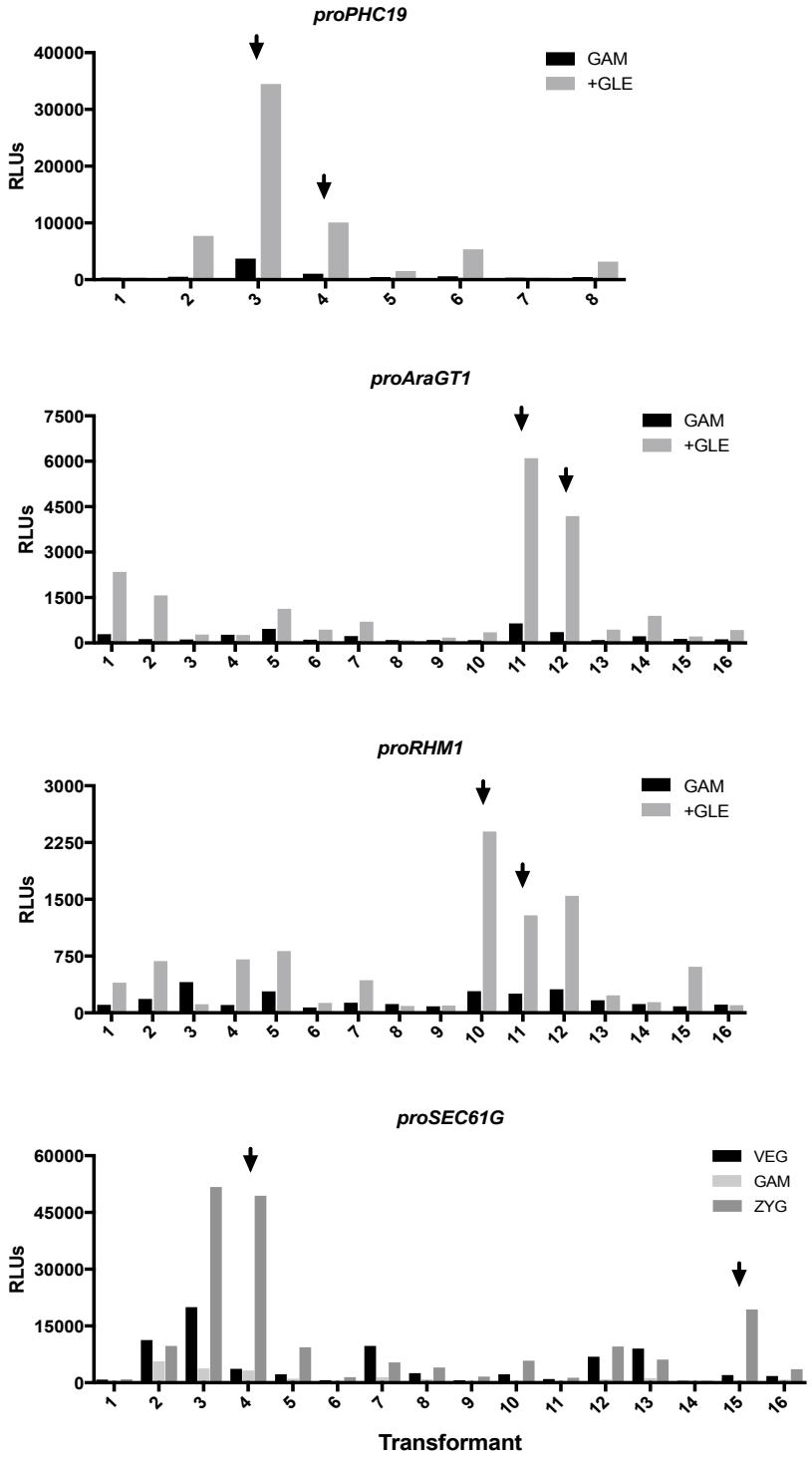


**Figure 2.2: Screening of promoter-luciferase transformed cell lines.** Bar graphs for each promoter-reporter construct, as labeled, show quantified luminescence in relative light units (RLUs). Legends indicate corresponding cell conditions: VEG, vegetative cells; GAM, gamete cells; ZYG, early zygote cells; +GLE, gametolysin-treated gamete cells. Arrows indicate cell lines exhibiting desirable luciferase activity pattern that were chosen for further experimentation.

Using the two selected transformants for each promoter-reporter construct (henceforth labelled as *proX-1* and *proX-2*) we quantified the change in promoter activity of cells treated with GLE. Following a 1 hour incubation of gamete cells with 50% GLE diluted in TAP, there was a distinct increase in activity of all promoters, as indicated by increased luminescence (Figure 2.3). The cell wall-related promoter, *proPHC19*, showed about 10x greater activity in both cell lines when treated with GLE compared to the untreated control (-GLE). *proRHM1* and *proAraGT1* showed modest increases in promoter activity, by about 5x and 7x respectively when treated with 50% GLE. *proSEC61G* exhibited a very large change in activity, with both promoter lines exhibiting a >100x increase compared to the untreated control. These results show that all our promoters are highly active following GLE treatment and indicates that our GOI are regulated at the transcript level.

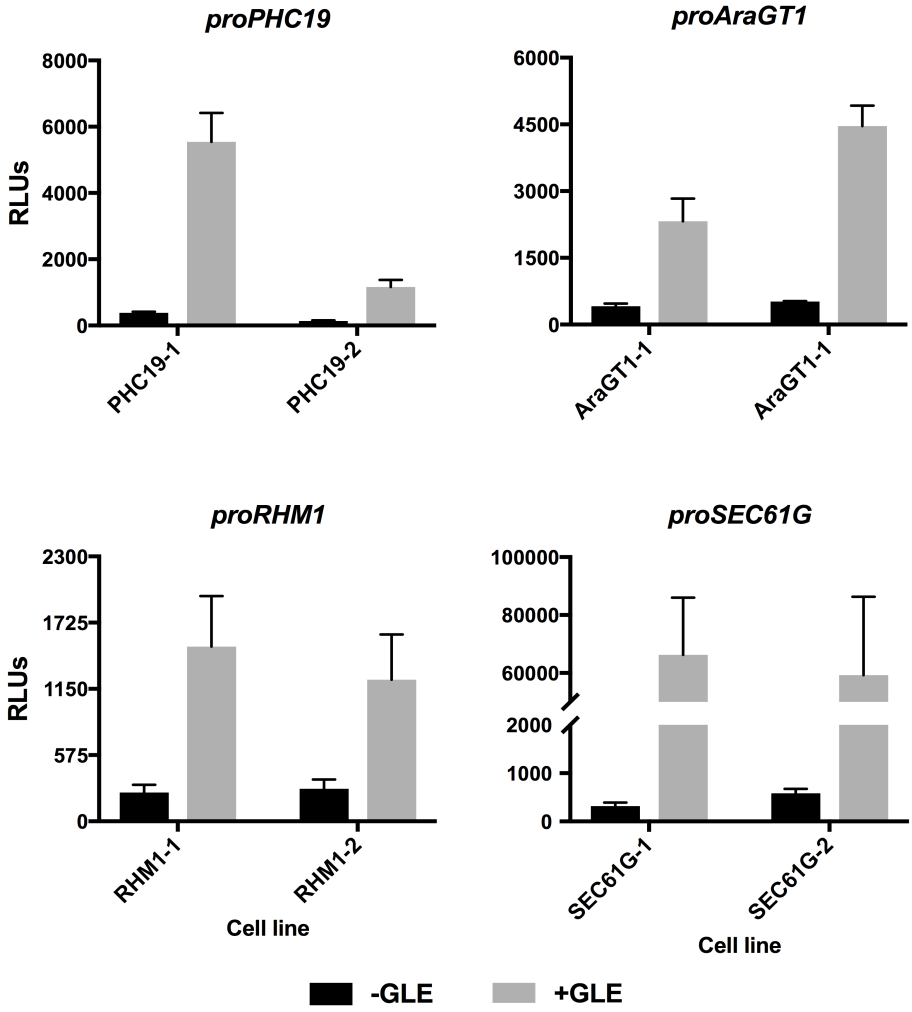


**Figure 2.3: Promoter-driven luciferase activity increases in response to gametolysin treatment.** Bar graphs represent the change in activity promoter activity of *PHC19, AraGT1, RHM1*, and *SEC61G* promoters, as labelled above, using two independent promoter-transformed lines. Luciferase activity is expressed as relative light units (RLUs) based on luminescence quantification. Untreated gamete control samples are represented by black bars. Grey bars represent gamete cells treated with 50% GLE. Error bars are one standard deviation from the mean of biological triplicate data.

### 2.2.4 Cycloheximide pretreatment inhibits transcription of several target genes

To further investigate the regulatory mechanisms involved cell wall reconstruction, we used the protein synthesis inhibitor cycloheximide (CHX) and observed changes in mRNA expression. By pretreating cells with CHX prior to GLE treatment, it is possible to determine if *de novo* protein synthesis is necessary for GLE-induced gene transcription (Hoffmann & Beck, 2005; Diaz-Troya et al., 2011). To allow for a comparison between conditions with and without CHX, mRNA levels were calculated as a difference in fold change compared to –CHX –GLE controls (Figure 2.4). Samples that had only CHX exposure (+CHX) were included to quantify any effect the pharmacological agent may have on the transcripts of interest.

We found CHX by itself to have a minimal effect on transcription, as a comparison of –GLE –CHX and +CHX conditions showed less than a 3-fold change in mRNA (Figure 2.4). Five out of seven of our transcripts of interest showed a significant decrease in the CHX pretreated GLE cells (+CHX +GLE) compared to the GLE-only cells (+GLE). *SEC61G* and *RHM1* exhibited about 8-fold and 20-fold less expression respectively when protein synthesis was inhibited before GLE treatment. Two of our cell wall-related genes, *GAS28* and *GAS30*, were almost entirely inhibited by CHX and *PHC19* showed about 450-fold less expression compared to the +GLE-only treatment. Interestingly, one transcript of each putative 'type', *AraGT1* and *GAS31*, showed significant increases in mRNA level when pretreated with CHX then treated with GLE. Cells exhibited approximately 20-fold and 1000-fold increases in expression for *AraGT1* and *GAS31* transcripts, respectively, when pretreated with CHX compared to cells treated only with GLE. These results suggest additional regulatory elements control the transcription of these GLE-responsive genes.

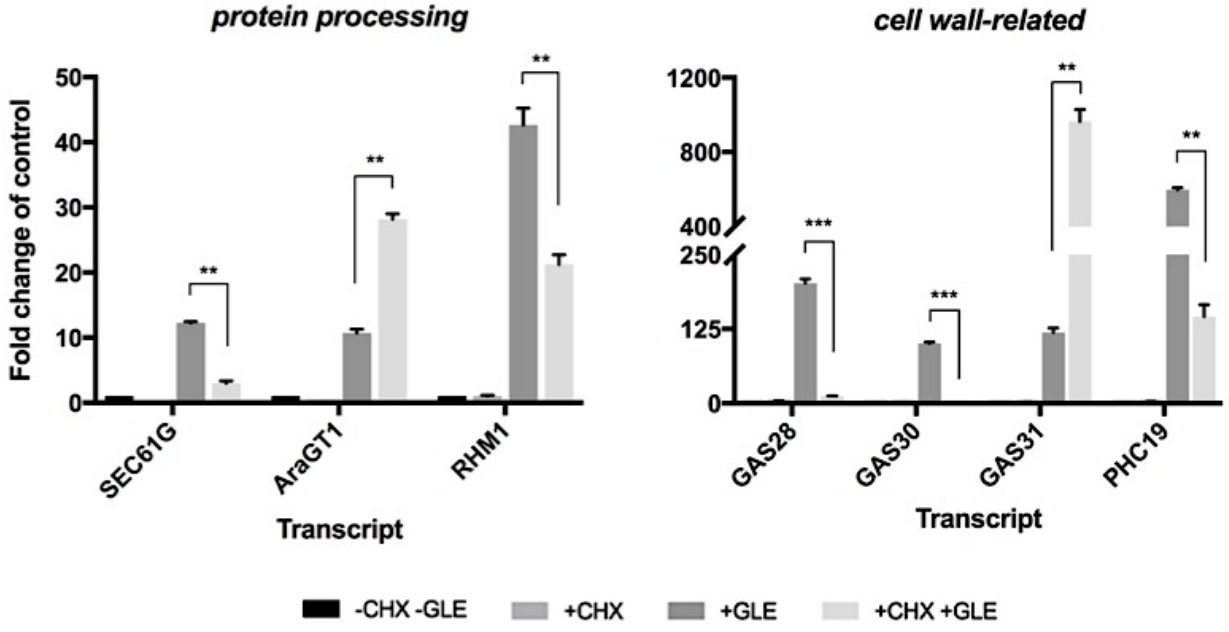


**Figure 2.4: Transcripts are differentially affected by cycloheximide pretreatment prior to gametolysin treatment.** Bar graphs represent the change in gene expression for protein processing-related and cell wall-related transcripts in wildtype (CC-125) cells. Untreated gamete control samples represented by black bars, -CHX –GLE; medium grey bars for cycloheximide treated cells, +CHX; dark grey bars for cells treated with 50% gametolysin, +GLE; light grey bars for both cycloheximide and gametolysin-treated cells, +CHX +GLE. Gene expression is quantified in terms of fold change of the untreated control condition (ΔΔCq). Error bars represent one standard deviation from the mean of biological duplicate samples. Welch's t-test indicates statistical significance at $p \leq 0.01$ (**), $p \leq 0.001$ (***); $\alpha = 0.05$.

### 2.2.5 Cell wall defective mutants are insensitive to GLE, but show constitutive gene expression

Because the hypotheses we generated for the GLE-induced cell wall response is dependent on the presence of an intact cell wall, it was important to understand how this

response functions in cells with an impaired or absent wall. The Lee lab has generated a vast insertional mutant library using a detergent sensitivity screen designed to isolate cell wall-defective (CWD) lines. After a lengthy curation of these CWD mutants with the help of colleague Ariel Shao, we chose a suite of mutants with varied cell wall phenotypes to observe their response to GLE. A summary of the CWD mutants selected is presented in Table 2.3, while a depiction of the morphological differences of these mutants is illustrated in Figure 2.5. An exception to these mutants is CW15, which is a mutant line not generated by the Lee lab and has been used in *C. reinhardtii* studies for decades.

Our selection of mutants was based on two criteria: 1) sensitivity to the detergent NP40, and 2) morphological appearance of the cell. NP40, a detergent which disrupts the lipid bilayer of the plasma membrane, cannot penetrate through an intact *Chlamydomonas* cell wall, so CWD mutants are often sensitive to NP40 and will burst in its presence. Mutant lines CW15, 24-119, 26-14, and 30-81 all showed high sensitivity (>90% cells burst) to NP40 while 15-39 and 38-11 showed medium sensitivity (70-90% cells burst).

From cells that were shown to exhibit either medium or high sensitivity to NP40, mutants with different observable phenotypes were selected to test if the amount or arrangement of cell wall material affects their response to GLE (Table 2.3). An early observational study of cell wall-defective mutants by Davies and Plaskitt (1971) categorized mutant phenotypes into 3 distinct groups: A) cells producing walls but not attached to plasma membrane, B) cells producing mostly normal walls attached to plasma membrane, and C) cells producing minute amounts of wall material not attached to plasma membrane. Through our screening process we did not identify any mutants of category A, but instead found representatives of categories B and C (Table 2.3).

**Table 2.3: Summary of selected cell wall defective mutants**

| Cell line | Significant characteristics | Cell wall phenotype | CWD classification[1] | NP40 sensitivity | Mutant genotype |
|---|---|---|---|---|---|
| CW15 | rounded cells | some wall material present; unattached | C | high | unknown |
| 15-39 | - teardrop shaped<br>- cell aggregates | unevenly distributed | B | medium | serine galactosaminyl-transferase |
| 24-119 | gap between membrane and cell wall | gap between membrane and wall | B | high | unknown |
| 26-14 | rounded cells | no wall material present | C | high | unknown |
| 30-81 | - rounded cells<br>- dented appearance | ruffled-looking edges | B | high | unknown |
| 38-11 | odd shaped, some have 'dented' appearance | ruffled or dented edges | B | medium | unknown |

[1]Davies & Plaskitt, 1971

To illustrate the selected mutant phenotypes, both micrographs and cartoons were generated for lines exhibiting a unique phenotype (Figure 2.5b). Wildtype *C. reinhardtii* are ovoid with an evenly distributed cell wall (Figure 2.5a). They also possess a distinctive cup-shaped chloroplast, posterior pyrenoid, central nucleus, and two anteriorly-located contractile vacuoles and flagella. Compared to wildtype, our CWD mutant morphologies are clear, from perfectly round cells lacking any cell wall (e.g. 26-14) to cells with an apparent uneven distribution of material (e.g. 30-81).

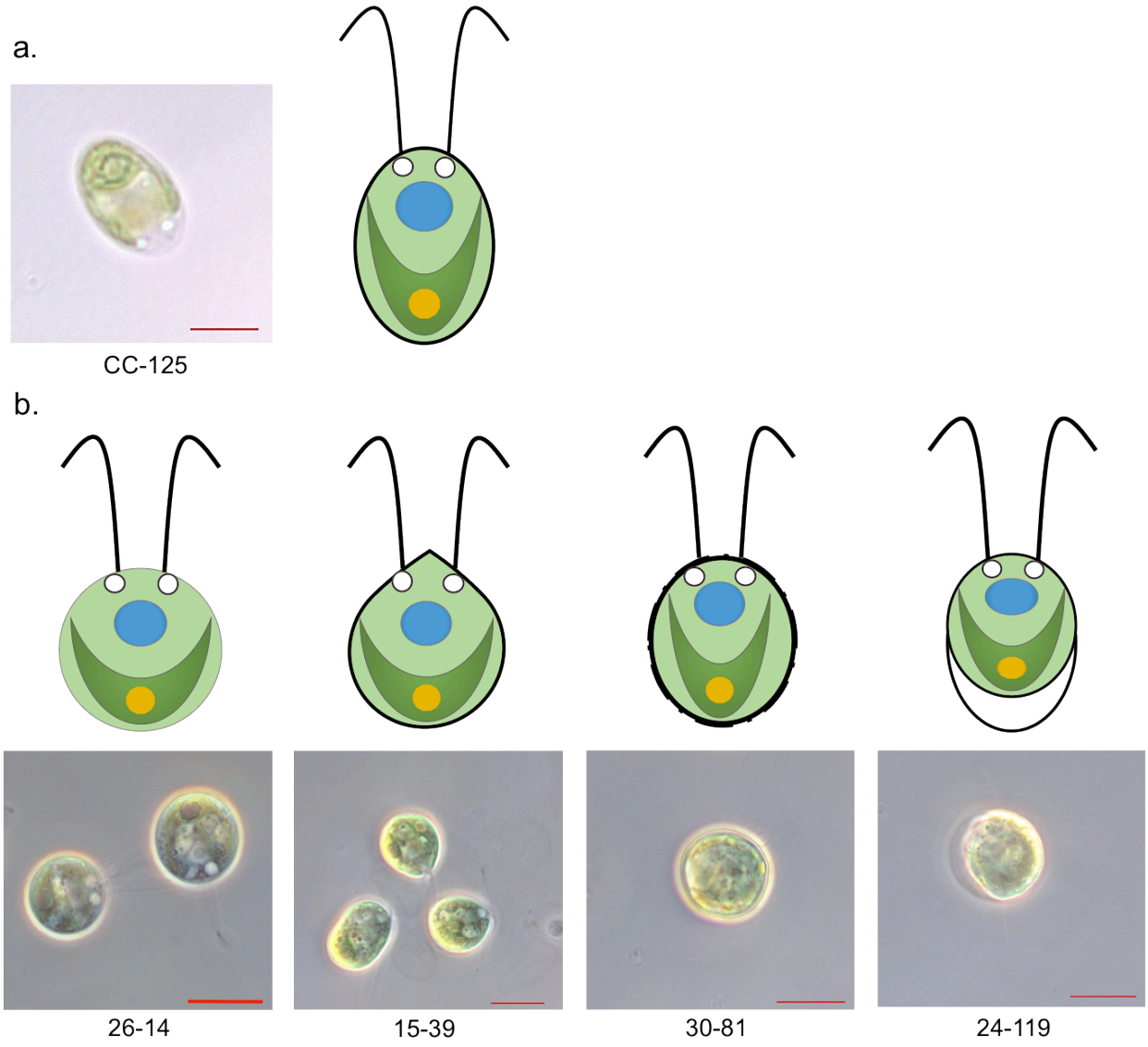


**Figure 2.5: Phenotypic characteristics of wildtype and selected cell wall-defective mutant lines.** a. Left image, micrograph of wildtype cell (labeled below), scale bar = 5μm; right image, illustration of wildtype cell showing typical morphology. b. Upper row, artistic renderings of CWD mutant cells illustrating variations in cell wall or lack of cell wall; bottom row, corresponding light micrographs of representative cell wall defective mutants (labeled below), scale bars = 10μm.

Once our CWD mutants were selected, we subjected cells to an exogenous 'fresh' GLE treatment. 'Fresh' GLE differs from the typically prepared GLE in that the extract is not highly purified and is used immediately after being harvested and not stored in -80°C. The rationale for

using this fresh GLE is that the extract should contain both GLE and fragmented cell wall proteins. This allows us to observe any response that these CWD mutants might have to either GLE or chopped wall proteins and is the first experiment to test our pherophorin hypothesis.

The real-time PCR results indicate that all CWD mutants have a diminished or absent response to fresh GLE compared to wildtype (Figure 2.6). *SEC61G* mRNA levels were about 15-fold lower in the mutant lines, some showing no expression above the control baseline. Even more dramatic was the 1300-fold and 200-fold reduction in expression for the cell wall-related transcripts *PHC19* and *GAS28.* Despite the largely impaired response to fresh GLE, some CWD lines showed mRNA levels that are modestly above the baseline. The CW15 mutant showed a 2-fold increase for *SEC61G*, an 8-fold increase for *PHC19*, and 4-fold increase for *GAS28*. Mutants 26-14 and 30-81 had similar transcript levels with a ~2.5-fold increase of *SEC61G*, a ~4-fold increase of *PHC19*, and ~4-fold increase of *GAS28*. The remainder of the mutants appeared to have no response to the fresh GLE treatment in terms of transcript expression. These mutant expression profiles appear to have no correlation with the mutant's phenotype. For example, CW15 and 26-14 mutants have no attached cell wall and we expected these lines to have the most diminished response to GLE, but the data does not support this expectation.

The data presented in Figure 2.6 fail to expose a key piece of information. While it is true that these CWD lines showed a diminished response to GLE, they were also constitutively transcribing our GOI. This constitutive expression is clear when we observe the ΔCq values rather than the ΔΔCq values presented in Tables 2.4-2.6. For example, in Table 2.5 the ΔCq for *PHC19* in wildtype (CC-125) is 13.91 in gametes and 2.3 in GLE-treated gametes. This means 13.91 is the baseline expression of *PHC19* and the large decrease to 2.3 following GLE treatment correlates to a large increase in transcript expression. The ΔCq values of transcripts in

our CWD mutants were very low in gametes, comparable to that of the GLE-treated gametes. In many instances, like in lines 24-119, 26-14, and 38-11, the ΔCq values of *PHC19* and *GAS28* in untreated gametes were even lower than wildtype gametes treated with GLE (Table 2.5 and 2.6). It is clear from this ΔCq data that nearly all our CWD lines exhibit constitutive expression of both cell wall transcripts *PHC19* and *GAS28* and to a lesser extent *SEC61G.* The exception was CW15, a line which is supposedly cell wall-less, but showed transcript induction by GLE. It may be possible that this line has been contaminated by another cell culture or that this expression pattern could be intrinsic to the unidentified mutation. The discrepancies between ΔCq and ΔΔCq data presented here impress the importance of representing data correctly and how different calculation methods can have a great impact on analysis outcomes.

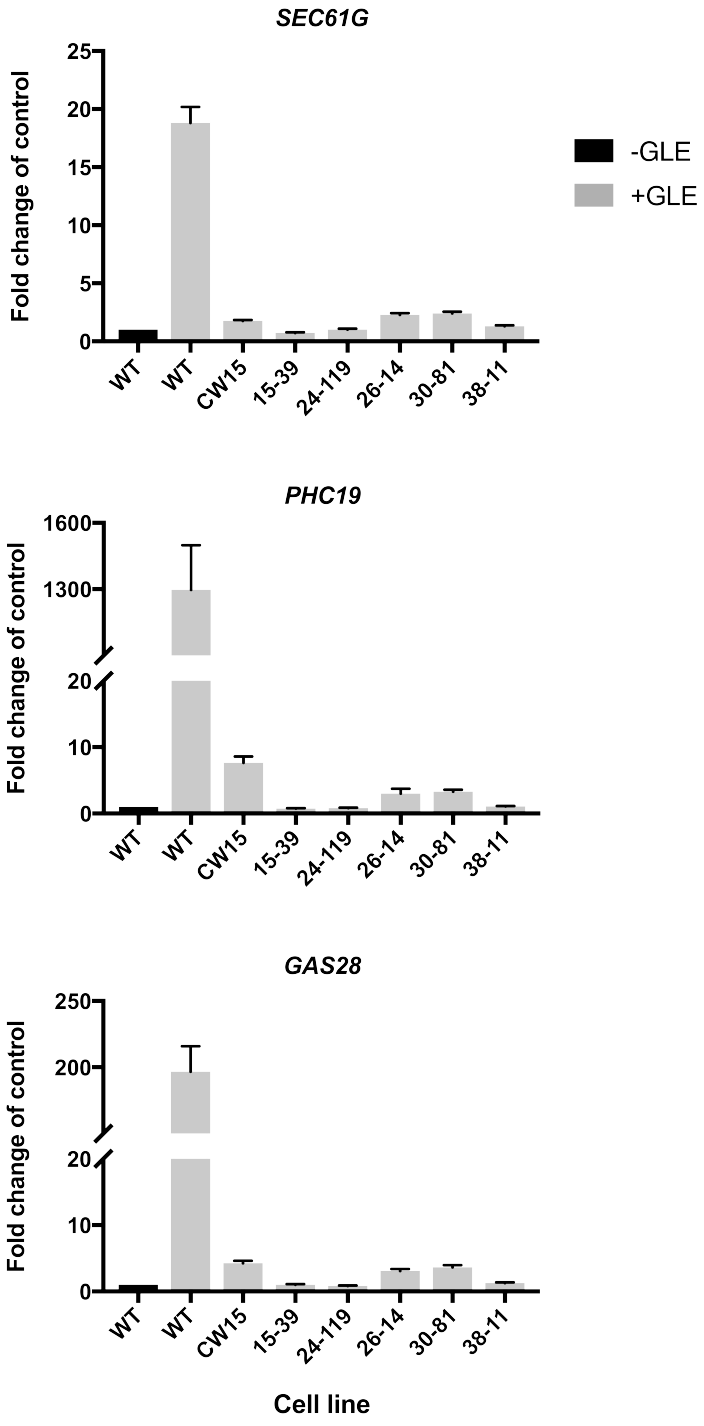


**Figure 2.6: Transcriptional response to fresh gametolysin appears muted in all cell wall-defective mutants.** Bar graphs show the expression of three transcripts, *SEC61G, PHC19, GAS29* (top to bottom) in cell wall defective mutants treated with GLE. Cell lines are labeled along X-axis. WT = wildtype line CC-125. mRNA expression is quantified in terms of fold change difference compared to the untreated control (WT, black bar) sample normalized to 1

(ΔΔCq). Error bars represent one standard deviation from the mean of biological duplicate samples.

**Table 2.4: *SEC61G* transcript expression data following GLE treatment in CWD lines**

| Cell line | Ct value (average RCK1 Ct) | | ΔCq target – RCK1 | | Average ΔΔCq (1 SD) | |
|---|---|---|---|---|---|---|
| | GAM | GLE | GAM | GLE | GAM | GLE |
| CC-125 (WT) | 20.04 (14.83) | 16.94 (16.49) | **5.21** | **0.45** | 1.00 (0) | 19.78 (1.46) |
| CW15 | 19.56 (16.11) | 18.99 (16.46) | **3.44** | **2.54** | 1.00 (0) | 1.81 (0.60) |
| 15-39 | 18.71 (15.35) | 18.31 (16.32) | **3.37** | **1.99** | 1.00 (0) | 2.39 (0.25) |
| 24-119 | 17.17 (16.16) | 17.68 (16.23) | **1.01** | **1.45** | 1.00 (0) | 0.76 (0.00) |
| 26-14 | 18.89 (16.31) | 18.67 (16.19) | **2.58** | **2.49** | 1.00 (0) | 1.08 (0.31) |
| 30-81 | 18.38 (15.19) | 18.79 (17.03) | **3.19** | **1.76** | 1.00 (0) | 2.51 (0.31) |
| 38-11 | 18.22 (16.08) | 18.21 (16.54) | **2.14** | **1.67** | 1.00 (0) | 1.35 (0.07) |

GAM, gamete cells; GLE, GLE-treated cells

**Table 2.5: *PHC19* transcript expression data following GLE treatment in CWD lines**

| Cell line | Ct value (average RCK1 Ct) | | ΔCq target – RCK1 | | Average ΔΔCq (1 SD) | |
|---|---|---|---|---|---|---|
| | GAM | GLE | GAM | GLE | GAM | GLE |
| CC-125 (WT) | 28.75 (14.83) | 18.79 (16.49) | **13.91** | **2.30** | 1.00 (0) | 1440.0 (203.2) |
| CW15 | 25.32 (16.11) | 22.33 (16.46) | **9.20** | **5.88** | 1.00 (0) | 8.32 (3.19) |
| 15-39 | 19.78 (15.35) | 18.84 (16.32) | **4.44** | **2.52** | 1.00 (0) | 3.54 (1.59) |
| 24-119 | 16.83 (16.16) | 17.30 (16.23) | **0.68** | **1.07** | 1.00 (0) | 0.79 (0.12) |
| 26-14 | 17.15 (16.31) | 17.22 (16.19) | **0.84** | **1.04** | 1.00 (0) | 0.89 (0.07) |
| 30-81 | 17.54 (15.19) | 17.40 (17.03) | **2.35** | **0.37** | 1.00 (0) | 3.51 (0.19) |
| 38-11 | 17.50 (16.08) | 17.78 (16.54) | **1.43** | **1.24** | 1.00 (0) | 1.13 (0.02) |

GAM, gamete cells; GLE, GLE-treated cells

**Table 2.6: *GAS28* transcript expression data following GLE treatment in CWD lines**

| Cell line | Ct value (average RCK1 Ct) | | ΔCq target – RCK1 | | Average ΔΔCq (1 SD) | |
|---|---|---|---|---|---|---|
| | GAM | GLE | GAM | GLE | GAM | GLE |
| CC-125 (WT) | 25.00 (14.83) | 17.00 (16.49) | **10.17** | **0.51** | 1.00 (0) | 182.98 (58.90) |
| CW15 | 24.12 (16.11) | 21.86 (16.46) | **8.00** | **5.40** | 1.00 (0) | 3.99 (0.07) |
| 15-39 | 18.32 (15.35) | 17.52 (16.32) | **2.97** | **1.20** | 1.00 (0) | 2.86 (0.82) |
| 24-119 | 15.87 (16.16) | 16.11 (16.23) | **-0.29** | **-0.12** | 1.00 (0) | 0.95 (0.26) |
| 26-14 | 15.65 (16.31) | 16.04 (16.19) | **-0.66** | **-0.15** | 1.00 (0) | 0.76 (0.05) |
| 30-81 | 15.91 (15.19) | 15.81 (17.03) | **0.72** | **-1.22** | 1.00 (0) | 3.40 (0.13) |
| 38-11 | 16.16 (16.08) | 16.43 (16.54) | **0.08** | **-0.11** | 1.00 (0) | 1.17 (0.09) |

GAM, gamete cells; GLE, GLE-treated cells

## 2.3 Discussion

In this study, we manipulated cells by treating them with GLE extract and observed their responses using several target transcripts and their promoters as indicators. After curating the GLE-induced transcriptome, we selected 7 transcripts for investigation; 3 associated with protein processing and 4 associated with the cell wall. Our first experiment was a simple GLE treatment of wildtype cells, which aimed to confirm the GLE-inducible expression of our GOI and to set a transcript expression baseline. We saw obvious transcript up-regulation for all our GOI, sometimes as high as a 500-fold increase in our cell wall-related transcripts.

To better understand the regulation of our GOI, we generated promoter-luciferase transgenic cells for *SEC61G, AraGT1, RHM1,* and *PHC19* genes. All our promoter-reporter lines showed a clear increase in luminescence in response to GLE, indicating a significant increase in luciferase synthesis and, thus, promoter activity. This suggests our genes are regulated at the transcriptional level. However, the increase of promoter activity for a given gene did not always match the extent to which the transcript accumulated in response to GLE. For example, the

promoter for *SEC61G* was shown to have a very large increase in activity when treated with GLE, while the mRNA levels increased only very modestly (Figures 2.1 and 2.3). This discrepancy could indicate that *SEC61G* mRNA are degraded to some extent after being transcribed, suggesting that other regulatory proteins (e.g. proteases and other degradation machinery) are involved with the regulation of *SEC61G*. However, this could also indicate an experimental error in either the construction or transformation of the *proSEC61G:cgLUC* transgene, leading to excessive promoter activity. One such possibility could be the presence of multiple copies of the transgene in the transformed cells.

While it appears that these GOI are transcriptionally regulated, it was interesting to observe the varied effects of pretreating the cells with CHX. The levels of *SEC61G, RHM1, GAS28, GAS30*, and *PHC19* transcripts all decreased when cells treated with GLE were pretreated with CHX, which indicates that regulatory proteins for these transcripts are synthesized *de novo* following lysin treatment. However, *AraGT1* and *GAS31* levels were enhanced by CHX pretreatment, suggesting the presence of inhibitors or repressors that normally control *AraGT1* and *GAS31* transcription. Such repressors may act by binding to the promoter region (or a regulatory region near to the gene coding sequence), thereby inhibiting the RNA polymerase from proceeding with transcription. In the presence of CHX these repressor proteins are no longer synthesized and transcription will occur as normal, as evidenced by an increase in gene expression.

Another way we investigated the nature of the GLE response was by using a number of cell wall-defective mutants. Cell lines with varying degrees of observable cell wall defects were selected using phenotype screens discussed in section 2.2.5. We subjected these CWD lines to exogenous GLE treatments and found all of them to accumulate lower levels of the three

transcripts tested – *SEC61G, PHC19*, and *GAS28*. Some CWD lines, 15-39, 24-119, and 38-11 appeared to be altogether insensitive to the GLE treatment. However, upon closer inspection of the data it was clear that our CWD lines, with the exception of CW15, were constitutively expressing our GOI. Because the cell wall is an inherently beneficial structure, it is logical that these CWD cells are continuously attempting to rebuild their cell wall – but to no avail. While it remains unclear why CW15 showed some GLE-induced transcription, one possibility is cell line contamination from years of culturing. Another possibility could be the down-regulation or silencing of the constitutively active 'wall rebuilding transcription' since the rebuilding is a fruitless effort and requires considerable energy and molecular resources.

These investigations raise a few interesting points. 1) It is clear there are different regulatory mechanisms that control transcriptional activity, yet all promoters and transcripts are induced by GLE treatment. 2) Cells lacking a normal cell wall do not exhibit a significant response to GLE and that they continuously attempt to rebuild their cell wall as indicated by constitutive transcription of our GOI. This suggests that the 'turning on and off' of these genes is likely triggered by the absence of a cell wall, either through a change in osmotic environment or direct sensing of the cell wall absence.

## 2.4 Materials and methods

### 2.4.1 GLE extract preparation

CC-125(+) and CC-621(-) *C. reinhardtii* cells were each suspended in 1.0mL of liquid TAP to a concentration of ~$1x10^7$ cells/mL. 10 x 100µL aliquots of each cell line were then

spread on 10 TAP plates (20 plates total) using a glass spreader, then cells were grown under medium light (~50μE) for 10 days. On day 10, cells were collected by pouring 2 x 5mL liquid NF-TAP onto each plate and scraping cells off with a glass spreader into 2 x 500mL Erlenmeyer flasks, one for each cell line. Cells were then mixed thoroughly on a shaker at 140 RPM for 1 hour and then poured into petri dishes, then placed under high light (~200μE) for 4 hours to continue gametogenesis. After incubation, the mating activity of 125+ and 621- cells was checked by mixing 5μL of each line and observing activity using phase contrast microscopy at 100x total magnification. When mating was observed to be strong, all 100mL of 125+ and 621- cells were rapidly mixed in a new 500mL Erlenmeyer flask and put under high light to induce the mating and GLE secretion. 5 minutes after cells were mixed, cells were rapidly decanted into 8 centrifuge bottles and weight between bottles was equalized. 10 minutes after being mixed, cells were placed on ice to halt any further mating and to preserve GLE enzyme activity. Cells were spun down at 15000 RPM for 10 minutes in an Eppendorf 5804 high-speed centrifuge. Supernatants from each bottle were poured off into a chilled 500mL beaker on ice, then 0.1g/mL sterile BSA was added to final concentration of 0.5mg/mL and swirled to mix. Using a 50mL syringe with a .44um pore size filter, the GLE solution was filtered and 10mL aliquots were made in 15mL conical tubes. Prepared GLE was then frozen at -80°C until use.

### 2.4.2 GLE, 'fresh GLE', and CHX treatment conditions

Cells were grown on TAP (TAP+NIC for CW15) plates for 7 days under continuous medium light (~50μE). On day 7, cells were harvested and suspended in nitrogen-free TAP (NF-TAP), then counted and normalized to a concentration of 5 x $10^7$ cells/mL. Suspended cells were

incubated under high light (~200μE) for a minimum of 3 hours to induce gametogenesis. Sufficient gametogenesis was determined by a mating efficiency analysis as per the method discussed in Hoffman and Goodenough (1980), with 80% mating efficiency being the acceptable lower limit before continuing the experiment.

For GLE treatment, suspended cells were mixed with an equal volume of GLE extract (50% GLE) and put under ~200μE light for 1 hour to ensure cell wall removal. GLE efficiency was determined by NP40 test (see 2.4.8 "NP40 sensitivity testing"). Untreated control samples were mixed with an equal volume NF-TAP to maintain equal cell concentrations across samples. Cells were then incubated for 1 hour before harvesting RNA.

For 'fresh GLE' treatment, suspended cells were mixed with an equal volume of 'fresh GLE' extract in NF-TAP then put under ~200μE light for 1 hour to ensure cell wall removal. GLE efficiency was determined by NP40 test. Untreated control samples were mixed with an equal volume NF-TAP to maintain equal cell concentrations across samples. Cells were then incubated for 1 hour before harvesting RNA.

For CHX pretreatment, suspended cells were mixed with a small volume of 10mg/mL CHX in NF-TAP to a final concentration of 10ug/mL, then incubated under ~200μE light for 45 minutes. Following the pre-treatment, some cells were treated further with an equal volume of thawed GLE extract, while control samples were diluted with an equal volume of 10ug/mL CHX in NF-TAP to maintain equal cell concentrations. Cells were then incubated for 1 hour before harvesting RNA.

### 2.4.3 RNA extraction and cDNA synthesis

Total RNA was harvested from ~1x$10^8$ cells following incubation in control or treatment conditions (see section 2.4.2). Suspended cells were pelleted using a tabletop centrifuge at 6000 RPM for 3 minutes and supernatant was removed. Cell pellets were suspended thoroughly in 300µL SDS-Tris-EDTA lysis buffer and allowed to sit for 30m at RT. Subsequently, precipitation, binding, and washing of RNA was performed using the protocol and reagents provided in the RNA Extraction Kit (QIAGEN). Total RNA was eluted in 40µL pure water and maintained on ice. RNA concentration was quantified using a Nanodrop spectrophotometer (ThermoFisher) and quality was determined by running 1µL of RNA on a 1.5% w/v agarose gel. 5µg of sufficient quality RNA was treated with Turbo DNase I (Ambion) and purified as per the manufacturers protocol. DNase-treated products were then quantified again using the Nanodrop spectrophotometer.

2.5µg of input RNA was used for cDNA synthesis. The reagents, protocol, and thermocycling conditions provided in the SSIV reverse transcriptase (Invitrogen) were used to generate cDNA from total RNA. Successful cDNA synthesis was confirmed using 0.1µL cDNA in a standard 30 cycle PCR protocol (56°C annealing and 72°C denaturing temperatures) against *RPL17*, a constitutively expressed gene encoding part of the 60S ribosomal subunit. The presence of a single, well-amplified band for *RPL17* indicates successful cDNA synthesis.

### 2.4.4 Quantitative real-time PCR analysis

qPCR reactions were carried out using SensiFAST SYBR master mix (Bioline), primers designed against our transcripts of interest, and 1µL cDNA in 15µL reactions. Relative DNA amounts were quantified using the CFX-96 PCR detection system (Bio-Rad) with a basic 40 cycle protocol (56°C annealing and 72°C denaturing temperatures). Each qPCR run had technical duplicate samples to generate average quantification cycle (Cq) data per run. The relative amount of DNA was quantified for both the transcripts of interest and the housekeeping gene *RCK1*, a constitutively expressed component of the 40S ribosomal subunit. Cq data was converted into 'fold change of control (*RCK1*)' using equations provided in Pfaffl (2001). The Pfaffl method allows for relative mRNA expression to be quantified by comparing Cq values of target transcripts to a housekeeping gene to generate a ΔΔCq value. This method also accounts for errors that may be generated from differences in qPCR primer efficiency for the different transcript targets. Welch's t-test was applied to the analyzed expression data to check for statistical significance between treatment conditions.

### 2.4.5 Engineering of promoter-reporter constructs

Promoter regions for *SEC61G, AraGT1, RHM1*, and *PHC1*9 were amplified from *C. reinhardtii* genomic DNA by standard PCR using primers designed against 400-1000bp regions directly upstream of the 5' end of the coding sequence. Upstream sequences were scanned for the presence of a TATA box – a binding region for transcription factors and/or histones - to determine the length of the promoter region that needed to be cloned. Forward primers contained

an Xba1 enzyme restriction site to be added to the 5' end of the amplified promoter fragment, while reverse primers contained an EcoR1 restriction site. Amplified promoter DNA was run on a 1.5% w/v agarose gel and DNA was cut and eluted from the gel using the QIAquick Gel Extraction Kit (QIAGEN). Promoter fragments were then cloned into a *pGEM-T* Easy vector using the materials and protocol provided by Promega. Subsequently, *proX:pGEM-T* plasmids were transformed into DH5α *E. coli* (as per the protocol by Promega) and *E. coli* were plated onto LB-Amp media for antibiotic resistance selection. Surviving colonies were then picked and transferred to new LB plates to grow large numbers of cells. Plasmid DNA (pDNA) was extracted from *E. coli* using a plasmid DNA Miniprep kit (Bio-Basic) and concentration of purified pDNA was measured using the Nanodrop spectrometer (ThermoFisher). The *proX:pGEM-T* pDNA and destination vector *NIC7:ARpro:cgLUC:rbcSTer* were double-digested with Xba1/EcoRI as per the reagents and protocol by NEB and T4 DNA ligase from Promega. Ligation products were transformed into *E. coli* and positive colonies were grown and pDNA harvested. Positive promoter-reporter products were determined by EcoRI/XbaI restriction digest analysis and were confirmed by Sanger DNA sequencing provided by GenScript.

### 2.4.6 Transformation of *C. reinhardtii* cells

The 'in-lab' protocol for transformation is a modified version of the published method by Kindle (1990). The *nic7* mutant strain mt28- was grown in 100μL liquid TAP+NIC media to mid-log growth phase (~3 days). Cells were harvested by centrifugation at 3000 RPM for 3 minutes in a 50mL conical tube. Cells were then suspended in purified GLE extract (see "GLE extract preparation") and incubated under ~200μE light for 1 hour to remove the cell wall.

Confirmation of cell wall removal was conducted by an NP40 detergent test (see "NP40 sensitivity testing"). Following a successful NP40 test, GLE was removed from cells by centrifugation at 3000 RPM for 3 minutes. Cells were then suspended in (300uL x # of transformations) of TAP media, then 300µL of cells were aliquotted into a 6mL test tube containing 300mg of sterile 0.5mm glass beads. In a 1.5mL microfuge tube, 100µL of 2% polyethylene glycol (PEG) and 1µg of plasmid DNA were mixed together by brief vortexing. The PEG and pDNA mixture was added to cells and the test tube was vortexed at high speed for 25 seconds to allow pDNA to pass into the cells. Cells were suspended with 2 x 5mL aliquots of TAP and poured off into 15mL conical tubes. Cells were then washed twice with 10mL TAP media, spun down at 3000 RPM for 3 minutes between each wash, and supernatant poured off. Finally, 300uL TAP and 3mL melted Top agar (TAP + 0.4% w/v agar) at RT was added to pelleted cells. Cells and media were then mixed by inversion several times and poured evenly onto TAP + acetylpyridine (AP) plates. AP is a chemical analogue of nicotinamide and is used for the auxotrophic selection of *nic7* transformants. Plated cells were placed under high light (~200uE) and allowed to grow over several days. Surviving colonies were picked and transferred onto new AP plates and allowed to grow up before phenotype screening.

### 2.4.7 Luciferase activity assays

To induce promoter-reporter activity, *proX*-transformed lines were subjected to the same treatment conditions outlined in section 2.4.2. Secreted *Gaussia princeps (Gaussian)* luciferase enzyme was collected from samples with equal cell concentrations by aliquotting 100µL of sample into 1.5mL centrifuge tubes and centrifuging for 3 minutes at 10000 RPM. 40µL of

supernatant was then transferred from each sample to PCR strip tubes and mixed with 10µL *Renilla* luciferase lysis buffer (Promega). Prepared samples were then stored at -20C until luciferase assays were run.

Samples for luciferase assay were prepared by mixing 25µL luciferase assay reagent (luciferase assay buffer + 1x final concentration luciferase assay substrate) (Promega) with 5µL of thawed samples in a 384-well microtitre plate. The relative amount of luciferase enzyme in each sample was then quantified by amount of luminescence detected by a BioTek Synergy 2 microplate reader.

### 2.4.8 NP40 sensitivity testing

20µL of suspended cells were mixed with 20µL 0.1% NP-40 "Tergitol" detergent (Sigma) in a 1.5mL tube. Another 20µL of suspended cells were simply diluted with media as an untreated control. 10µL of mixture and 10µL of untreated cells were loaded onto either side of a hemocytometer and visualized using brightfield microscopy with 100x total magnification. Both treated and untreated cells were counted to compare cell concentrations and determine the number of cells that had burst from the NP-40. This detergent disrupts the lipid-based plasma membrane and will therefore cause any cells without an intact cell wall to lyse. A simple 'NP40 sensitivity' value was then calculated by dividing the untreated cell concentration by the treated cell concentration and multiplying by 100%. For example, if all cells in a sample treated with NP40 burst, the NP40 sensitivity would be 100%.

# Chapter 3: Involvement of the UP stress response in cell wall integrity signalling

## 3.1 Introduction

Intrinsic cellular stress response mechanisms are vital for the survival of all organisms. One such mechanism that is highly conserved across eukaryotes is called the unfolded protein response (UPR). UPRs are induced when the rate of transcription exceeds a cell's capacity for mRNA processing, translation, and protein processing, leading to the accumulation of misfolded proteins in the ER. This stress state occurs following large changes in transcription, for example, during exposure to abiotic stresses or cell cycle transition events. The consequence of an UPR is the up-regulation of synthesis of proteins involved with the endomembrane system, protein processing, and ER-associated protein degradation (ERAD) (Leber et al., 2004; Liu & Howell, 2010). This leads to higher rates of protein synthesis to match rates of transcription and a reduction of ER stress from the removal of misfolded proteins.

Key players have been identified in the UPR pathway, most of which are ubiquitous throughout eukaryotes. Originally discovered in the yeast *Saccharomyces cerevisiae*, the ER resident protein, Ire1p, was determined to be the master regulator of the UPR system (reviewed in Ma & Hendershot, 2001). Ire1p possesses both a signal-sensing domain within the ER lumen, for detecting misfolded proteins, and an RNA endonuclease domain, for initiating an UPR signal cascade (Guo & Polymenis, 2006). *IRE1* is highly conserved and possesses two homologs *IRE1a* and *IRE1b* in mammals and *Arabidopsis* and one homolog in *C. reinhardtii* (Ma & Hendershot, 2001; Liu et al., 2007). Upon sensing accumulation of misfolded proteins in the ER,

biochemically active IRE1 acts as a post-transcriptional modifier of some bZIP family transcription factors by splicing regions of the bZIP mRNA with its endonuclease domain, which produces functionally active bZIP transcripts (Guo & Polymenis, 2006).

Basic leucine zipper (bZIP) transcription factors are found in all eukaryotes and are regulators of many important processes including plant defense, cell differentiation, and abiotic stress responses (reviewed in Corrêa et al., 2008). In *C. reinhardtii*, there are no published studies that have characterized any bZIP transcription factors. However, using BLAST alignment software with a conserved bZIP domain sequence as the search query, we found 17 genes possessing bZIP domains in *C. reinhardtii* (data not shown). In *Arabidopsis*, one study described a distinct role for the mRNA of bZIP factor *bZIP60*, which was found, using a protein-protein interaction assay, to interact directly with *IRE1* and stimulate a generalized stress response during pathogen attack (Moreno et al., 2012). They also found *IRE1/bZIP60* activity to function as part of a general UP response, and could be induced by tunicamycin (TM), a chemical that prevents proper protein folding. The use of TM to 'artificially' induce an ER stress response has been used extensively to study stress response systems and identify their key components, and can be also be used in *Chlamydomonas* (reviewed in Deng et al., 2013; Perez-Martin et al., 2014).

As the transcriptome analysis by Joo et al. (2017) reported a large number of protein processing-related transcripts to be up-regulated by GLE, we hypothesized that an ER stress response might be associated with the lysin-induced cell wall recovery. Cellular events like total wall reconstruction require many transcripts to be expressed simultaneously, which can overload the cell's capacity for processing mRNA and lead to the accumulation of misfolded proteins. We first treated both wildtype and bZIP mutant cells with tunicamycin to see 1) if tunicamycin

induces a UPR, and 2) if an UP response affects the transcription of cell wall-related genes by itself. We then treated both cell lines with GLE and compared transcript expression of our GOI. In doing this, we could assess the impact that an ER stress response might have on cell wall recovery.

## 3.2 Results

### 3.2.1 Tunicamycin induces an UPR but not in the bZIP mutant

To determine if an unfolded protein response is facilitating increased expression of our transcripts of interest, it was necessary to artificially induce an UPR in *C. reinhardtii*. By incubating cells in liquid culture containing 0.5μM TM, we could determine effects of the UP response on gene expression. We treated both wildtype and bZIP mutant cells with TM to observe any differences in gene expression. This bZIP mutant is an insertional knockout of Cre05.g238250, which encodes a protein that shares significant identity with *AtbZIP60* – a gene known to be an important transcription factor for the UPR in *A. thaliana* (Moreno et al., 2012). A local protein sequence alignment between *AtbZIP60* and Cre05.g238250 indicates 43% identity for a 65 amino acid region that encodes a predicted bZIP domain (data not shown). Based on the bZIP mutant profile, we might expect that these cells will exhibit an impaired UPR.

Quantitative PCR showed observable effects of TM treatment on transcription of protein processing-related transcripts in wildtype cells but not in the bZIP mutant (Figure 3.1). Both *SEC61G* and *RHM1* showed approximately 2.5 and 5 times greater expression when wildtype cells were treated with TM, which is indicative of UPR-mediated transcription. There was no

change in the levels of *AraGT1,* which means simply that this transcript is up-regulated in response to UP stress. The bZIP mutant showed no increase in protein processing transcript levels when treated with TM, confirming that this mutant has an impaired UPR. In both cell lines, cell wall-related transcripts showed no difference between untreated and TM treated samples. This lack of change in the transcript levels for cell wall-associated transcripts suggests that an UPR has no direct effect by itself.

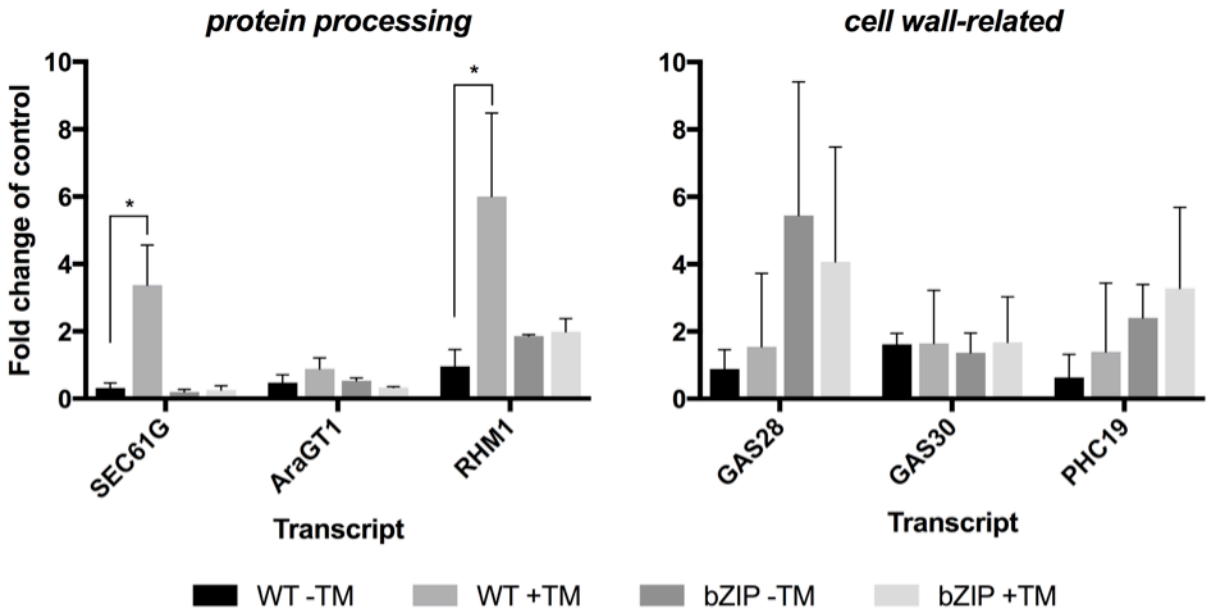


**Figure 3.1: Tunicamycin-induced UPR is impaired in a bZIP mutant.** Bar graphs represent the change in gene expression for protein processing-related and cell wall-related transcripts in wildtype (CC-125) and bZIP mutant cells. Samples represented are both wildtype untreated (black) and treated with 0.5μM tunicamycin (medium grey), and bZIP mutant untreated (dark grey) and treated with 0.5μM tunicamycin (light grey). Gene expression is quantified in terms of fold change compared to control condition (ΔΔCq). Error bars represent one standard deviation from the mean of biological triplicate samples. Welch's t-test indicates statistical significance at $p \leq 0.05$ (*); $\alpha = 0.05$.

### 3.2.2 A bZIP mutant shows reduced transcriptional response to GLE

To delve deeper into the question of whether an UP response plays a role in the regeneration of the cell wall, we treated the UPR-impaired bZIP mutant with GLE and compared the transcriptional response to wildtype cells. Figure 3.2 shows a side-by-side comparison of transcript levels for our GOI in both wildtype and bZIP mutant cells treated with GLE. Nearly all our GOI in the bZIP mutant were found to have a lower up-regulation when treated with GLE in comparison to the wildtype. This lower expression is especially pronounced for the cell wall-related transcripts, as all showed between 50 to 150-fold lower induction in the bZIP cells treated with GLE. These results suggest that this bZIP transcription factor might play a role in the regulation of these transcripts following cell wall removal. This role would likely be secondary to the initial GLE-induced transcription event and is probably necessary to maintain high levels of cell wall-related transcripts. It is, however, curious to note that *RHM1* shows no change in expression between wildtype and mutant treated with GLE in Figure 3.2, when there is a large increase in expression when wildtype cells were treated with TM. This observation suggests that perhaps *RHM1* might be regulated by another branch of the UPR system and not by this mutated bZIP gene.

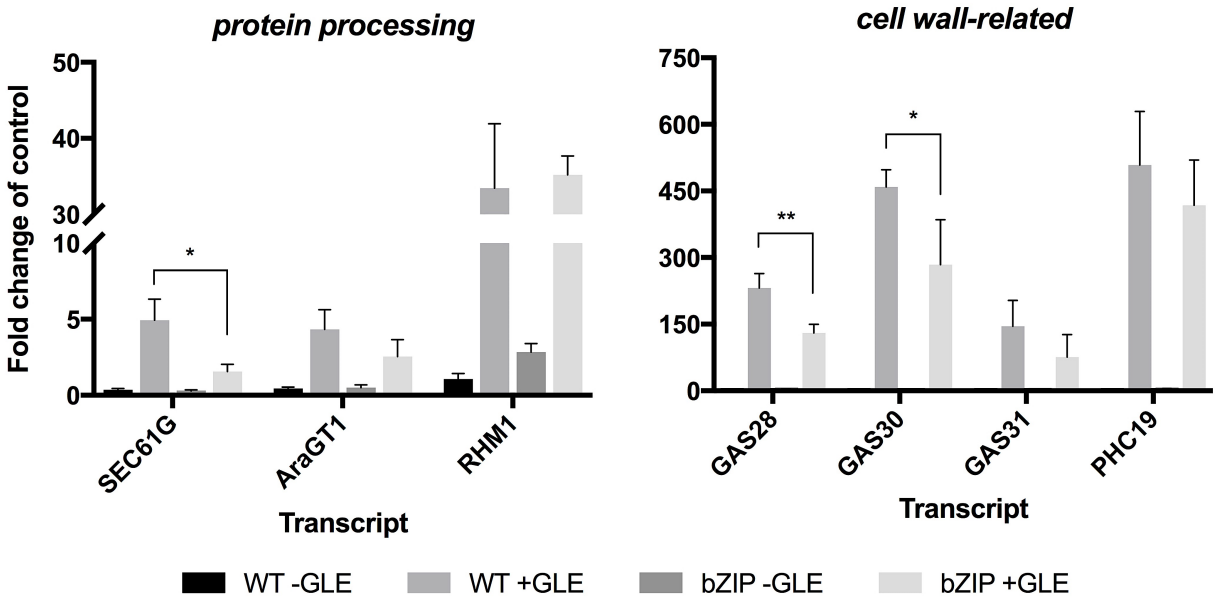


**Figure 3.2: Transcript levels following gametolysin treatment show a diminished response in bZIP mutant vs. wildtype.** Bar graphs represent the change in gene expression for protein processing-related and cell wall-related transcripts. Samples represented are both wildtype, CC-125, untreated (black) and treated with 50% tunicamycin (medium grey), and bZIP mutant untreated (dark grey) and treated with 50% tunicamycin (light grey). Gene expression is quantified in terms of fold change compared to control condition (ΔΔCq). Error bars represent one standard deviation from the mean of biological triplicate samples. Welch's t-test indicates statistical significance at $p \leq 0.05$ (*), $p \leq 0.01$ (**); $\alpha = 0.05$.

## 3.3 Discussion

In this study, we questioned if an ER stress response – in the form of an UPR – was involved in the regulation and mounting of the transcriptional response to GLE treatment. When cells undergo a large-scale transcriptional event, it is not uncommon for misfolded proteins to accumulate in the ER as the rate of transcription exceeds the rate of protein processing. When gamete cells are treated exogenously with GLE, or naturally shed their cell walls during sexual mating, we expect many transcripts to be expressed and the transcriptome analysis in Joo et al.

(2017) has supported this claim. To see if an UPR alone had effects on the expression of our GOI, wildtype and bZIP mutant cells were treated with tunicamycin, an N-glycosylation inhibitor and UPR-inducer. We observed that two protein processing-related transcripts, *SEC61G* and *RHM1,* were affected by the UPR in wildtype cells. This result suggests a UPR has been stimulated given that the accumulation of protein-processing machinery is known to increase during an UPR (Liu & Howell, 2010). The bZIP mutant line showed no increase in transcript levels when treated with TM, which confirms an impaired UPR. In wildtype cells there was no observable effect of TM treatment on cell wall related transcripts, meaning that an UPR alone does not influence the expression of cell wall components.

To observe the effects of an UPR on the GLE transcriptional response, we treated the UPR-impaired bZIP mutant with GLE and compared transcript levels of our GOI to wildtype cells. We found that transcript levels for our GOI were significantly reduced in the bZIP mutant compared to wildtype, with the exception of *RHM1*, which appeared to have unchanged expression. When considering that the artificial UPR induced by TM had no effect on the expression of cell wall-related transcripts, it was interesting to find that these transcripts exhibited a diminished response to GLE in the bZIP. These results could likely be explained as: a) an UPR contributes to cell wall-related transcriptional up-regulation during GLE treatment as a secondary response to keep up with the transcriptional demand, or b) the bZIP transcription factor is involved with another regulatory network that coincidentally contributes to cell wall-related gene regulation.

## 3.4 Materials and methods

### 3.4.1 GLE and tunicamycin treatment conditions

Cells were grown on TAP (TAP+NIC for CW15) plates for 7 days under continuous ~50μE light. On Day 7, cells were harvested and suspended in NF-TAP, then counted and diluted to a concentration of 5 x $10^7$ cells/mL. Suspended cells were incubated under high light (~200μE) for a minimum of 3 hours to induce gametogenesis.

For GLE treatment, suspended cells were mixed with an equal volume of GLE extract (now 50% GLE) and put under ~200μE light for 1 hour to ensure cell wall removal. GLE efficiency was determined by NP40 test (see 2.4.8 "NP40 sensitivity testing"). Untreated control samples were mixed with an equal volume NF-TAP to maintain equal cell concentrations across samples. Cells were then incubated for 1 hour before harvesting RNA.

For tunicamycin treatment, suspended cells were mixed with an equal volume of 5μg/mL tunicamycin in NF-TAP and put under ~200μE light for 1 hour before harvesting RNA.

### 3.4.2 RNA extraction and cDNA synthesis

See section 2.4.3 "RNA extraction and cDNA synthesis".

### 3.4.3 Quantitative real-time PCR analysis

See section 2.4.4 "Quantitative real-time PCR analysis".

# Chapter 4: The potential role of osmoregulation in cell wall integrity signalling

## 4.1 Introduction

As *C. reinhardtii* is primarily a soil and freshwater-dwelling organism, tight osmoregulation is necessary for survival and to carry out cellular processes. This regulation is maintained by a number of mechanisms, most notably the pumping of water out of the cell via the contractile vacuoles (CVs) and the selective uptake of solutes from the environment. In higher plants, turgor pressure generated in the periplasmic space between the plasma membrane and cell wall is also known to contribute to osmoregulation. Whether turgor pressure plays a role in osmoregulation in *C. reinhardtii* is unclear. Early researchers assume that it does (Hoffmann & Beck, 2005), whereas others state this is not the case considering the flexibility of the cell wall (Komsic-Buchmann et al., 2014). The model in Figure 4.1a is an illustration demonstrating how *Chlamydomonas* cells are likely to maintain a suitable intracellular osmolarity. In a hypotonic environment, water will tend to move into the cell via osmosis. Anytime the intracellular water volume is too high (solute concentration is too low), the contractile vacuoles will pump the excess out of the cell. In a hypertonic environment, it is hypothesized that cells will pump solutes into the cell and/or accumulated solutes in the cytosol avoid water loss, but direct evidence for this mechanism is lacking.

A measurement of the osmotic balance in cells is known as the water potential – the tendency of water to enter or leave a cell – and can be understood as the sum of many pressure potentials. The pressures exerted on cells include: solute potential ($\Psi_s$), pressure potential ($\Psi_p$),

gravimetric potential ($\Psi_g$), and matrix potential ($\Psi_m$) (Taiz et al., 2015). In an aquatic environment, gravimetric and matrix potentials are negligible, so pressure potential and solute potential are crucial to aquatic organisms (Taiz et al., 2015). It remains unclear how much of an effect the pressure potential has on *Chlamydomonas.* Certainly, the aqueous environment will exert a certain amount of hydrostatic pressure on the cell, but whether a turgor pressure is generated between the plasma membrane and cell wall is unknown. Given the specific water potential of a *C. reinhardtii* cell, the cell's surface area, and the plasma membrane permeability for water, one can generate an understanding about the rate of water movement into or out of the cell, called water flux (see equation in Figure 4.1b).

A limited number of studies have been published about osmoregulation in *Chlamydomonas*. Existing research has focused primarily on the organization and role of the CVs in water efflux. Early studies by Luykx et al. (1997a) demonstrated CV formation to be the dynamic amalgamation of scores of smaller water-containing vesicles, which eventually fuse with the plasma membrane as a large contractile vacuole and force water out of the cell. They also found several osmoregulatory mutants to have contractile vacuole defects, whose poor viability confirmed the importance of the CV in maintaining cytosolic osmolarity (Luykx et al., 1997b). More recent studies have found and characterized specific components of the CV system, such as the vesicular membrane protein SEC6 that mediates CV membrane fusion and the aquaporin MIP1, which facilitates the passive movement of water from the cytosol into the CV vesicles (Komsic-Buchmann et al., 2012; Komsic-Buchmann et al., 2014).

A study by Hoffmann & Beck (2005) considered the effects of cell wall removal on osmoregulation using three previously identified transcripts *GAS28, GAS30,* and *GAS31.* Their results indicated that in both hypo- and hyperosmotic media, cells will accumulate high levels of

all three transcripts within 2 hours. However, this study may contain inaccuracies as the reported osmolarities for the test conditions and cytosol of *C. reinhardtii* differ greatly from the more recently calculated values presented in Komsic-Buchmann et al. (2014).

This limited research has focused almost solely on the cell's response to hypotonic environments and how cells actively remove excess water to maintain a water balance. We know very little about the cellular responses to hypertonic environments, aside from the cessation of contractile vacuole activity and decreased expression of *SEC6* and *MIP1* (Komsic-Buchmann et al., 2012; Komsic-Buchmann et al., 2014). One study observed the intracellular accumulation of glycerol in cells exposed to a hypertonic environment (León & Galván, 1995), which was presumed to prevent water loss in cells by raising the intracellular solute content. What we aim to know is whether osmotic stress sensing contributes to the GLE-induced transcriptional event. It is possible that during the removal of the cell wall, the water balance is disrupted and the cell sense this change and elicit an osmotic stress response.

To determine if sensing of osmotic stress or change in osmotic environment affects transcription of our GOI, we began an investigation of cellular responses to changing osmotic environments. First, we observed CV cycling rates in wildtype and a cell wall defective mutant to see if these cell lines respond similar to media with different osmolarities. We then looked at the gene expression of our GOI in wildtype cells in these different media to see if there is any cellular response at the transcript level. These preliminary studies provide useful tools and information that we can use to further determine the role of an osmotic stress response in cell wall integrity signalling.

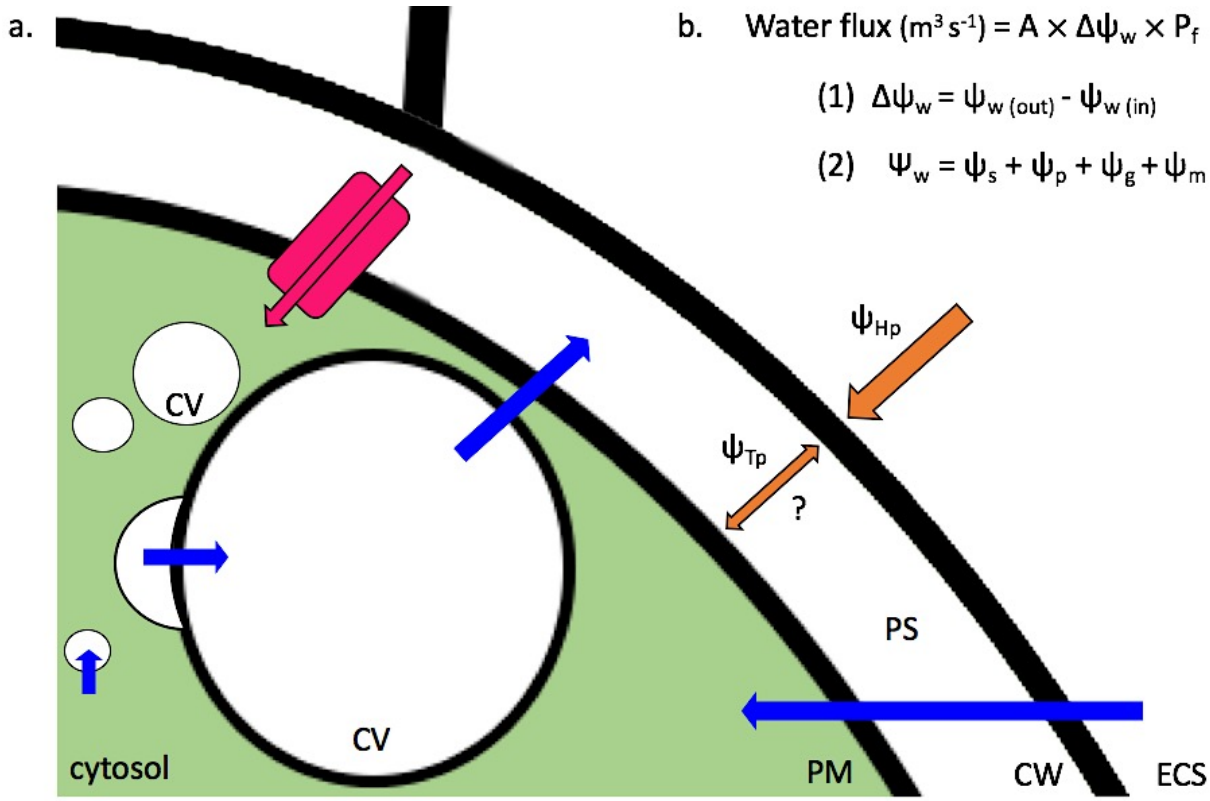


**Figure 4.1: Diagrammatic representation of osmoregulation in *C. reinhardtii* cells.** a. 2D visualization of water flow through cells near the periphery. Important cellular structures labelled: CV, contractile vacuole; PS, periplasmic space; PM, plasma membrane; CW, cell wall; ECS, extracellular space. Water flux in/out of cell represented by blue arrows. Pressure potentials represented by orange arrows; $\psi_{Tp}$, turgor pressure; $\psi_{Hp}$, hydrostatic pressure. Solute transporter represented by pink shape and arrow. b. Equation and derivation for total cellular water flux: A, surface area ($m^2$); $P_f$, permeability coefficient (m/s); $\psi_w$, water potential (Pa); $\psi_s$, solute potential (Pa); $\psi_p$, pressure potential (Pa); $\psi_g$, gravitational potential (Pa); $\psi_m$, matrix potential (Pa).

## 4.2 Results

### 4.2.1 Contractile vacuole dynamics adjust to changing osmolarity of environment

As a means of testing the effects of changing osmotic conditions and verifying the protocol used by Komsic-Buchmann et al. (2012), we measured contractile vacuole cycling times. An example of how CV cycle times were measured is demonstrated in Figure 4.2 showing a CV of a CW15 cell progressing through the systolic phase of a CV cycle.

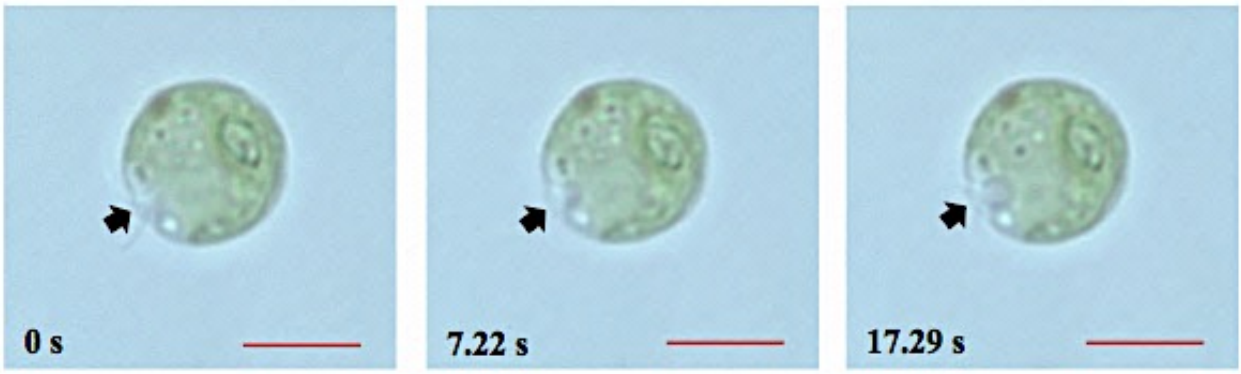


**Figure 4.2: Visualization of contractile vacuole cycling in CW15 cells in water.** Micrographs are of a single CW15 cell at different time points of one systolic phase. Time in seconds is indicated in bottom left of each image. Black arrow highlights one contractile vacuole as it grows through systole. Scale bar = 5μm.

The data presented in Table 4 clearly show that increasing osmolarity in the medium increases the time of CV cycles. This trend appeared to be proportional for both cell lines tested, wildtype and cell wall-less CW15. Interestingly, CW15 showed a much longer baseline CV cycle time, nearly double the length of wildtype. For example, in pure water (0 mOsm) WT cells had a CV cycle time average of 9.56s ± .40s whereas CW15 had an average time of 17.66s ± 1.90s. Also, both cell lines exhibited CV activity in the TAP+S condition but none in the

TAP+SS condition, indicating that the TAP+SS environment is hypertonic to the cell. This observation suggests that the intracellular osmolarity of *C. reinhardtii* is between 144 and 204mOsm, which is in agreement with the published value of ~171mOsm determined by Kosmic-Buchmann et al. (2014).

**Table 4.1. Contractile vacuole cycling times in varying osmotic conditions.**

| | Media condition (osmolarity) | | | | |
|---|---|---|---|---|---|
| **Cell line** | **H2O (0 mOsm)** | **½ TAP (32 mOsm)** | **TAP (64 mOsm)** | **TAP +S (144 mOsm)** | **TAP +SS (204 mOsm)** |
| CC-125 | 9.56 ± .40s | 10.90 ± .50s | 16.64 ± 1.12s | 22.91 ± 1.28s | NCV |
| CW15 | 17.66 ± 1.90s | 21.24 ± 0.73s | 27.16 ± 1.31s | 38.80 ± 1.65s | NCV |

NCV, no contractile vacuole cycling observed

### 4.2.2 Change in environmental osmolarity induces modest transcriptional response

To evaluate the effect of osmolarity on transcription of our GOI, cells were subjected to varying osmotic conditions then mRNA levels for the GOI were quantified. Using the same osmotic conditions as defined in Komsic-Buchmann et al. (2012), cells were suspended in either hypotonic media, ½ TAP (32 mOsm) and TAP (64 mOsm), or hypertonic media, TAP +SS (204 mOsm) and incubated for an hour before harvesting mRNA. Compared to control (TAP) conditions, all transcripts showed increased expression in both more hypotonic and hypertonic environments (Figure 4.3). The changes in expression in ½ TAP conditions were modest and insignificant at the level tested, but a few transcripts in the hypertonic TAP +SS environment showed modest but significant up-regulation. An explanation for why we observed a greater difference in transcript accumulation between TAP and TAP +SS compared to TAP and ½ TAP

could be the larger difference in osmolarity. The difference between TAP and TAP +SS is 140 mOSm whereas the difference between TAP and ½ TAP is 32 mOsm, thus the greater change in osmolarity is likely to generate more osmotic stress and induce a larger increase in transcription.

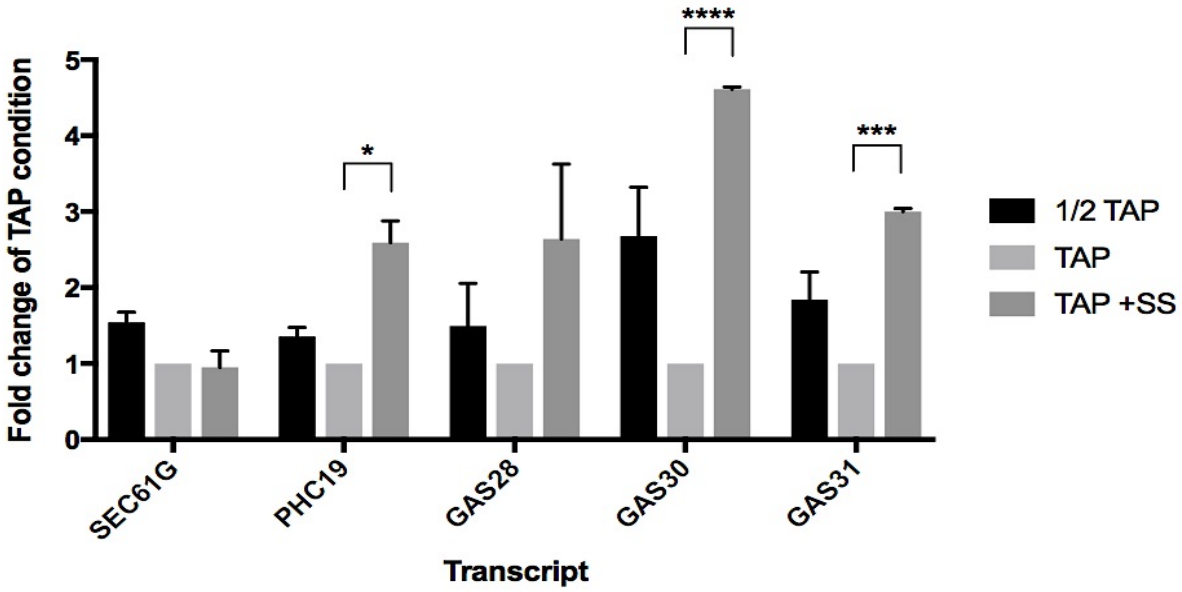


**Figure 4.3: Transcript expression suggests modest cellular response to changing osmotic environment.** Bar graphs represent the change in transcript activity in half-diluted liquid media (1/2 TAP, black bars) and liquid media plus sucrose (TAP +SS, medium grey bars) compared to the normal TAP media (light grey bars). mRNA levels are quantified as fold change of the TAP condition with TAP normalized to 1 (ΔΔCq). Error bars represent one standard deviation from the mean of biological duplicate samples. Welch's t-test indicates statistical significance at $p \leq 0.05$ (*), $p \leq 0.001$ (***), $p \leq 0.0001$ (****); $\alpha = 0.05$.

## 4.3 Discussion

If the cell wall aids in the maintenance of a water balance in *C. reinhardtii*, it is logical that the removal of the cell wall would have an impact on the osmoregulation of a cell. The detection of a sudden change in the environment could elicit an osmotic stress response, which could in turn result a transcription event.

Based on measurements of CV cycling times, it appears that regardless of the presence of a cell wall, CVs will respond in a similar manner to changing environmental osmolarity (Table 4). However, it was notable that the cell wall-less CW15 line showed a much longer CV cycling time than WT. This finding is rather curious as it may be expected that a cell with no wall might be more susceptible to their environment, and in, say, water, CW15 might experience a greater tendency for water to rush into the naked cell and increase its water flux. In a normal *Chlamydomonas* cell, the cell wall may reduce the hydrostatic pressure exerted on the cell by the medium, thereby slowing the movement of water into the cell. In the CW15 line, the lack of a wall means the cell is completely susceptible to the pressures of the surrounding medium. However, it may be possible that the difference in CV cycling is intrinsically related to the unknown mutation of CW15 and not related to the presence or absence of the cell wall.

When wildtype CC-125 cells were subjected to environments with varying osmolarity, we saw a minor transcriptional change for many of our GOI. Most notably, cell wall-related transcripts increased a modest but significant amount when placed into hypertonic media. In the hypertonic media cells' CVs are not functional but water may tend to leave the cell passively if the environment is very different than the iso-osmotic point (~171 mOsm). In this condition where the media was 204 mOsm, water is likely leaving the cell, which is then detected by the

cell and a stress response is invoked. Why these transcripts are up-regulated in increasingly hypo- and hypertonic environments remains unclear (an opinion shared by Hoffmann & Beck, 2005), but is likely due to an intrinsic osmotic stress response. A cellular response to this stress may be the synthesis of a more resilient cell wall, to further shelter the cell from the environment.

## 4.4 Materials and methods

### 4.4.1 Contractile vacuole visualization and timing

Liquid media with varying osmolarities were made by either adding amounts of sucrose to liquid TAP(+NIC) media or by diluting the media with water. ½ TAP(+NIC), 1:1 water to media; TAP(+NIC) +S, 60mM sucrose; TAP(+NIC) +SS, 120mM sucrose. Cells were suspended in 100µL of corresponding media and incubated for 1 hour under ~200µE light. 8µL of cell suspension was loaded onto glass slides and cells were viewed under 1000X total magnification using Zeiss Axioscope A1. Contractile vacuole cycling was measured manually by timing consecutive systole and diastole cycles of a single contractile vacuole per cell. A minimum of 3 cells per cell line per osmotic condition were measured.

### 4.4.2 Osmotic stress treatment conditions

Cells were grown on media plates for 7 days under continuous ~50µE light. On Day 7, cells were harvested and suspended in TAP, then counted and diluted to a concentration of 5 x

$10^7$ cells/mL. Suspended cells were incubated under ~200μE light for 1 hour to acclimate cells to the liquid culture. Cells were then diluted according to the following conditions: ½ TAP, 1:1 water to TAP; TAP; or TAP +SS, 120mM sucrose in TAP. Cells were then put under ~200μE light for 1 hour before harvesting RNA.

### 4.4.3 RNA extraction and cDNA synthesis

See section 2.4.3 “RNA extraction and cDNA synthesis”.

### 4.4.4 Quantitative real-time PCR analysis

See section 2.4.4 “Quantitative real-time PCR analysis”.

# Chapter 5: Conclusions

## 5.1 Major findings of thesis and contribution to science

While previous research has identified a number of genes and key factors involved with the cell wall and its modification during the mating reaction, it remains unclear how these factors are regulated. At a surface level, a wealth of transcriptome data has provided clues about this regulation, but mRNA expression data on a large scale has a limited capacity for understanding complex gene regulatory networks.

To better understand how genes that are up-regulated in response to GLE, we selected several genes of interest and determined the levels at which they are regulated during this response. As described in Chapter 2, promoter-reporter and mRNA expression analyses showed our GOI to be strongly transcriptionally induced by GLE. When cells were exposed to the protein synthesis inhibitor CHX prior to GLE treatment, we found our GOI to be effected differently. *AraGT1* and *GAS31* transcripts showed a marked increase in expression with the CHX pretreatment, while the other transcripts decreased considerably. This strongly suggests that *AraGT1* and *GAS31* are normally under the regulation of another element (e.g. a repressor protein) that is limiting their normal GLE-induced expression. This also suggests that the other transcripts are under the control of other regulatory proteins that are synthesized *de novo* following GLE treatment.

The enrichment of protein processing-related transcripts in the GLE-induced gene cluster as defined in Joo et al. (2017) led us to question if an ER stress response contributes to cell wall reconstruction. To test this hypothesis, we used the glycosylation inhibitor tunicamycin to induce

ER stress – in the form of an UPR – and observe the change in transcription of our GOI (Chapter 3). While we observed up-regulation of *SEC61G* and *RHM1* transcripts, indicative of an UPR, there was no effect of the ER stress on cell wall-related transcript accumulation. However, when we treated an UPR-impaired bZIP mutant line with GLE, six of seven transcripts showed lower up-regulation when compared to wildtype. As the effect of GLE on the transcription of cell wall-related transcripts was impacted by the bZIP mutation, this suggests that a bZIP-mediated UPR contributes to their up-regulation. Together these results indicate that an UPR plays a secondary role in the regulation of cell wall synthesis by allowing cells to meet the demands of widespread transcript up-regulation.

To further examine the hypotheses we generated about how cells are mounting a response to cell wall removal and/or what the trigger might be, we examined how cell wall-defective lines respond to GLE. When we observed the responses of several CWD lines to freshly prepared GLE, we obtained two interesting results: 1) CWD lines have a significantly diminished or absent response to fresh GLE, and 2) CWD lines are constitutively expressing our GOI. It is reasonable to assume that these cells continuously transcribe these GOI in a fruitless attempt to build a cell wall, but it remains unclear how the cells are sensing the lack of cell wall. This is a question that we are actively pursuing. One possible explanation is that the cell is sensing and responding to the osmotic environment. Our early investigation into the effects of an osmotic stress response as part of the GLE-induced response has suggested only a minimal role of osmotic stress. While our GOI show modest up-regulation in increasingly hypo- and hypertonic environments, the expression change was quite small compared to the change we see following GLE treatment.

The information gained from this project is important for understanding how both cell wall development and the haploid-to-diploid transition are regulated in *C. reinhardtii.* By building upon the research presented in Joo et al. (2017) we have gained greater insight into the gene regulatory mechanisms upstream of GSM1:GSP1 activity during the sexual mating event. We have shown that cell wall regeneration following shedding involves large-scale transcription, requires *de novo* synthesis of regulatory elements, and appears to employ an ER stress response mechanism to accommodate increased transcription. How exactly these processes are triggered remains unclear, but the hypotheses we have generated for the 'trigger' of the GLE-induced response can be used to design further experiments.

## 5.2 Remaining questions and future directions

Gene regulation in any organism is a complex process. As such, there remain many questions regarding how gene regulation occurs in *C. reinhardtii* during times of cell wall reconstruction. Much of the research presented here can be used as a starting point to generate further questions regarding the regulatory processes of cell wall development.

### 5.2.1 Which factor(s) is/are the ultimate trigger for the GLE-mediated response?

The three hypotheses presented for the normal initiation of the GLE-induced genetic response are still potential explanations. At this point it is unclear if one or more of these hypotheses are true and will require further investigation. To determine if pherophorin signalling exists as a trigger for cell wall regeneration, we need to revisit the idea of 'fresh GLE'

treatments. The current study has applied fresh GLE extracts to both wildtype and cell wall defective strains, with the intention of observing any notable changes in the wall defective samples. However, the fresh GLE extracts also contain GLE enzyme itself and so can skew the results if the wall defective cells exhibit a response to the enzyme. Moving forward, this experiment should be repeated with fresh GLE extracts treated with EDTA to chelate the enzyme, thereby rendering the GLE inactive and isolating the effects of cleaved pherophorin proteins alone. However, the general lack of response to the 'fresh GLE' treatment in CWD cells in Chapter 2 makes this hypothesis a less likely candidate.

Another method that will help distinguish between osmotic stress and wall detachment as the trigger of lysin-induced response is to treat wildtype cells with preparations of iso-osmotic GLE. If osmotic stress is the major trigger then cells in this condition should result in a minimal response to cell wall removal, as the water flux of cells should be maintained. Conversely, if wall detachment is the trigger, we should see a genetic response similar to the GLE treatments reported in Chapter 2 and 4.

### 5.2.2 Is there a master regulator for the GLE-mediated gene response?

Another question to be addressed is whether there exists any "master" regulator(s) that initiate these gene regulatory responses. Because our research investigated promoter and transcript activity as a proxy for these regulatory networks, information about upstream elements that initiate these processes is still lacking. Using a bioinformatics approach, it would be possible to scan the promoter regions of the lysin-inducible genes and search for any consensus sequences or known binding sites upon which a regulator could act. By doing this, one can make

predictions about how these genes are regulated and may provide clues as to what the identity of these master regulators might be. A similar investigation was discussed in Joo et al. (2017), which found a ZYRE domain in the promoter regions of many early zygote-expressed genes.

If we could identify candidates for these ‘master regulators’ we could then search the vast insertional mutant libraries to see if a corresponding mutant can be obtained for further study.

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